



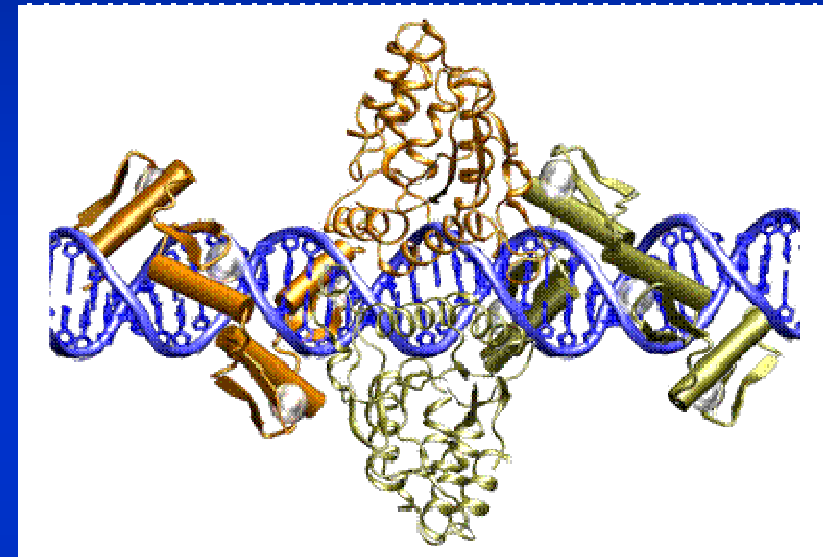
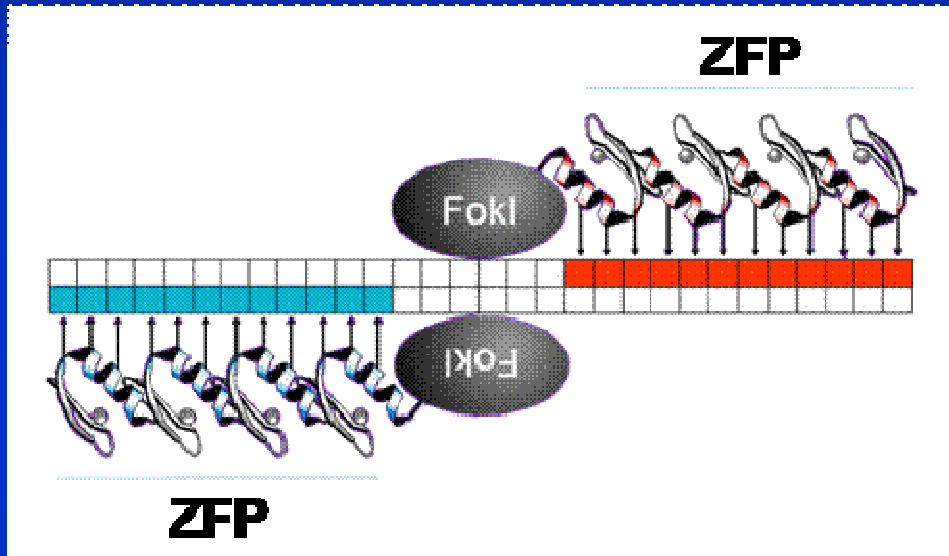
Zinc-Finger Nuclease-Mediated Gene Targeting in Maize

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Designed Zinc Finger Nucleases (ZFNs)



