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High frequency modification of plant genes using engineered zinc finger nucleases

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Abstract

An efficient method for making directed DNA sequence modifications to plant genes (gene targeting) is presently lacking, thereby frustrating efforts to dissect plant gene function and engineer crop plants that better produce the world's burgeoning need for food, fiber and fuel. Zinc finger nucleases (ZFNs) - enzymes engineered to create DNA double strand breaks at specific loci - are potent stimulators of gene targeting,^{1,2} including at engineered reporter genes in plants.^{3,4} Here we demonstrate high frequency ZFN-stimulated gene targeting at endogenous plant genes, namely the tobacco acetohydroxyacid synthase (*SuRA* and *SuRB*) genes, for which specific mutations are known to confer resistance to imidazolinone and sulfonylurea herbicides.⁵ Herbicide-resistance mutations were introduced into *SuR* loci by ZFN-mediated gene targeting at frequencies exceeding 2% of transformed cells for mutations as far as 1.3 kb from the ZFN cleavage site. More than 40% of recombinant plants had modifications in multiple *SuR* alleles. The observed high frequency of gene targeting indicates that it is now possible to efficiently make targeted sequence changes in endogenous plant genes.

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Author information DNA sequence of the *SuRB* locus has been deposited with GenBank under accession number FJ649655. Reprints and permissions are available at www.nature.com/reprints. Requests for OPEN reagents should be addressed to J.K.J (jjoung@partners.org).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

ZFNs were engineered that recognize *SuR* loci using publicly available resources provided by the Zinc Finger Consortium.^{6,7} The Consortium-sponsored ZFN architecture uses two zinc finger arrays (ZFAs), each with three zinc fingers that collectively recognize a nine bp target site (Supplementary Fig. 1A). The ZFAs are fused to a *FokI* nuclease domain, and a 5-7 bp spacer separates the target sites for the two arrays, allowing the nuclease to dimerize and cleave within the spacer. ZFA engineering is most robust for G-rich sequences,⁸ and four such target sites were selected in *SuRB* for constructing ZFAs by modular assembly, namely the joining together of individual zinc fingers with predetermined specificities (sites 815, 1071, 1853 and 1947) (Fig. 1A, Supplementary Table 1)⁹. Thirty-two ZFAs were constructed, and electrophoretic mobility shift assays identified three arrays with DNA binding activity, two of which bind half sites for the 815 target. This low success rate is consistent with previous findings that ZFAs constructed by modular assembly are often non-functional.⁸

Oligomerized Pool Engineering (OPEN) - a method developed by the Consortium - employs genetic selections in bacteria to identify ZFA variants that recognize specific target sequences⁶ (Supplementary Fig. 1B). ZFAs made by OPEN typically show higher activity than those made by modular assembly, likely because the process of selection accommodates context-dependent interactions among neighboring zinc fingers in the array.^{6,10,11} OPEN was used to generate ZFNs for four sites (sites 865, 1853, 1947, 2163), including two that had been targeted by modular assembly (Supplementary Table 2). Functional left and right ZFAs were obtained for the 1853 target, for which modular assembly had failed, as well as for target 2163. The OPEN-derived ZFAs showed activity in bacterial two-hybrid (B2H) assays,⁷ in which binding of ZFAs upstream of a *lacZ* reporter gene activates expression (Supplementary Table 2).

To test whether the ZFAs function as ZFNs, an assay was developed that measures ZFN activity in yeast (Supplementary Fig. 2A). This assay uses a *lacZ* reporter gene with a 125 bp internal DNA sequence duplication. The ZFN target site is cloned between the duplicated sequences, and cleavage of the target site creates a functional *lacZ* gene through repair of the break by single strand annealing. ZFN activity is assessed by quantitative measurements of β -galactosidase activity. The six ZFAs for the 815, 1853 and 2163 target sites functioned effectively as ZFNs (Fig. 2A). The 815 left and 1853 right arrays showed the most activity, comparable to activity observed with a ZFN designed from the well-characterized Zif268 ZFA.²

