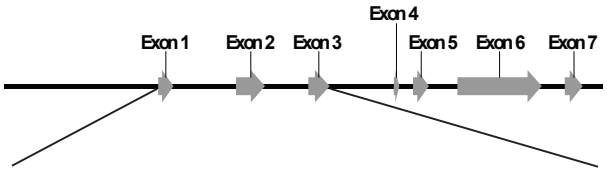
a

ZmIPK1

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CACTTGACTT TAGGGGCTCA ATGTGTGATT CACGGGTGCC GCGGCGCCAT
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GGACGATCAC GTGGTCACTT GAAAATTACG TGAGGTCCGG GGCGACGATG
TACGGCGCGG CGAACTCTCA AACACTCACA CAACCAAAAC CGCTTCGTGT
TCGTCTTTGT TCCAAGCGAC TGTGTGAGTG TTTGAGAGTT CGCCAGCGCG
ACATCGCCCG ATCTGACAAA TTAAGCTTTC GTTGCTTTTC CATGATTGTG
CATTTTGTGA GCATGCACTG AATACTATGA TGGATATGTT TGGAGGAAGC
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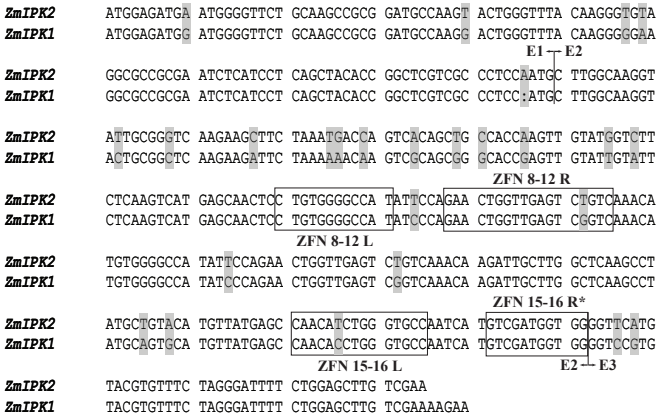
GCAAGGTACT GCGGCTCAAG AAGATTCTAA AAAACAAGTC GCAGCGGGCA
CCGAGTTGTA TTGTATTCTC AAGTCATGAG CAACTCCTGT GGGGCCATAT
CCCAGAACTG GTTGAGTCGG TCAAACAAGA TTGCTTGGCT CAAGCCTATG
CAGTGCATGT TATGAGCCAA CACCTGGGTG CCAATCATGT CGATGGTGGG
GTATGGTTCA GATTCAGTTC ATTTATGTCC TGTTATTGTG ATTTTGATTG
GTAACATATT GACAACCTCG ACACTTGGGA TCAGATTCAG TTCACTTATG
GAAGAAATTG GAGAATTGTT ATAATTTATC TATAATCACC CCTACTGAAA
TAGAAATAAC ATGGCATCAA TGTGCATGCT ATTGGATTTT GACACGAATA
TGCTTTATTC TATCATATGT TGGTAATTCC AGCAGGCAGC AGGCACTACT
CTTTGGATCC ACGTGACTTG ACAAAGAAAT CATGCCATCT TTCCACAATG
CAGGTCCGTG TACGTGTTTC TAGGGATTTT CTGGAGCTTG TCGAAAAGAA
TGTTCTTAGC AGCCGTCCTG CTGGGAGAGT AAATGCAAGT TCAATTGATA
ACACTGCTGA TGCCGCTCTT CTAATAGCAG ACCACTCTTT ATTTTCTGG
```

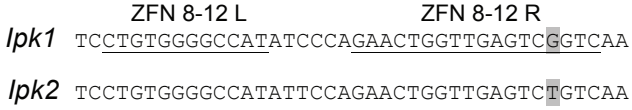
b



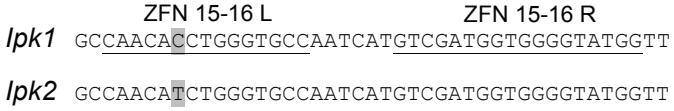
* The binding site overlaps with the exon-intron boundary; it is partially represented in the context of the CDS.

c

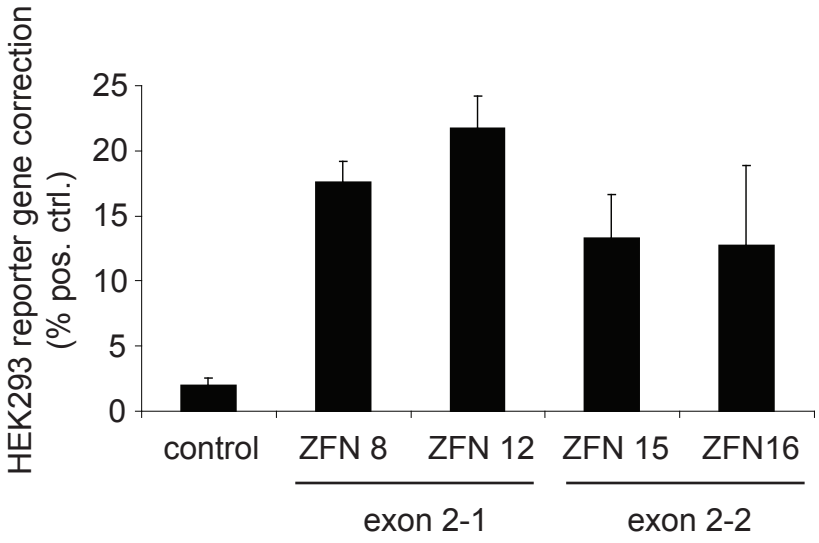
Exon 2-1



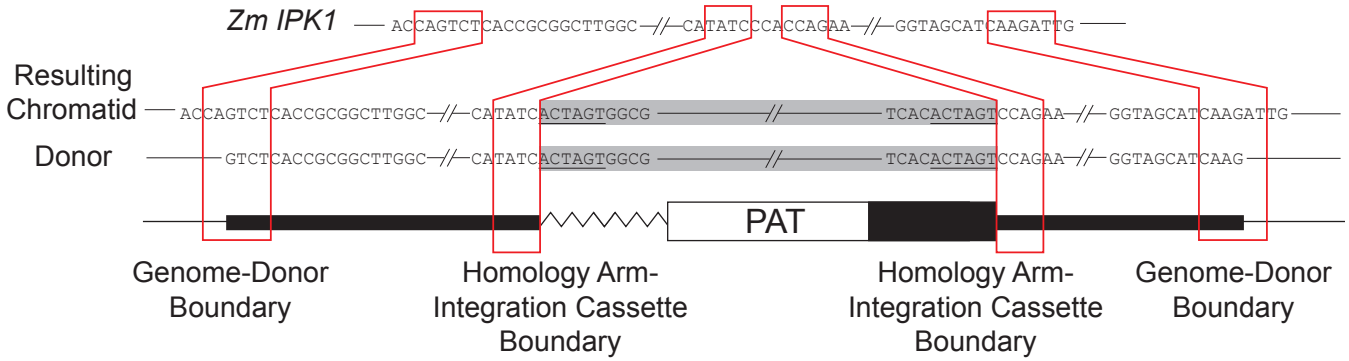
Exon 2-2



Supplementary Figure 1. The *ZmIPK1* Sequence From HiII Reveals Polymorphisms Relative to *the ZmIPK2* Paralog.