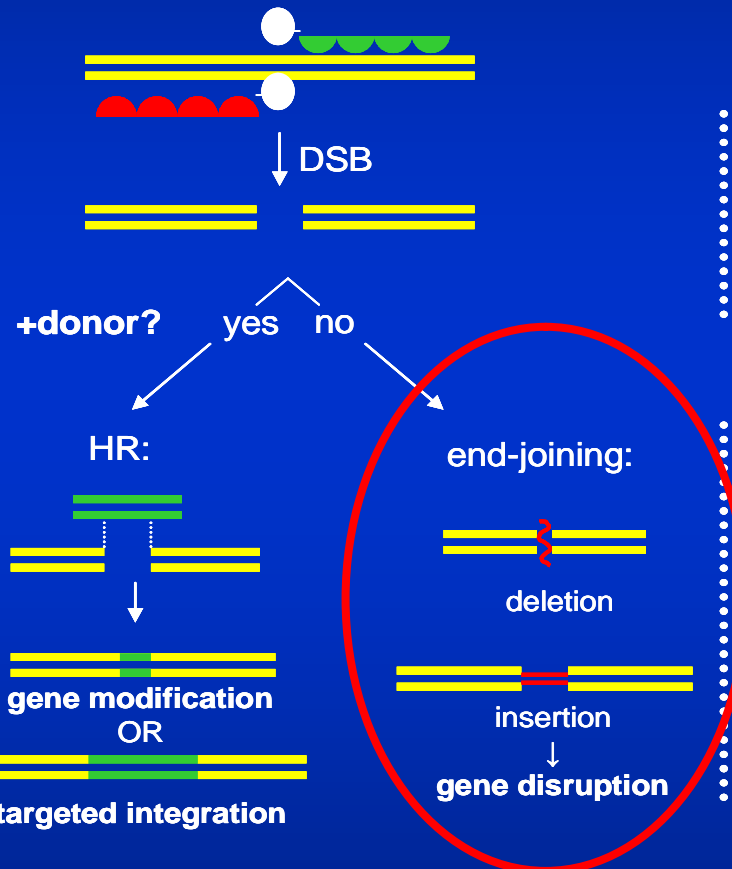
Contains two domains:

- Designed zinc finger protein (ZFP)
 - FokI Nuclease domain
- Cleaves as a dimer and creates a double-strand break
 - May be engineered to cleave virtually any sequence

Repair of Zinc Finger Nuclease-Mediated Cleavage in Living Cells



Two zinc finger proteins target a nuclease domain to a specific locus in vivo



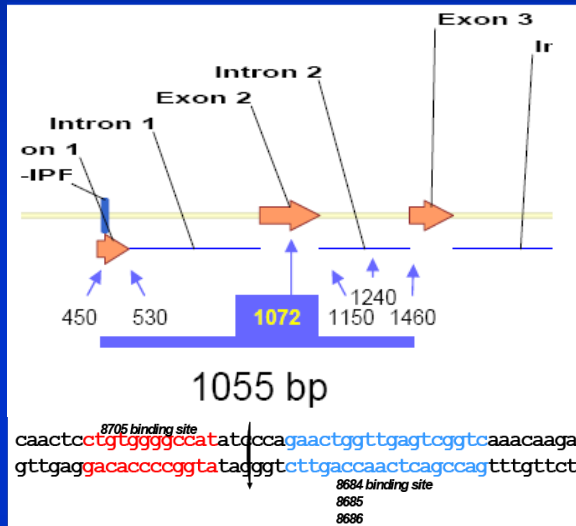
The mechanism for double-strand break repair depends on the availability of homology-containing donor

The donor may provide (insert) a minor sequence alteration or large insertion at the position of the break.

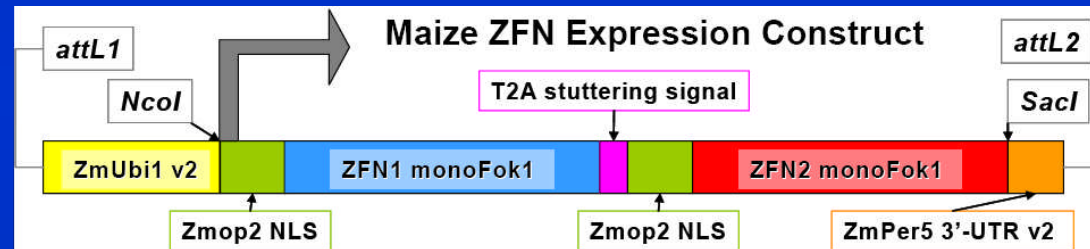
NHEJ repair generates small deletions and insertions in the endogenous gene.

•DSBs are repaired by both NHEJ and homologous recombination mechanisms in plants (Puchta H. (2005) J.Exp.Bot. 46:1-14 and others)