ZFNs were tested against their endogenous targets in tobacco by measuring whether they create mutations by non-homologous end-joining (NHEJ) (Supplementary Fig. 2B). ZFN-encoding constructs were electroporated into tobacco protoplasts, and the relevant target sites in *SuRA* and *SuRB* were amplified by PCR and subjected to high-throughput pyrosequencing.¹² The fraction of unique sequence reads showing size-polymorphisms (consistent with imprecise repair by NHEJ) was normalized to controls (Fig. 2B, Supplementary Table 3). Mutation frequencies were significantly higher for ZFN 815 at both *SuR* loci. Interestingly, the highest mutation frequencies were not at the intended target in *SuRB*, but rather at the corresponding sequence in *SuRA*, which differs by two nucleotides. Not only does the ZFN 815 (which was created by modular assembly) lack

specificity, but the higher level of mutagenesis at *SuRA* relative to *SurB* suggests other factors such as chromatin or DNA methylation influence access of this enzyme to target sites. In contrast to ZFN 815, the OPEN-designed ZFN 1853 only showed enhanced mutagenesis at its intended *SuRB* target, which differs in sequence from the *SuRA* site by a single nucleotide. This suggests that the genetic selections used in OPEN yield ZFNs with high specificity. No enhancement of mutagenesis was observed with ZFN 2163.

To measure whether the engineered ZFNs could stimulate incorporation of specific DNA sequence changes at *SuR* loci by homologous recombination (HR) (Supplementary Fig. 2B), three donor templates were constructed, each with a missense mutation that confers resistance to one or more herbicides (P191A, chlorsulfuron; S647T, imazaquin; W568L, chlorsulfuron and imazaquin)^{5,13} (Fig. 1A). Silent nucleotide changes were introduced into codons adjacent to each mutation to distinguish the donor template from the native locus and spontaneous mutants from those generated by recombination (Fig. 1B). An additional set of donor templates was made in which the ZFN recognition sites were altered to prevent cleavage (Fig. 1C).

To test for gene targeting by HR, plasmids encoding the 815 ZFNs were electroporated into tobacco protoplasts with donor templates bearing the P191A, W568L or S647T mutations (Table 1, rows 1-3). The mean ZFN-induced herbicide resistance ranged from 5.3% for the P191A donor to 2.4% for the S647T donor. Both *SuRA* and *SuRB* were PCR-amplified from 12 randomly selected resistant calli derived from each treatment, using primers specific for the target locus (Fig. 1A). DNA sequence analysis revealed that in 9 of 12 lines generated with both the P191A and W568L donor template, resistance was due to HR (P191A: 5 in *SuRA* and 4 in *SuRB*; W568L: 4 at *SuRA*, 5 at *SuRB*) (Table 2, rows 1-3, Supplementary Table 4). With the S647T donor template, only 1 of the 12 herbicide resistant lines had evidence of HR (at *SuRA*) and nine were spontaneous *SuRB* mutants. For 8 of the 36 resistant lines in the gene targeting experiments, no mutations were observed in *SuRA* or *SuRB*, and so the molecular basis for the resistance is unknown. This resistance could be due to genotypic and phenotypic variation (somoclonal variation) typically observed when plant cells are grown in culture.¹⁴ Based on the number of recombinants recovered, the estimated gene targeting frequencies range from 4.0% for P191 to 0.2% for S647 (Table 1).

One surprising outcome of the above experiment was that gene targeting frequencies exceeding 2% were obtained at a distance more than 1.3 kb from the cleavage site. This suggests that plant genes can be modified even when DNA sequence composition precludes engineering ZFNs near the desired site of modification. The high frequencies of recombination observed at both *SuRA* and *SuRB* with ZFN 815 are consistent with the pyrosequencing data indicating that this enzyme cuts promiscuously at both targets (Fig. 2B). *SuRA* and *SuRB* differ at the nucleotide sequence level by 4%,⁵ and it is notable that high efficiency gene targeting could be achieved at *SuRA* using the *SuRB*-derived donor template.

We next tested the ability of the 1853 and 2163 ZFNs to stimulate HR and incorporate amino acid sequence changes near their respective target sites. Donor templates were used with mutations in the ZFN target site that prevent cleavage. ZFN 815 was used as a control,