S1A. Genomic structure of *ZmIPK1* from maize variety HiII (top) and nucleotide sequence spanning exons 1-3 (zoom view, bottom). The indicated sequence was used as input template for ZFN design. **S1B.** Sequence comparison of the coding regions in exons 1-3 from *ZmIPK2* (top line) and *ZmIPK1* (bottom line) in HiII. Polymorphic nucleotides such as those exploited for differential ZFN binding site design are shaded in gray. Exon/exon boundaries are indicated. The boxed sequences indicate binding sites for ZFNs used in this study. **S1C.** ZFN target sequences within *ZmIPK1* and *ZmIPK2*. The complete sequences of the ZFN binding sites are underlined and differential SNPs are shaded in gray.



Supplementary Figure 2. ZFNs designed to target *ZmIPK1* induce DSBs in an integrated chromosomal reporter.
Representative results of ZFN reporter gene correction activity performed in two distinct HEK293 reporter lines. ZFNs were tested for efficiency of chromosomal reporter gene correction using HEK293 reporter lines, constructed as described (Porteus Science 2003), each engineered to carry a short fragment of the *IPK1* gene. Reporter constructs included a minor modification: the GFP reporter gene was disabled with the recognition site not only of the ZFNs targeting *IPK1*, but also ZFNs that target the *CCR5* gene (Perez Nat. Biotechnol. 2008), to allow normalization of reporter gene correction activity across different lines. The same ranking was observed in three independent experiments. Each datapoint represents the reporter gene correction activity of the indicated ZFNs, normalized to activity exhibited in the same reporter line by control ZFNs. The data are presented as “percent gene correction activity” of control ZFNs.



Supplementary Figure 3. Consensus sequence of target locus analysis of *ZmIPK1* PCR-genotyping amplicons. High fidelity integration of the non-autonomous PAT donor was diagnosed based on integrity of the indicated genome/donor boundary sequences, homology arm integration cassette boundaries, preservation of diagnostic sequences in the donor molecule (SpeI site, underlined), and PAT gene cassette components.

a

Zinc finger nuclease expression constructs

↓

Site-selection *in vitro*

↓