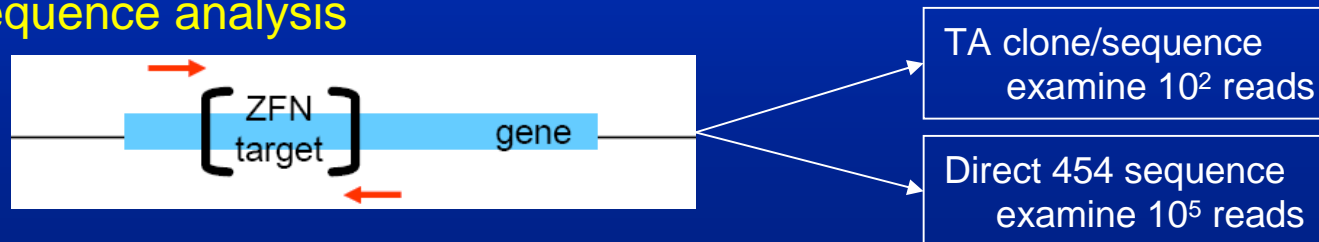
Inducing DSBs in an Endogenous Maize Gene with Designed ZFNs



- Design ZFNs to bind at different regions of our target gene (*ZmIPK1: Plant Physiol. (2007) 144: 1278-1291*)
- Design multiple heterodimeric ZFN pairs to bind at each unique sequence



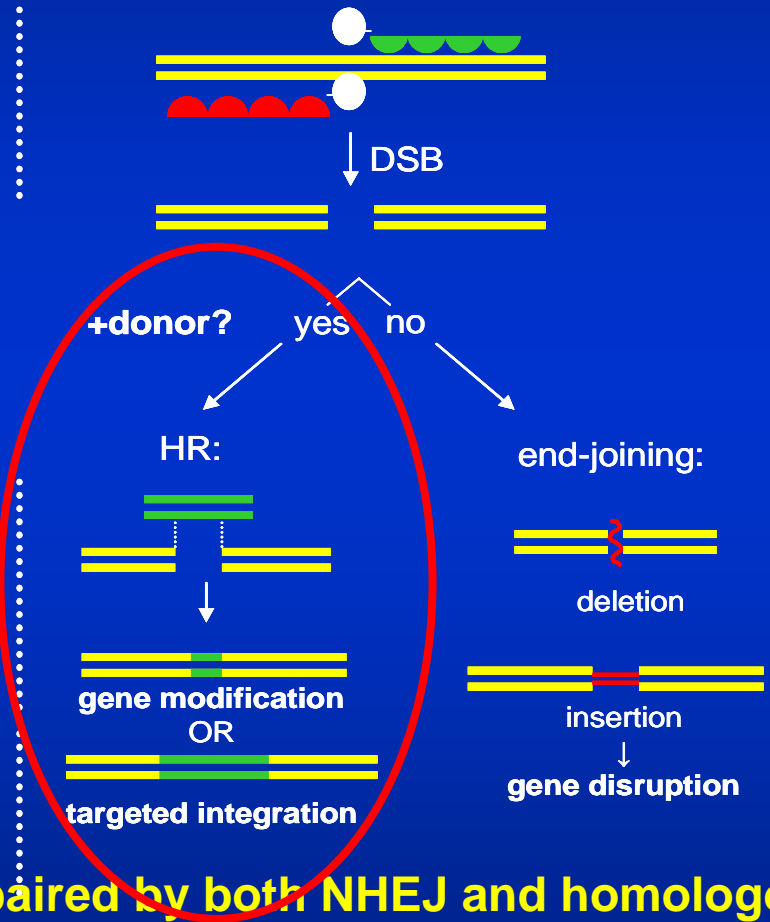
- Express each ZFN pair under control of a single constitutive promoter
- Deliver expression construct -> "Hill" embryogenic callus via Whiskers™
- Harvest callus after 2-4d transient expression, isolate gDNA & interrogate via PCR/sequence analysis