and the mutated donor did not substantially alter the overall frequency of herbicide resistance or gene targeting (Table 1, compare rows 1 & 4). The mutated P191A donor template did, however, cause an increase in the proportion of gene targeting events at *SuRB* relative to *SuRA* (Table 2, compare rows 1 & 4). Why inability to cleave the donor template influences the outcome of recombination is unclear. For the 1853 ZFN, the mean number of herbicide resistant events at W568L (281 bp from the cut site) was 0.6% (Table 1, row 5), more than 5-fold lower than gene targeting observed with ZFN 815 at much greater distances from the cut site. The 2163 ZFN yielded only three, non-targeted herbicide resistant calli in twelve separate experiments, two of which had mutations at sites in *SuRB* previously known to confer herbicide resistance¹³ (Supplementary Table 5 and data not shown). The activity of all three ZFNs in HR parallels activities of these enzymes in the yeast and NHEJ assays (Fig. 2). Among the 47 herbicide resistant calli analyzed in the various gene targeting experiments, 19 (40.4%) showed modifications at multiple *SuR* loci, including mutations introduced by NHEJ at the ZFN cleavage site (Supplementary Tables 4 & 5). This indicates that transformed cells that sustain a ZFN-induced modification often incur changes at multiple alleles. Ten plants were regenerated from herbicide resistant calli and shown to carry the *SuRA* and *SuRB* mutations (Supplementary Tables 4 & 5), indicating that ZFN-assisted gene targeting can be used to engineer genetically modified whole plants.

Based on the high frequency of gene targeting observed at *SuRA* and *SuRB*, we reasoned that populations of cells transformed with ZFNs might be screened directly for gene targeting events. To test this, protoplasts were transformed with plasmids encoding the 815 ZFN, the P191A donor template, as well as a neomycin phosphotransferase II (NPTII) gene that confers resistance to kanamycin. In this experiment, NPTII was used merely to identify cells that had been transformed. Approximately 1000 transformed cells were transferred to media with herbicide, and two calli displayed herbicide resistance, both of which carried mutations introduced by the donor template (Supplementary Table 4). Although this experiment identified HR events at the *SuR* loci by examining herbicide resistance, its success suggests that screens, perhaps using high throughput DNA sequencing, could be used to identify recombinants among populations of transformants for any genetic modification introduced by recombination, regardless of whether a selection exists for its associated phenotype.

Current methods for modifying plant genomes are limited to decades-old methods of DNA transformation that lack precision and control over the outcome of the modified chromosome. Plant biologists have long sought a method to make directed mutations in plant genes with high efficiency, as evidenced here with the use of ZFNs. Gene targeting offers numerous opportunities for studying plant gene function, and it also enables biosynthetic pathways to be harnessed to better produce much-needed plant-derived products. The ability to engineer highly functional ZFNs using publicly available reagents and to recover HR-induced mutations even at considerable distances from the ZFN cleavage site demonstrate that targeted mutagenesis in plants is now practical.

Methods Summary

ZFA engineering and testing

Engineering of ZFAs by modular assembly or OPEN followed protocols established by the Zinc Finger Consortium.^{6,7} Assessments of ZFA function are described in greater detail in the supplementary material online and are summarized in Supplementary Table 6.

Plant cell transformation and culture

Tobacco protoplasts were prepared from aseptically grown plants and transformed by electroporation as previously described⁴. Prior to transformation, donor, ZFN-, and NPTII-encoding DNAs were linearized by digestion with *Bgl*II, *Alw*NI, or *Fsp*I, respectively. Electroporation experiments used 20 µg each of donor template and ZFN-encoding DNA. Chlorsulfuron (5 ppb) and imazaquin (0.5 ppm) were used for selection, the former for the P191A and W568L donor templates and the latter for the S647T donor. Transformation frequencies were assessed using *SuRB* genes with herbicide resistance mutations. Numbers of resistant calli were scored 30 days post treatment. All of the gene targeting experiments used obligate heterodimeric *Fok*I domains fused to the left and right ZFAs.^{15,16} Experiments with homodimeric (wildtype) *Fok*I domains reduced plating efficiency relative to experiments with the heterodimeric domains, and no gene targeting events were recovered, consistent with high levels of ZFN-induced toxicity (Supplementary Table 7).

Characterization of recombinant plant material

DNA was isolated from calli with the PowerPlant DNA isolation kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA). The entire coding regions of both *SuRA* and *SuRB* were PCR-amplified using primers specific to the 5' end of each gene and a common 3' primer (Fig. 1A). The Expand Long Template PCR System (Roche, Indianapolis, IN, USA) was used to ensure fidelity of the PCR reactions and to minimize strand transfers during amplification. PCR products were sequenced in their entirety to identify mutations that confer herbicide resistance and modifications at the ZFN cut site.

Online Methods

DNA donors

Tail-PCR was used to amplify and clone a 7.8 kb genomic DNA fragment encompassing the *SuRB* locus. Oligonucleotide-directed mutagenesis was used to introduce mutations that confer herbicide resistance and silent nucleotide changes that distinguish HR events from spontaneous mutants (Fig. 1). Numbers specifying mutations and target sites are referenced with respect to GenBank accession X07645.