Binding site consensus and position weight matrix

↓

Maize genome search to identify potential off-target sites

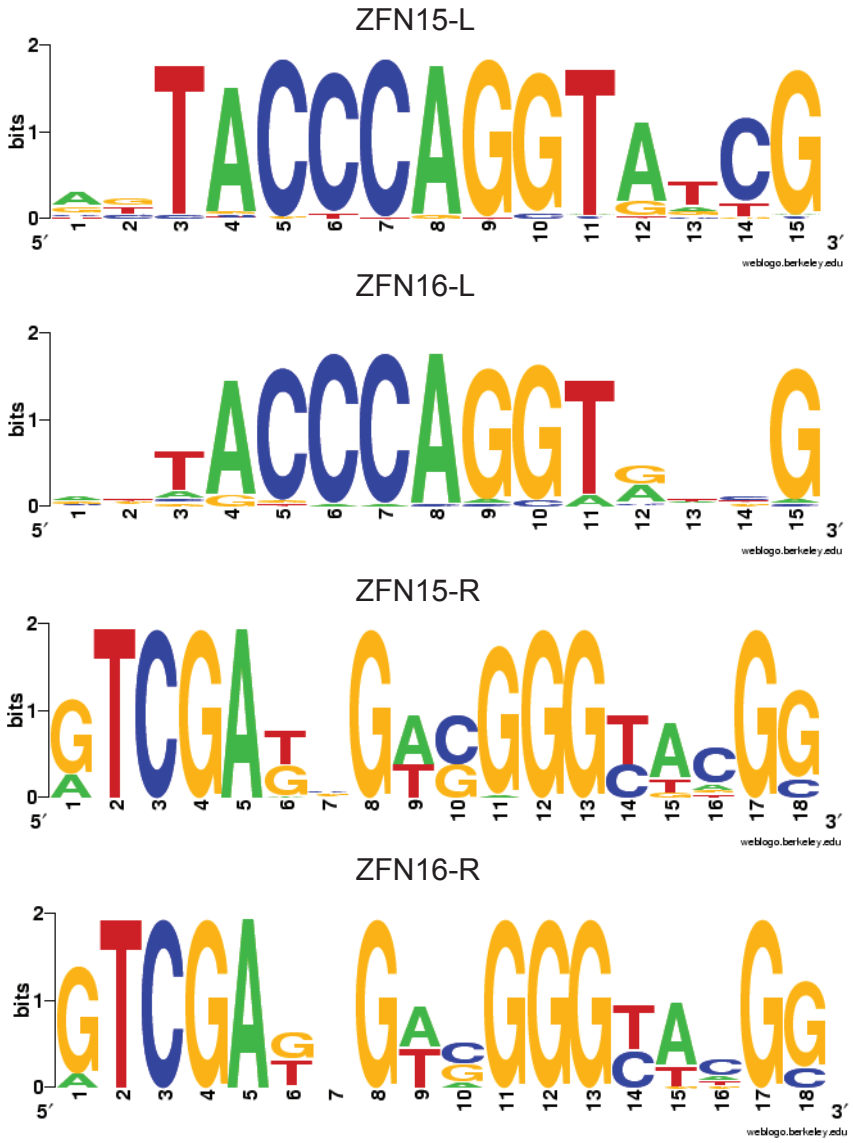
↓

List of off-target sites, ranked with respect to position weight matrix for ZFN

↓

Experimental evaluation of off-target action by direct genotyping of potential off-target sites in ZFN-treated T0 regenerated plants

c



b

GTCGAKNGWSGGGYACGG ZFN16-R

GTCGAKNGWSGGGYACGG ZFN15-R

site selection

CCAACACCTGGGTGCCAATCATGTCGATGGTGGGGTATGGT

GGTTGTGGACCCACGGTTAGTACAGCTACCACCCCATACCA

site selection

ZFN15-L GCTATGGACCCATNR

ZFN16-L GNNRTGGACCCATNN

d

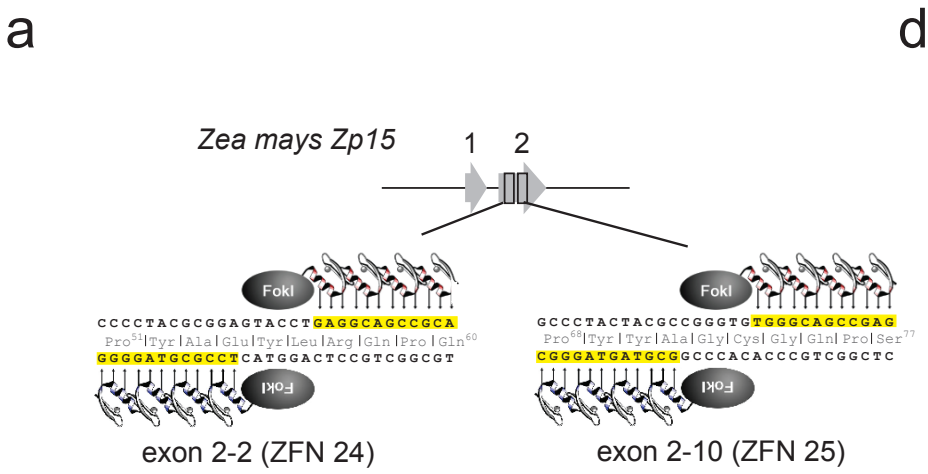
ZFN15-L	ZFN16-L	ZFN15-R	ZFN-16-R
ACTACCCAGGTATCG	ATTGCCCAGGTGGGG	GTCGAGCGACGGGTAAGG	GTCGAGAGACGGGCACGG
ATTACCCAGGTGCTG	GTTACCCAGGTGCTG	GTCGATAGACGGGTGCGG	GTCGAGCGACGGGCATGC
GGTACCCAGGTATCG	AATGCCCAGGACGGG	GTCGAGGGACGGGCACGC	GTCGAGCGACGGGTTAGG
GGTACCCAGGTATCG	TATGCCCAGGTGACG	ATCGATAGTGGGGCACGG	GTCGAGTGTAGGGTTAGG
AGTACCCAGGTATCG	TCTACCCAGGTAGAG	ATCGATGGTGGGGCACGG	GTCGAGTGTGGGGTTAGG
ACTACCCAGGTATTG	GTTACCCAGGTACTG	GTCGAGCGACGGGCACGC	GTCGATAGTGGGGCACGG
ATTACCCAGGTAACG	CCGACCCAGGTACTG	GTCGAGCGACGGGCACGG	GTCGATAGTGGGGCATGC
CTTTCTCAGCTGACC	GGTACAAACCTTAAC	GTCGAGCGACGGGTATGG	GTCGATAGTGGGGCATGG
ACTACCCAGGTATCG	ATTACCCAGGTATCG	GTCGAGCGACGGGTGCGG	GTCGATGGACGGGCACGC
ATTACCCAGGTAGCG	ATAACCCAGGTGACG	GTCGAGCGTGGGGTAAGG	GTCGATGGACGGGTAGGG
GATACCCAGGTATTG	GGTACCCAGGTGTTG	GTCGAGCGTGGGGTTCGG	ATCGAGCGACGGGCTCGG
ATTACCCAGGTATCG	ATTACCCAGGTATCG	GTCGAGTGACGGGTTGGG	GTCGACCGACGGGCACGG
GCTACCCAGGTATCG	GTGAGCCCAGTCAGG	GTCGAGTGTGGGGTACGG	GTCGAGAGACGGGTTAGG
GGTACCCAGGAGGTG	AGTACCCAGGTATCG	GTCGATAGTGGGGCGCGG	GTCGAGAGACGGGTTCGG
CGTACCCAGGTGGCG	AAAACCCAGGTATGG	GTCGATAGTGGGGTTAGG	GTCGAGCGTGGGGTAGGG
ACTACCCAGGTATCG	ACTACCCAGGAGGAG	GTCGATCGTGGGGCACGG	GTCGAGTGAAGGGCACGG
CGTGCCCGTCTATCG	GGTACCCAGGTGTCA	GTCGATCGTGGGGCACGG	GTCGAGTGTAGGGCACGG
CTTGGTCAGCTTTTA	GGAGCCCAGGTGCCG	GTCGATCGTGGGGTATGG	GTCGATAGTGGGGCACGC
TGTACCCAGGTATCG	ATTACCCAGGTATCG	GTCGATGGACAGGTATGG	GTCGATAGTGGGGCATGG
ATTACCCAGGTAGCG	CTTACCCAGGTATGG	GTCGATGGACGGGTTAGG	GTCGATAGTGGGGTAAGG
ATTACCCAGGTGCTG	GGTACCCAGGTGCCG	ATCGAGTGACGGGTAGGG	GTCGATAGTGGGGTATGG
GTTACCCAGGTATCG	CGTACCCAGGTAACG	ATCGATAGTGGGGTACGC	GTCGATGGAAGGGTACGG