Repair of Zinc Finger Nuclease-Mediated Cleavage in Living Cells



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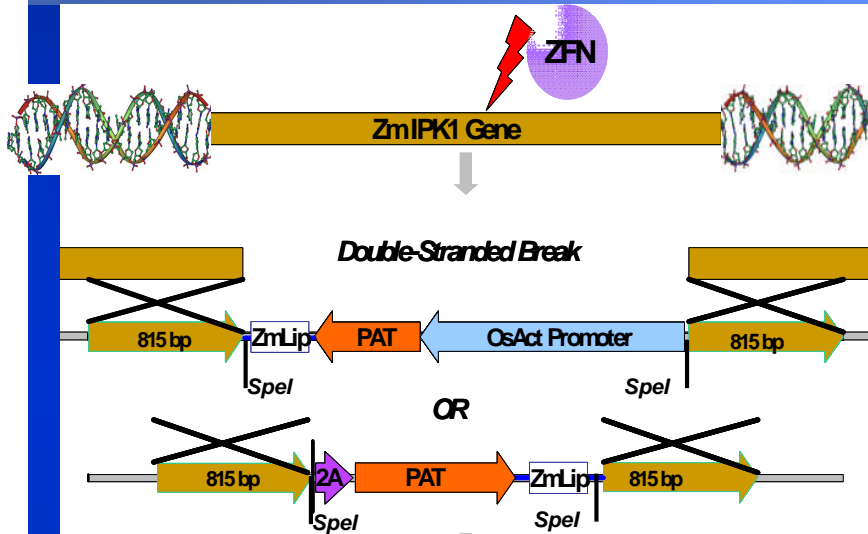
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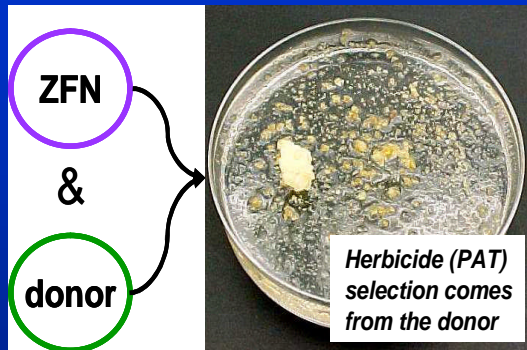
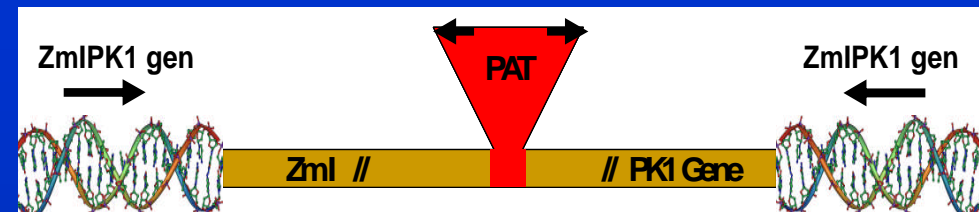
•DSBs are repaired by both NHEJ and homologous recombination mechanisms in plants (Puchta H. (2005) J.Exp.Bot. 46:1-14 and others)

Inducing Homology-Driven Repair of DSBs With a Donor DNA



Donor DNA design:

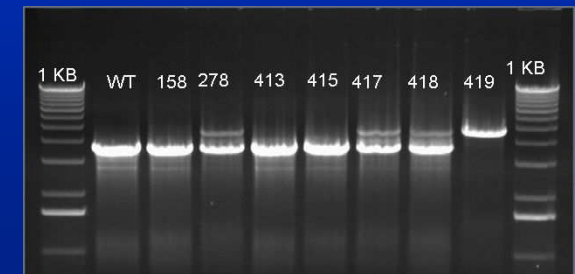
- PAT cassette (herbicide tolerance) selects both targeted and random integration of donor
- Targeting is mediated by segments of gene homology in donor molecule
- Both autonomous and non-autonomous donors



Whiskers™ mediated DNA delivery
 Embryogenic callus "Hill" selected on bialaphos
 Callus events have varying degrees of chimerism

Differentiating targeted vs random integration:

- PCR anchored in the genome & donor (in-out) or genome only (out-out) yields +/- amplification or size differences



Frequency of Targeted Integration in T0 Callus Events is Relatively High

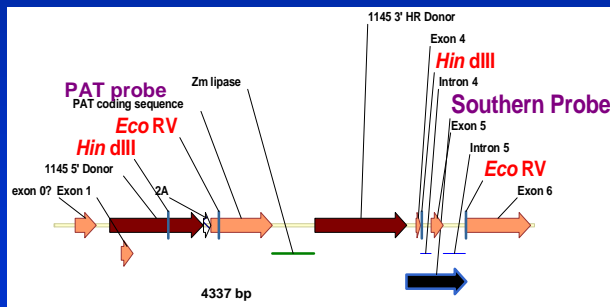


PCR/Sequence Sorting of Targeted Integration Events ZmIPK1 / PAT Cassette Insertion in Z. mays "Hill" Callus (T0)