Three donor DNA pairs were constructed, each truncated 59 bp into the coding sequence. Truncation prevented herbicide resistance due to random integration of the donor DNA. pDW1963 and pDW1964 carry the P191A mutation; the latter has the 815 ZFN cut site mutated. pDW1927 and pDW1968 carry the W568L mutation; the latter has the 1853 ZFN cut site mutated. pDW1969 and pDW1972 have the S647T mutation; the latter has the 2163

ZFN site mutated. Donor DNAs are referred to by the mutation they carry (e.g. W568L); 'm' indicates donors with mutated cut sites (e.g. W568L-m).

Yeast assay

To test whether zinc finger arrays (ZFAs) function as zinc finger nucleases (ZFNs), a yeast-based recombination assay was developed similar to ones previously reported.^{17,18} Our assay uses a derivative of the *E. coli lacZ* gene with a 125 bp duplication of coding sequence. The sequence duplication flanks a *URA3* gene and a polylinker for cloning the target sequence being tested (Supplementary Fig. 2A). After cleavage of the target sequence by the ZFN, recombination between the *lacZ* sequence duplication results in loss of *URA3* (conferring 5-fluoroorotic acid resistance). Recombination also reconstitutes a functional *lacZ* gene, enabling quantitative measurements of enzyme activity that reflect frequencies of recombination.

A two-step process is used to construct yeast reporter plasmids. First, complementary oligonucleotides corresponding to the target site are cloned into the polylinker of plasmid pDW1666, which has the 3' half of the *lacZ* gene. Secondly, a 2.5 kb *NcoI* to *ApaI* fragment from the pDW1666 derivative is inserted into the *BspHI* and *ApaI* sites of either plasmid pDW1714 or pDW1742. These latter plasmids contain the 5' half of the *lacZ* gene, a *TRP1* marker, and either the 2 μ origin of replication (pDW1714) or a yeast centromere (*CEN*) (pDW1742). Reporter plasmids are introduced into the yeast strain YPH500 (*MAT α*) and grown on synthetic complete medium lacking tryptophan and uracil (SC-W-U). The medium is adjusted to pH 7.0 and includes X-gal as previously described.¹⁷

pDW1789 is used to express ZFNs in yeast from the *TEF1* promoter. ZFAs are cloned into a polylinker to generate *FokI* nuclease fusions. The expression plasmid has a *HIS3* gene and a *CEN*, and is introduced into YPH499 (*MAT α*). The transformed yeast strain is plated on SC medium lacking histidine (SC-H).

Quantitative β -galactosidase assays are performed by growing a white yeast colony carrying the target plasmid and a colony with the ZFN expression plasmid overnight at 30 C in liquid SC-H or SC-W-U media, respectively. The cultures are adjusted to the same OD₆₀₀, and 100 μ l of each are added to 10 ml of yeast peptone dextrose (YPD) medium and cultured at 30 C for 20 to 26 hours. An aliquot of cells is harvested and quantitative β -galactosidase assays are performed as described¹⁷. Enzyme activity is normalized to cell number¹⁷.

Plant ZFN expression vectors

ZFA::*FokI* fusions that recognize left and right half-sites of a given target are expressed in plant cells from a single plasmid. Expression plasmids are constructed in two steps: one ZFA is cloned as an *XbaI/BamHI* fragment into a polylinker in pDW1876; the second ZFA is cloned as an *XbaI/BamHI* fragment into a polylinker in pDW1895. The polylinkers in both pDW1876 and pDW1895 are flanked by CaMV 35S promoters and NOS transcriptional terminators. To make a dual ZFN expression plasmid, both plasmids are digested with *ApaLI* and *AvrII*. The fragment encoding the ZFN from the pDW1895 derivative is then cloned into the *ApaLI* to *AvrII* sites of the pDW1876 derivative. The

above plasmids use the wild type *FokI* nuclease, and two variants (pDW2000 and pDW2001) express the obligate heterodimeric form of *FokI* to reduce cellular toxicity¹⁸. All the *FokI* nucleases were codon-optimized for expression in plants. pDW998 was used to identify transformed tobacco protoplasts. It carries a neomycin phosphotransferase gene (NPTII) expressed from a CaMV 35S promoter.