ACTACCCAGGTGCCG	ACGACCCAGGTGTCG	ATCGATGGACGGGCACGC	GTCGATGGACGGGCATGC
GCTACCCAGGTATCG	CCTACCCAGGTGCCG	ATCGATGGACGGGCACGC	GTCGATGGACGGGTTAGG
CGTACCCAGGTATCG	CATACCCAGGTGACG	ATCGATGGACGGGCACGC	GTCGATGGTGGGGCACGG
TCTACCCAGGTATCG	GGTACCCAGGTAGTG	ATCGATGGACGGGTACGG	GTCGATGGTGGGGTAAGG
ACTACCCAGGTATCG	ATTACCCAGGTATTG	ATCGATGGACGGGTTCGG	ATCGAGAGTGGGGTACGG
AGTACCCAGGTATCG	GTTACCCAGGTGATG	GTCGAACGACGGGCACGG	ATCGAGCGACGGGTACGG
AGTACCCAGGTATCG	ACTACCCAGGTGTCG	GTCGAGAGTGGGGTACGG	ATCGAGCGACGGGTGCGG
GGTACCCAGGTATCG	ATTACCCAGGTATCG	GTCGAGCGACGGGCACGG	ATCGATAGTGGGGTACGC
ATTACCCAGGTATCG	GGCATCCAGGTAGTG	GTCGAGCGACGGGTTGGG	GTCGAAAGTGGGGCACGG
GTTACCCAGGTATCG	TTCACCCAGGAGTAG	GTCGAGCGTGGGGCACGG	GTCGAGCGACGGGCACGC
AGTACCCAGGTATGG	ATTACCCAGGTGTGG	GTCGAGGGACGGGTTAGG	GTCGAGCGACGGGCACGG
AATACCCAGGTAGCG	CGTACCCAGGTATCG	GTCGAGTGACGGGTACGC	GTCGAGCGACGGGTTCGG
AGTACCCAGGTATCG	AACACCCAGCACCCG	GTCGATAGTGGGGTACGG	GTCGAGTGACGGGTTAGG
GGTACCCAGGTGTGG	TTAACCCAGGTGATG	GTCGATGGACGGGTACGG	GTCGAGTGTGGGGCACGC
ATTACCCAGGTAGCG	ATTACCCAGGTATGG	GTCGATGGTGGGGCACGG	GTCGAGTGTGGGGTACGG
TCTACCCAGGTAACG	GGAACCCAGGTGTGG		GTCGATAGACGGGTACGG
ACTACCCAGGTAACG	AGTACCCAGGTATCG		GTCGATAGACGGGTTCGG
TTTACCCAGGTATCG			GTCGATAGTAGGGCACGG
AGTACCCAGGTATCG			GTCGATGGTGGGGTAGGG
ATTACCCAGGTAGCG			
ACTACCCAGGTAACG			
ACCACCTGGGCTCCG			
ATTACCCAGGTATCG			
GGTACCCAGGTATCG			
ATTACCCAGGTATCG			
TGTACCCAGGTATCG			
CTTACCCAGGTATCG			
GCTACCCAGGTGCTG			
GGTACCCAGGTATCG			
GGTACCCAGGTATCG			
GGTACCCAGGTAACG			
ATTACCCAGGTATCG			
ATTACCCAGGTATCG			
GCTACCCAGGTGTCG			
ATTACCCAGGTATCG			
TTTACCCAGGTAGTG			
AGTACCCAGGTAACG			
ATTACCCAGGTAACG			
AGTACCCAGGTAACG			
GGCCCTCAGGTGCTG			
ATTACCCAGGTATCG			

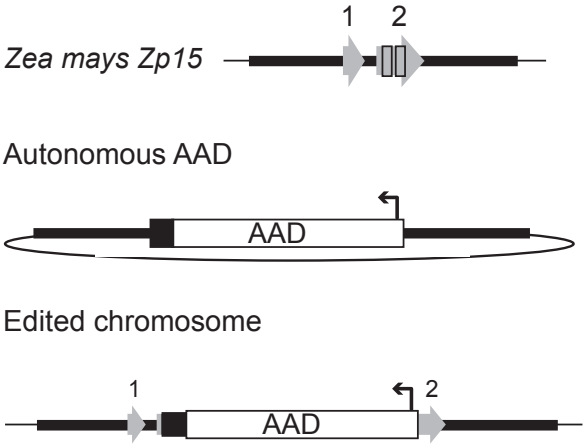
Supplementary Figure 4. Analysis of ZFN 15 and 16 action at potential off-target sites.

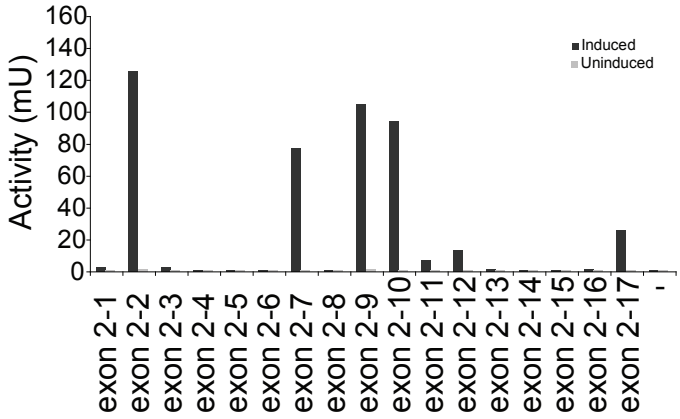
S4A. Experimental scheme to evaluate ZFN action at potential off-target sites.

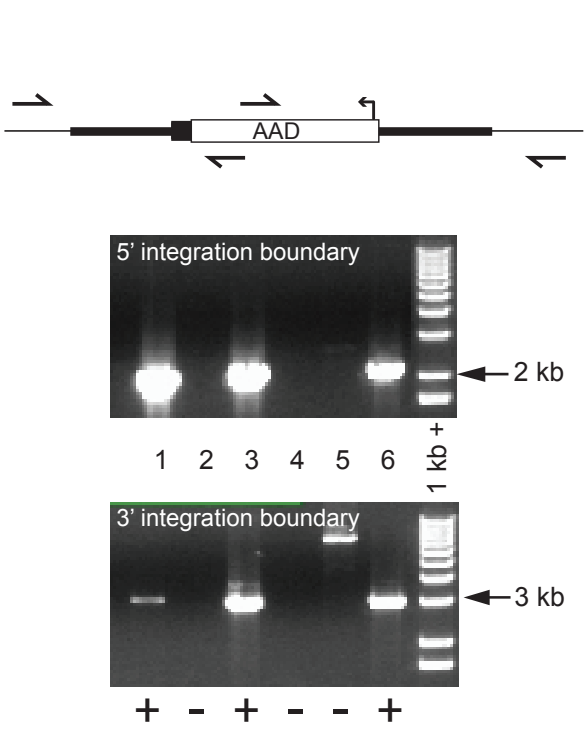
S4B. Illustration showing the ZFN 15-16 recognition sites in the *IPK1* gene (shaded), and a qualitative representation of *in vitro* SELEX results yielding the ZFN recognition sites determined experimentally.**S4C.** Summary of ZFN site selection experiments depicted via standard logograms (http://weblogo.berkeley.edu/logo.cgi).