ZFN design #	ZF-binding position	Donor Configuration	Total # HT Events Recovered	# Targeted Integration Events	Percent Targeted Integration
8	1072	autonomous (P+)	29	1	3.4
12	1072	autonomous (P+)	31	4	12.9
15	1145	autonomous (P+)	195	43	22.1
16	1145	autonomous (P+)	216	46	21.3
12	1072	combo ZFN / (P+)	39	6	15.4
n/a	n/a	autonomous (P+)	25	0	0.0
			535	100	sum
8	1072	non-autonomous (P-)	1	1	100.0
12	1072	non-autonomous (P-)	5	3	60.0
15	1145	non-autonomous (P-)	16	11	68.8
16	1145	non-autonomous (P-)	8	4	50.0
12	1072	combo ZFN / (P-)	30	5	16.7
			60	24	sum

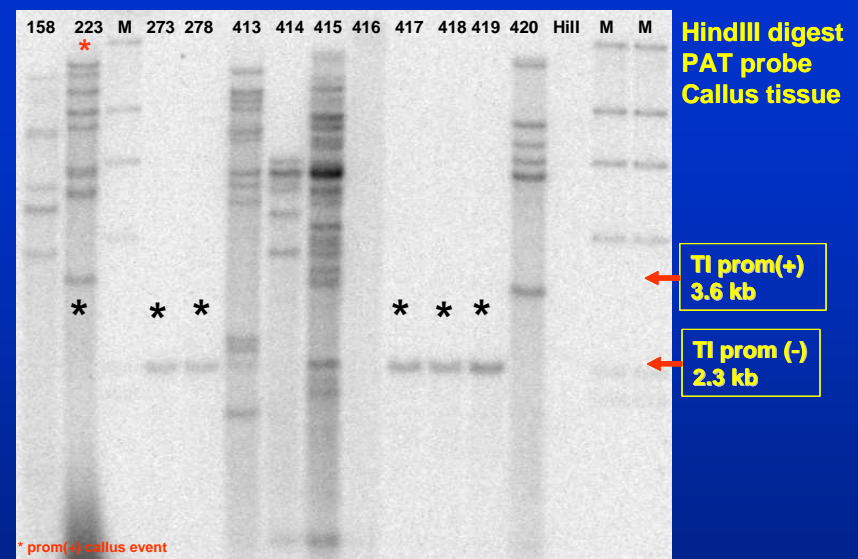
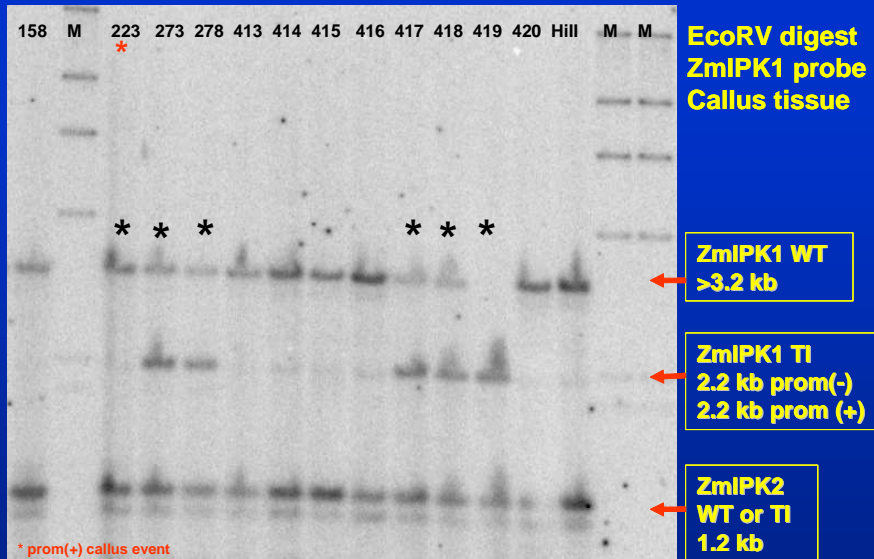
- This assay does not tell us if PAT or ZFN expression cassettes are *also* present elsewhere in the genome (random integration).