Tests of ZFN toxicity

To evaluate whether ZFN toxicity impacts the recovery of gene targeting events, aliquots of protoplasts were transformed with plasmids expressing the 815 ZFN with either the homodimeric or heterodimeric form of *FokI* nuclease (see Methods Summary and Supplementary Table 7). The 815 ZFNs were separately electroporated with the three donor DNAs. Selection was imposed on the cells (see Methods Summary) to identify possible recombinants. An aliquot of the culture was grown in the absence of selection, and plating efficiency was calculated by determining the number of calli that formed in the absence of selection relative to the total number of protoplasts treated.

Pyrosequencing to monitor NHEJ

Tobacco protoplasts were transformed with DNA encoding the 815, 1853 or 2163 ZFNs as described in the Methods Summary, except that 30 µg of ZFN-encoding DNA was used. For each of the target sites, four aliquots (experimental replicates) of protoplasts were transformed. An aliquot of protoplasts was also transformed with a ZFN derived from Zif268 to serve as a control. Forty-eight hours after transformation, DNA was prepared and the target sites were PCR-amplified with primers specific for given target sites and that amplify both *SuRA* and *SuRB*. The primers were bar-coded to distinguish the target sites, each of the experimental replicates, and the control. PCR products were gel purified and quantified using a Quant-iT PicoGreen kit (Invitrogen, Carlsbad, CA, USA). Sequencing was carried out at the University of Iowa DNA Facility using the Roche 454 sequencer, yielding a total of 422,077 sequence reads. Sequences were parsed according to locus (*SuRA*, *SuRB*), target site (815, 1853, 2163), experimental replicate (Rep1 - Rep4), and control (Zif268). Analysis was limited to sequence reads showing size polymorphisms (indels) consistent with NHEJ-induced mutations. The frequency of indels was normalized to the Zif268 control as described in the text and the legend to Supplementary Table 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

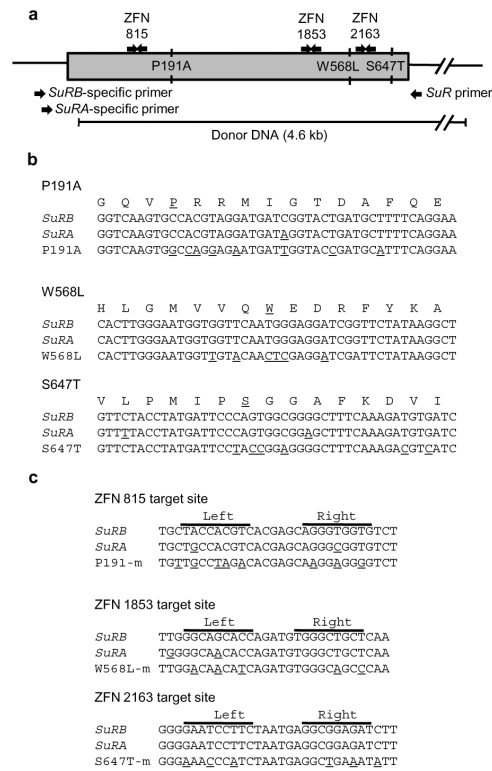
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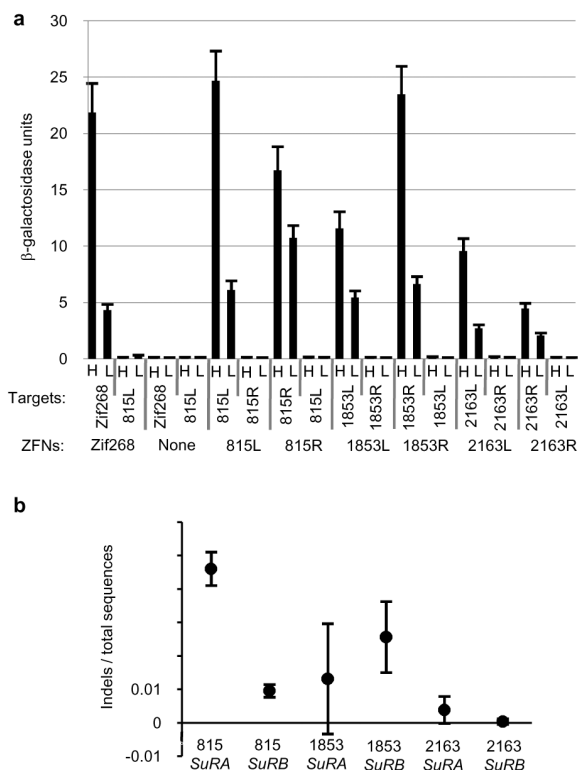
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**Figure 1.**