S4D. Sequences obtained from the SELEX experiment used to generate the logograms.









c

ZFN 24

ACCCCTACGCGGAGTACCTGAGGCAGCCGCAG(wt)

ACCCCTACGCGGAG:::CTGAGGCAGCCGCAG(Δ3)

ZFN 25

CGCCCTACTACGCCG::::::GGTGTGGGCAGCCGAGC(wt)

CGCCCTACTACGCCG**GCAATT**GGTGTGGGCAGCCGAGC(+6)

Supplementary Figure 5. Homology-directed repair of a ZFN-induced DSB results in targeted gene addition to the *Zp15* gene. S5A. Schematic representation of the *ZmZp15* gene, with exons indicated as arrows. Gene segments used as templates for ZFN design are indicated by boxes. The zoom-in view illustrates the binding site for two ZFN pairs (Smith NAR 2000) designed to induce DSBs at 2 distinct positions.

S5B. Results of normalized ZFN reporter gene correction activity in a budding yeast proxy screening system, constructed as described (Doyon Nat biotechnol 2008). **S5C.** Identification of representative deletions (ZFN 24, top) and insertions (ZFN 25, bottom) relative to wild-type at the endogenous Zp15 locus. Modifications induced by NHEJ occur at the predicted ZFN cleavage sites delimited by the ZFN binding sites (underlined).**S5D.** *Zp15* donor design. Top, schematic of the *ZP15* gene region targeted by ZFNs. Middle, schematic of the *Zp15* donor construct comprised of an autonomous or AAD gene cassette flanked by short segments of *Zp15* sequence identity shown as thick black lines. The AAD gene cassette included a promoter from the rice actin gene (OsAct) and terminator from the maize lipase gene (black box). Bottom, integration of the donor at the intended site is expected to yield an edited chromatid with a disrupted Zp15 gene.**S5E.** Representative results of PCR-based genotyping of the *Zp15* locus. Primers hybridizing to *Zp15* gene sequences not contained on donor constructs and the AAD1 transgene coding region, spanning the predicted integration boundaries (schematic, top) were used to amplify gDNA from all herbicide tolerant calli. Gel, top: 5'-integration boundary. Gel, bottom: 3'-integration boundary. The presence of appropriately sized bands (5'-boundary, 2 kb; 3'-boundary, 3 kb; arrows left) indicates integration of AAD into *Zp15* (lanes marked as +). Lanes 1-6, *Zp15* events 1-6, 1-7, 148, 149, 151, 155.

Supplementary Table 1. Amino Acid Sequences of *ZmIPK1* Zinc-Finger Binding Domains

ZFN #	ZFN Target Position	ZF Domain Left*	ZF Domain Right*
8	exon 2-1	AAMAERPFQCRICMRNFSRSDHLSEHIRTHTGEKPFACDICGRKFAQSATRKKHTKIHTGSQKPFQCRICMRNFSERGTLARHIRTHTGEKPFACDICGRKFARSDALTQHAQRCGGLR	AAMAERPFQCRICMRNFSDRSALSRHIRTHTGEKPFACDICGRKFARNDDRKKHTKIHTGGQRPFQCRICMRNFSRSDNLARHIRTHTGEKPFACDICGRKFATSGSLTRHTKIHTGSQKPFQCRICMRNFSRSDVLSEHIRTHTGEKPFACDICGRKFAQSGNLARHAQRCGGLR
12	exon 2-1	AAMAERPFQCRICMRNFSRSDHLSEHIRTHTGEKPFACDICGRKFAQSATRKKHTKIHTGSQKPFQCRICMRNFSERGTLARHIRTHTGEKPFACDICGRKFARSDALTQHAQRCGGLR	AAMAERPFQCRICMRNFSDRSALSRHIRTHTGEKPFACDICGRKFARNDDRKKHTKIHTGGQRPFQCRICMRNFSTSGNLTRHIRTHTGEKPFACDICGRKFATSGSLTRHTKIHTGSQKPFQCRICMRNFSRSDVLSEHIRTHTGEKPFACDICGRKFAQSGNLARHAQRCGGLR
15	exon 2-2	AAMAERPFQCRICMRNFSRSDSLSAHIRTHTGEKPFACDICGRKFARSAALARHTKIHTGGQRPFQCRICMRNFSRSDNLSEHIRTHTGEKPFACDICGRKFAASKTRTNHTKIHTGSQKPFQCRICMRKFADRSHLARHAQRCGLR	AAMAERPFQCRICMRNFSRSDHLSTHIRTHTGEKPFACDICGRKFAQSGSLTRHTKIHTGGQRPFQCRICMRNFSRSDHLSEHIRTHTGEKPFACDICGRKFAQNHHRINHTKIHTGSQKPFQCRICMRNFSTSGNLTRHIRTHTGEKPFACDICGRKFADRSALARHAQRCGLR
16	exon 2-2	AAMAERPFQCRICMRNFSRSDSLSAHIRTHTGEKPFACDICGRKFARSAALARHTKIHTGGQRPFQCRICMRNFSRSDNLSEHIRTHTGEKPFACDICGRKFAASKTRTNHTKIHTGSQKPFQCRICMRKFADRSHLARHAQRCGGLR	AAMAERPFQCRICMRNFSRSDHLSTHIRTHTGEKPFACDICGRKFAQSGSLTRHTKIHTGGQRPFQCRICMRNFSRSDHLSEHIRTHTGEKPFACDICGRKFAQNHHRINHTKIHTGSQKPFQCRICMRNFSTSGNLTRHIRTHTGEKPFACDICGRKFADRSALARHAQRCGGLR

* DNA recognition helicies in each domain of the ZFN pair are underlined

ZFN15 (TI events # 278, 417, 418, 419)
[event # 418 = phytate content analysis; Fig 5 b-c-d -- # 419 = TI homozygote]

ZF domain left homodimerization sites

#	Loc	Pos*	Sequence	Region	Coding?	Events	Wild-type chromatids	Mutant chromatids
1	chr7	190,548,543	ACGtTACaaGGGcACCTCCTCAAGTACCCAGtTAagGT	AC199903.3	Yes	418, 419	8/8	0/8