Genomic Southernblots Confirm Targeted Integration in a Subset of Callus Events

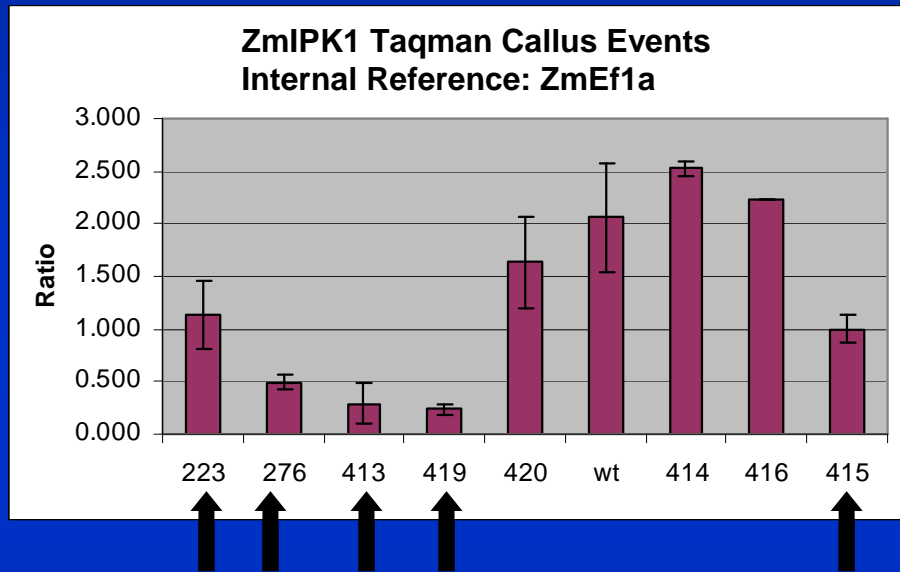


- *ZmIPK1* probe detects both WT and TI-mutated forms of the gene
- PAT probe detects insertion @ *ZmIPK1* and elsewhere in the genome

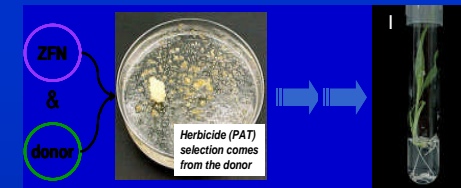
		Southern Blot Predicted Band Size (kb)			
		<i>ZmIPK1</i>		<i>ZmIPK2</i>	
PROBE	gDNA	EcoRV	HindIII	EcoRV	HindIII
<i>ZmIPK1</i>	WT	>3.2	1.3	1.2	2.3
	TI (P+)	2.2	3.6	1.2	2.3
	TI (P-)	2.2	2.3	1.2	2.3
PAT	WT	n/a	n/a		
	TI insertion	2.2	2.3		
	random insertion	?	?		



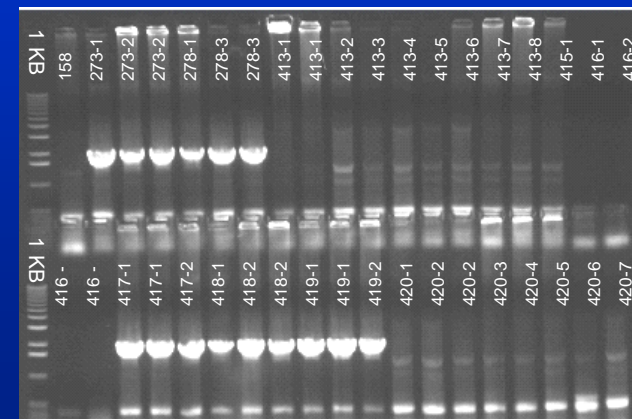
TI Disrupts *ZmIPK1* Expression & Is Genetically Preserved in Regenerated Plants



Targeted integration events display reduced transcription of *ZmIPK1* in callus relative to WT or non-TI transgenic controls



- Fertile Plants are Self-Pollinated or Out-crossed in the Greenhouse
- T1/F1 Progeny Analysis is Underway



3'- In/Out PCR
T0 Regenerated Plantlets

Zinc-Finger Nuclease-Mediated Gene Targeting in Maize



We have recapitulated these maize studies:

- @ *ZmIPK1* using a different donor & additional ZFN designs.
- @ another distinct maize locus using 2 different donors.
- Results are consistent.

Summary:

- Designed ZFNs cleave with sequence specificity.
- Homology-driven repair can be mediated at high frequencies.
- Targeted integration is precise and stable in callus tissue.
- Targeted integration is retained during plant regeneration.

Next Steps:

- Assess meiotic stability in T1 and T2 plants
- Assess segregation behavior of T1 locus