The tobacco *SuRB* locus. a) The diagram is drawn to scale and annotated with ZFN sites, amino substitutions that confer herbicide resistance, PCR primers used to characterize recombinants, and the region used as a donor template. b) ZFN target sites. Left and right denote bases recognized by each ZFA. Underlined bases are either *SuRA* sequences that differ from *SuRB* or mutated bases in the donor (bottom row) that prevent cleavage by the ZFN. c) Sequences at the sites of introduced mutations. The targeted amino acid is underlined, as are sequences in *SuRA* that differ from *SuRB* and silent nucleotide changes in the donor template that distinguish recombinants from spontaneous mutants.

**Figure 2.**

Activity of engineered ZFAs and ZFNs. a) ZFAs as stimulators of recombination in yeast. Target sites for each ZFA are listed in vertical text below the chart; H, high-copy plasmid; L, low-copy plasmid. Error bars denote s.d.; n = 3. b) Engineered ZFNs as stimulators of mutagenesis by NHEJ in tobacco. ZFNs were expressed in protoplasts, and the *SuRA* and *SuRB* target sites were analyzed by pyrosequencing. The number of sequences with insertions/deletions (indels) was divided by the total number of reads for a given target and normalized to a Zif268 control. Values above the X axis indicate a higher proportion of sequences with indels than the control. Error bars denote 95% confidence intervals; n = 4.

Table 1

Gene targeting frequencies at *SuRA* and *SuRB*.

| Row | ZFN | Donor DNA ¹ | Distance to mutation | Number herbicide resistant | | | Freq. ZFN-induced resistance ³ | Freq. gene targeting ⁴ |
|-----|------|------------------------|----------------------|----------------------------|--------------|--------------------------|---|-----------------------------------|
| | | | | Neg. control | Pos. control | ZFN + donor ² | | |
| 1 | 815 | P191A | 188 bp | 0 | 3461 | 184 | 5.3% | 4.0% |
| 2 | 815 | W568L | 1319 bp | 0 | 2217 | 75 | 3.4% | 2.6% |
| 3 | 815 | S647T | 1541 bp | 0 | 3600 | 88 | 2.4% | 0.2% |
| 4 | 815 | P191A-m | 188 bp | 0 | 1804 | 46 | 2.5% | 2.4% |
| 5 | 1853 | W568L-m | 281 bp | 0 | 1577 | 10 | 0.6% | 0.5% |

¹The letter m in columns 4 and 5 denotes donors with mutated ZFN recognition sites.

²Values are the mean resistant calli obtained in three separate experiments.

³Values are the mean resistant calli divided by the number of transformed cells (based on data obtained from the positive control construct) and expressed as a percentage.

⁴Values are the percentage of ZFN-induced herbicide resistance adjusted according to the frequency of HR as determined through molecular analyses of randomly sampled calli (Table 2).

Table 2

Molecular basis for herbicide resistance in gene targeting experiments.

| Row | ZFN | Donor DNA | SuRA | | | SuRB | | |
|-----|------|-----------|--|--------------------------------|--|--|--------------------------------|--|
| | | | ZFN target NHEJ indels/ alleles examined | HR events/ alleles examined | Mutation site Spont. mutants/ alleles examined | ZFN target NHEJ indels/ alleles examined | HR events/ alleles examined | Mutation site Spont. mutants/ alleles examined |
| 1 | 815 | P191A | 0/24 | 5/24 | 0/24 | 8/24 | 4/24 | 0/24 |
| 2 | 815 | W568L | 0/24 | 4/24 | 0/24 | 3/24 | 5/24 | 0/24 |
| 3 | 815 | S647T | 0/24 | 1/24 | 0/24 | 2/24 | 0/24 | 9/24 |
| 4 | 815 | P191A-m | 3/36 | 0/36 | 0/36 | 3/36 | 20/36 | 1/36 |
| 5 | 1853 | W568L-m | 0/22 | 0/22 | 1/22 | 2/22 | 9/22 | 1/22 |

NHEJ-induced mutations, HR events, and spontaneous (Spont.) mutations are expressed in terms of the number of alleles of *SuRA* or *SuRB* analyzed (compiled from Supplementary Tables 4 and 5). Note that some plants sustained HR at more than one allele and that herbicide resistant somaclonal variants were recovered with no mutations in *SuRA* or *SuRB*. The letter m in columns 4 and 5 denotes donors with mutated ZFN recognition sites.