2	chr2	241,214,062	ACctTAaCTGGGTACTTGAGGAGGTgCCCttGTAatGT	AC197242.3	Yes	418, 419	8/8	0/8
	chr2	241,018,416		AC194208.3	Yes			
3	chr7	49,025,336	TCGATgCgTGGGTAATAGATTTATTACCtAGaTcTaGG	AC212685.2	No	418, 419	7/7	0/7
	chr7	49,150,133		AC198132.3	No			
4	chr5	97,762,802	TCagTAgCTGaGTAGCTGAGCGCTACgCgGGTgctGG	AC212095.2	No	418, 419	8/8	0/8
5	chr1	114,392,019	AtatgACgTGGGTACTGTATAAGGTAgCCAGGgAgCGC	AC190694.3	No	418, 419	ND°	ND°

ZF domain right homodimerization sites

#	Loc	Pos*	Sequence	Region	Coding?	Events	Wild-type chromatids	Mutant chromatids
1	chr7	41,879,634	AggGgACCCGACGCTCGAgCGGTGGTaGAGCtTGGGGCAaGGC	AC214430.2	No	418, 419	7/7	0/7
2	chr6	202,077,821	CCCtTACCCGTCCAgCGAtCCAGCGGTgGgcTGTGGGcCttGGT	AC204876.2	No	418, 419	8/8	0/8
3	chr7	41,888,298	AggGgACgCGACGCTCGAgCGGTGGTaGAGCtTGGGGCAaGGC	AC214430.2	Yes	418, 419	7/7	0/7
4	chr9	74,538,149	CCCGaGCCtGACGtTCGcCATACGGcCGAaAcgCGGGCAgGGA	AC203748.3	Yes	418, 419	8/8	0/8
	chr9	74,303,084		AC205255.3	Yes			
5	chr4	235,580,893	GagcaGCCCGTgCATCGAgGAGCTGTCGAGGGACGGtCAgGGA	AC194903.3	Yes	418, 419	5/5	0/5

ZF domain heterodimerization sites

#	Loc	Pos*	Sequence	Region	Coding?	Events	Wild-type chromatids	Mutant chromatids
1	chr2	4,503,501	CCaAcAtCTGGGTgCCAATCATGTCGATGGTGGGGTAtGGT	AC193307.3 (IPK2)	Yes	278, 417, 418, 419	15/15	0/15
2	chr10	147,570,681	GCCATgCCTGGGcACGAAGAGGTtGAGCcAGGGGTgCGGC	AC198492.2	Yes	418, 419	8/8	0/8
	chr10	147,886,806	GCcATgCCTGGGcACgAAGAGGTtGAGCcAGGGGTgCGGC	AC199218.2	Yes			
3	chr2	37,321,203	GCcATgCCTGGGcACgAAAAAGGTtGAGCcAGGGGTgCGGC	AC204299.2	No	418, 419	ND°	ND°
4	chr1	231,112,029	CCGcTgCCTGGGTtGaACGTCGagCGAGGGTGaGGTAgGGG	AC203826.4	No	418, 419	7/7	0/7
5	chr10	49,474,665	ACcATAgaTcGGTAGaTGTAGGGTCGAGAGAaGGtCACGGT	AC197428.3	No	418, 419	8/8	0/8
	chr10	49,652,355		AC206269.2	No			

* www.maizesequence.org Release 2a.50 (October, 2008)
° We could not get a specific PCR product for this locus

Supplementary Table 2. Analysis of ZFN 15 action at potential off-target sites.
A listing of the potential homodimerization and heterodimerization off-target sites for ZFN15 in the non-repetitive portion of the maize genome; the mismatches relative to the ZFN consensus sites are shown in lowercase and the potential binding sites are highlighted. Leaf gDNA was extracted from T0 regenerated plants and the sites were amplified by PCR followed by TOPO cloning and sequencing analysis of multiple independent clones.

ZFN16 (TI event # 273)

ZF domain left homodimerization sites

#	Loc	Pos*	Sequence	Region	Coding?	Event	Wild-type chromatids	Mutant chromatids
1	chr1	206,282,173	CCACCACCTGGGcACCTCCATGGTggCgAGGTGCCGT	AC212040.1	Yes	273	4/4	0/4
2	chr10	5,168,217	CCAGaACCTGGGcACCCGAAACCTagCCCAGGTGACcA	AC188612.3	Yes	273	4/4	0/4
3	chr1	118,906,910	TCCGCACCTGGGcAGGGGATGAGAcACCCAGcaGGGGC	AC195543.3	Yes	273	4/4	0/4
4	chr6	58,313,525	GCCAagtCTGGGTACTTGTGACGTTACCCcGGTGGAGA	AC205041.2	No	273	4/4	0/4
	chr6	58,198,472		AC205397.3	No			
	chr6	58,118,855		AC205397.3	No			
5	chr7	72,155,807	GCCAagtCTGGGTACTTGTGGCGTTACCCcGGTGGAGA	AC207566.2	No	273	4/4	0/4

ZF domain right homodimerization sites

#	Loc	Pos*	Sequence	Region	Coding?	Event	Wild-type chromatids	Mutant chromatids
1	chr7	41,879,634	AggGgACCCGACGCTCGAgCGGTGGTaGAGCtTGGGGCAaGGC	AC214430.2	No	273	4/4	0/4
2	chr6	202,077,821	CCCtTACCCGTCCAgCGAtCCAGCGGTgGgcTGTGGGcCttGGT	AC204876.2	No	273	4/4	0/4
3	chr6	8,595,095	GCCGTGCaCGgaGCTCGcCCAGGAtTCGcGCGTaGGGTtCGGA	AC200868.3	No	273	4/4	0/4
	chr6	8,378,531		AC211622.3	No			
	chr6	8,712,818		AC194149.3	No			
	chr8	99,043,796		AC212370.3	No			
	chr8	99,311,115		AC202068.2	No			
	chr3	199,367,460		AC209909.2	No			
	chr3	199,194,688		AC209909.2	No			
4	chr7	41,888,298	AggGgACgCGACGCTCGAgCGGTGGTaGAGCtTGGGGCAaGGC	AC214430.2	Yes	273	4/4	0/4
5	chr7	28,499,159	GCCGcGCCaGTCTATacACGTCTGGTCGATTGcCGGGTttGaA	AC194902.3	No	273	ND°	ND°

ZF domain heterodimerization sites

#	Loc	Pos*	Sequence	Region	Coding?	Event	Wild-type chromatids	Mutant chromatids
1	chr2	4,503,501	CCaAcAtCTGGGTgCCAATCATGTCGATGGTGGGGTAtGGT	AC193307.3 (IPK2)	Yes	273	4/4	0/4
2	chr1	72,608,255	CCTTCtCCgGGGTgAAGGTGAGGTCGAGCGTCGGGgACGaG	AC205071.2	Yes	273	3/3	0/3
3	chr10	49,474,665	ACcATAgaTcGGTAGaTGTAGGGTCGAGAGAaGGtCACGGT	AC197428.3	No	273	4/4	0/4
	chr10	49,652,355		AC206269.2	No			
4	chr9	42,980,450	ACCacGCCCtTCCATCGACGACGACCTACCCAcGcGCTGC	AC216802.2	No	273	ND°	ND°
5	chr10	147,570,681	GCCATgCCTGGGcACGAAGAGGTtGAGCcAGGGGTgCGGC	AC198492.2	Yes	273	4/4	0/4
	chr10	147,886,806	GCCATgCCTGGGcACGAAGAGGTtGAGCcAGGGGTgCGGC	AC199218.2	Yes	273	4/4	0/4

* www.maizesequence.org Release 2a.50 (October, 2008)
° We could not get a specific PCR product for this locus

Supplementary Table 3. Analysis of ZFN 16 action at potential off-target sites.
A listing of the potential homodimerization and heterodimerization off-target sites for ZFN16 in the non-repetitive portion of the maize genome; the mismatches relative to the ZFN consensus sites are shown in lowercase and the potential binding sites are highlighted. Leaf gDNA was extracted from T0 regenerated plants and the sites were amplified by PCR followed by TOPO cloning and sequencing analysis of multiple independent clones.

ZFN15
ZF domain left homodimerization sites

#	Loc	Pos*	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
1	chr7	190,548,543	GCT TAC TGC GGT TTT GTT AAT G	CGA TAA ACC CGC CAA TTT TA
2	chr2	241,214,062	ACGCTACATGGTCAGCGAATCAGGAAG	AACGATCGATGACCGCTTCAAGG
	chr2	241,018,416		
3	chr7	49,025,336	GCG AGA TTT TTA GTT CAG TCG	TCA GCT CTT CAC TGT TTG TC
	chr7	49,150,133		
4	chr5	97,762,802	ATT AGT AGT CCG TAT TTG CTA C	TGG ATT CAA TTG CGC GGG CG
5	chr1	114,392,019		

ZF domain right homodimerization sites

#	Loc	Pos*	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
1	chr7	41,879,634	GCA CCG AGG AAG CAA GGA TG	GAT CTC CGG CTG ATA TTT AT
2	chr6	202,077,821	CAT GAG CTT TAA GAC TAA CAA C	CAA GCT CAA AGC CCA ATG CG
3	chr7	41,888,298	CAT CAC CAG CGA GCT CCA AG	CAG CTC CCT CGA AAA ATC TC
4	chr9	74,538,149	CAC GTC CTC TCT CTC CCT CAT C	GCG AGC TAA CCG TAG AAC GG
	chr9	74,303,084		
5	chr4	235,580,893	GCG AGA AAG CTG GCC CGC AG	CCA AGA TGG CGC GCA CGG AG

ZF domain heterodimerization sites

#	Loc	Pos*	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
1	chr2	4,503,501	TTGGAGGAAGCATTGCTTCACTTTG	GTCGCAATTCTCCAATTTCTTCC
2	chr10	147,570,681	AGGGACCCCTCTTTATCTACCA	GGGCAGCTGAGCTATCTGAG
	chr10	147,886,806		
3	chr2	37,321,203		
4	chr1	231,112,029	ACC GTC CGA TCT TCA ATC AGT G	ACG CCC CAA TTC AAG GTG AG
5	chr10	49,474,665	CTG CAC CAA TCG CTC GCT AG	GGG TAT TGG TGT GTC CTG GCC
	chr10	49,652,355		

ZFN16
ZF domain left homodimerization sites

#	Loc	Pos*	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
1	chr1	206,282,173	TGAGAAAGCTTTAAACATAACAACCGC	GCTACTTAGCTGTAAAAATTAACTGATGAGC
2	chr10	5,168,217	TCGTCGTCGTTTGTAAATGCGGC	GGTGTATCCCGGAGCTTTTGTGC
3	chr1	118,906,910	CAGCAGGGGAATGCAGTGGGAATC	TCAGTCTCGTCGGACAGCCCTGC
4	chr6	58,313,525	GAATCTCCGGGCGCAACAGAAATC	TCCCCAGCTATTCTAACCCGACG
	chr6	58,198,472		
	chr6	58,118,855		
5	chr7	72,155,807	CGTAGAAATCTCCGGGCGCAACAG	TCCCCAGCTATTCTAACCGACGC

ZF domain right homodimerization sites

#	Loc	Pos*	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
1	chr7	41,879,634	GCA CCG AGG AAG CAA GGA TG	GAT CTC CGG CTG ATA TTT AT
2	chr6	202,077,821	CAT GAG CTT TAA GAC TAA CAA C	CAA GCT CAA AGC CCA ATG CG
3	chr6	8,595,095	ACTTACCTGGGCCGCCAGATGGAATTC	TCTCGGCGCAATACGGCAAGAAG
	chr6	8,378,531		
	chr6	8,712,818		
	chr8	99,043,796		
	chr8	99,311,115		
	chr3	199,367,460		
	chr3	199,194,688		
4	chr7	41,888,298	CAT CAC CAG CGA GCT CCA AG	CAG CTC CCT CGA AAA ATC TC
5	chr7	28,499,159		

ZF domain heterodimerization sites

#	Loc	Pos*	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
1	chr2	4,503,501	TTGGAGGAAGCATTGCTTCACTTTG	GTCGCAATTCTCCAATTTCTTCC
2	chr1	72,608,255	ACGACCTCGATGCCGTTGAGAAAG	TCCACTTCTACCCGGCCAACTAC
3	chr10	49,474,665	CTG CAC CAA TCG CTC GCT AG	GGG TAT TGG TGT GTC CTG GCC
	chr10	49,652,355		
4	chr9	42,980,450		
5	chr10	147,570,681	AGGGACCCCTCTTTATCTACCA	GGGCAGCTGAGCTATCTGAG
	chr10	147,886,806		

* www.maizesequence.org Release 2a.50 (October, 2008)

Supplementary Table 4. Primer sequences used to genotype potential off-target sites for ZFN action.

Supplementary Table 5. Amino Acid Sequences of *Zp15* Zinc-Finger Binding Domains

ZFN #	Target Position	ZF Domain Left*	ZF Domain Right*
24	exon 2-2	AAMAERPFQCRICMRNFSRSDHLSRHIRTHTGEKPFACDICGRKFARSDNLTTHTKIHTGSQKPFQCRICMRNFSRSDDLTRHIRTHTGEKPFACDICGRKFADSSDRKKHAQRCGLR	AAMAERPFQCRICMRNFSQSGDLTRHIRTHTGEKPFACDICGRKFADRSDLSRHTKIHTGSQKPFQCRICMRNFSQSGDLTRHIRTHTGEKPFACDICGRKFARSDNLTRHAQRCGLR
25	exon 2-10	AAMAERPFQCRICMRNFSDRSHLTRHIRTHTGEKPFACDICGRKFARSDNLTTHTKIHTGSQKPFQCRICMRNFSRSDNLSTHIRTHTGEKPFACDICGRKFARSADLSRHAQRCGLR	AAMAERPFQCRICMRNFSRSDNLARHIRTHTGEKPFACDICGRKFADRSDLSRHTKIHTGSQKPFQCRICMRNFSQSGSLTRHIRTHTGEKPFACDICGRKFARSDHLTTHAQRCGLR

* DNA recognition helicies in each domain of the ZFN pair are underlined

Supplementary Table 6. ZFN engineering and testing statistics

Gene target	ZFNs generated	ZFN pairs	Pairs tested in proxy systems	Tested in maize	Active in editing at endogenous locus
IPK1	66	63	38	4	4
Zp15	41	33	17	2	2

Note 1. ZFN pairs can be formed by mix-and-matching several ZFNs that recognize the same site and for that reason the number of ZFN pairs is greater than the number of ZFNs generated / 2.

Note 2. Of all the ZFNs generated, not all bind DNA as gauged by an ELISA assay; only pairs in which both ZFNs bind DNA are tested in proxy systems.

Supplementary Discussion

Design of ZFNs to discriminate between the ZmIPK paralogs: a structural-biology based rationale.

In general, designing zinc finger proteins to robustly discriminate against a single mismatch can be challenging. In the case of *ZmIPK1* versus *ZmIPK2*, which contained several SNPs in the regions targeted (Supp. Fig. 1), we generated many potential ZFN pairs that bound at least one of the relevant mismatches (Supp. Table 6) and we were able to choose designs that we felt had the greatest chance of discriminating against the unwanted sequence.

In the case of ZFN15-L and ZFN16-L we felt the recognition helix pair RSAALAR RSDNLSE had a good chance of discriminating between the hexamer 3'-GTGGAC-5' in IPK1 and the hexamer 3'-GTAGAC-5' in IPK2. This situation seemed analogous to fingers 1 and 2 of Zif268 which use RSDELTR RSDHLTT to recognize 3'-GCGGGT-5'. In the 1.6 A Zif 268 structure (Elrod-Erickson et al., *Structure* 1996), the guanine analogous to our SNP is bound by Arg24 (at position +6 of RSDELTR) via two hydrogen bonds and the cytosine base-paired to this guanine is bound by Asp48 (at position +2 of RSDHLTT). These contacts likely explain the extremely high specificity for a guanine in this position observed in SELEX experiments with Zif268 (Wolfe et al., *J Mol Biol* 1999 and Miller et al., *J Mol Biol* 2001). Our SELEX experiment with ZFN15-L yielded 63 usable sequences; at the position of the SNP we found 60 guanines and 3 cytosines. A similar SELEX experiment with ZFN16-L yielded 39 usable sequences; at the position of the SNP we found 37 guanines and 2 cytosines. These results are consistent with a ZFN pair that can discriminate between a guanine and an adenine at this position.

For ZFN12-R, we felt that RNDDRKK had a good chance of discriminating between 3'-GCT-5' in IPK1 and 3'-TCT-5' in IPK2. Presumably the Arg at position -1 of this helix recognizes the guanine in 3'-GCT-5' in roughly the same way that Arg18, Arg46 and Arg74 of Zif268 recognize the relevant guanines in 3'-GCGGGTGCG-5'. Based on SELEX experiments with Zif268 (Wolfe et al., *J Mol Biol* 1999) this interaction should result in a strong preference for a guanine.

Theoretical Calculation of ZFN density needed to effectively target all known genes in the maize genome.

The most recent release of the maize genome (release 3a.50; Dec. 2008, http://www.maizesequence.org) indicates that the genome consists of 2,778.9 Mb of DNA, comprising 59,052 genes of average length equal to 2,605 base pairs. Our current ZFN design platform uses a large number of interchangeable 1-finger and 2-finger subunits that are mixed and matched to generate individual 4-finger, 5-finger and 6-finger ZFNs (use of ZFNs with longer recognition sequences yields, in our hands, more effective ZFNs, and, furthermore, allows greater specificity in binding and action). The total number of unique proteins we can build with our current platform is extremely large (~2x10^10), but some of these subunit-combinations will target the same DNA sequence. When we adjust the calculations to address that fact and restricting ourselves to the best-characterized modules in our archive, our current archive enables targeting of approximately 32 unique sites per kilobase of random DNA sequence. Based on the average maize gene having a coding sequence of approximately 2,600 bp, we are currently able to design ZFNs to target approximately 83 unique locations per gene. Assuming at least one of these designs per gene is effective, in theory we should be able to modify the vast majority of genes in the maize genome with the current archive.