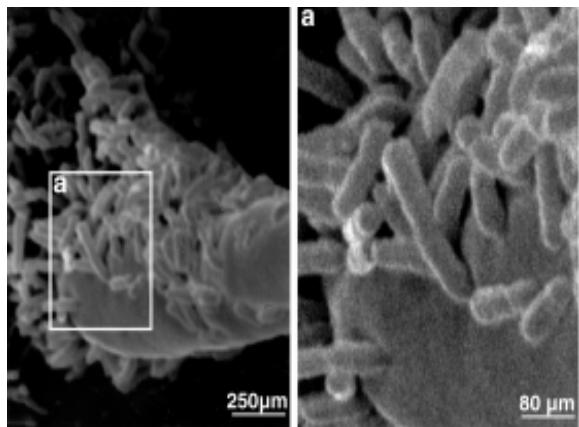


**Creating tools for the control of transgene integration
and for
genome editing in plant species**

*Department of Molecular, Cellular and Developmental Biology, University
of Michigan, Ann Arbor, MI*

These days, virtually every plant species can be transformed...



... using various biological and physical means...



But ...

several technical and biological barriers do not (in most cases) allow controlling the outcome of a transformation event ...

What we got:

- random integration
- integration of plasmid DNA, scrambled DNA, genetic ‘contamination’

What we want:

- ‘clean’ integration
- gene targeting (replacement, insertion, deletion)
- gene stacking

Novel tools are needed for controlled DNA integration and genome editing

Double strand breaks can act as ‘traps’ for T-DNA integration (via NHEJ)

“Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells.”

(Salomon 1998 EMBO J)

“Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration.”

(Chilton 2003 Plant Physiol)

“Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates.”

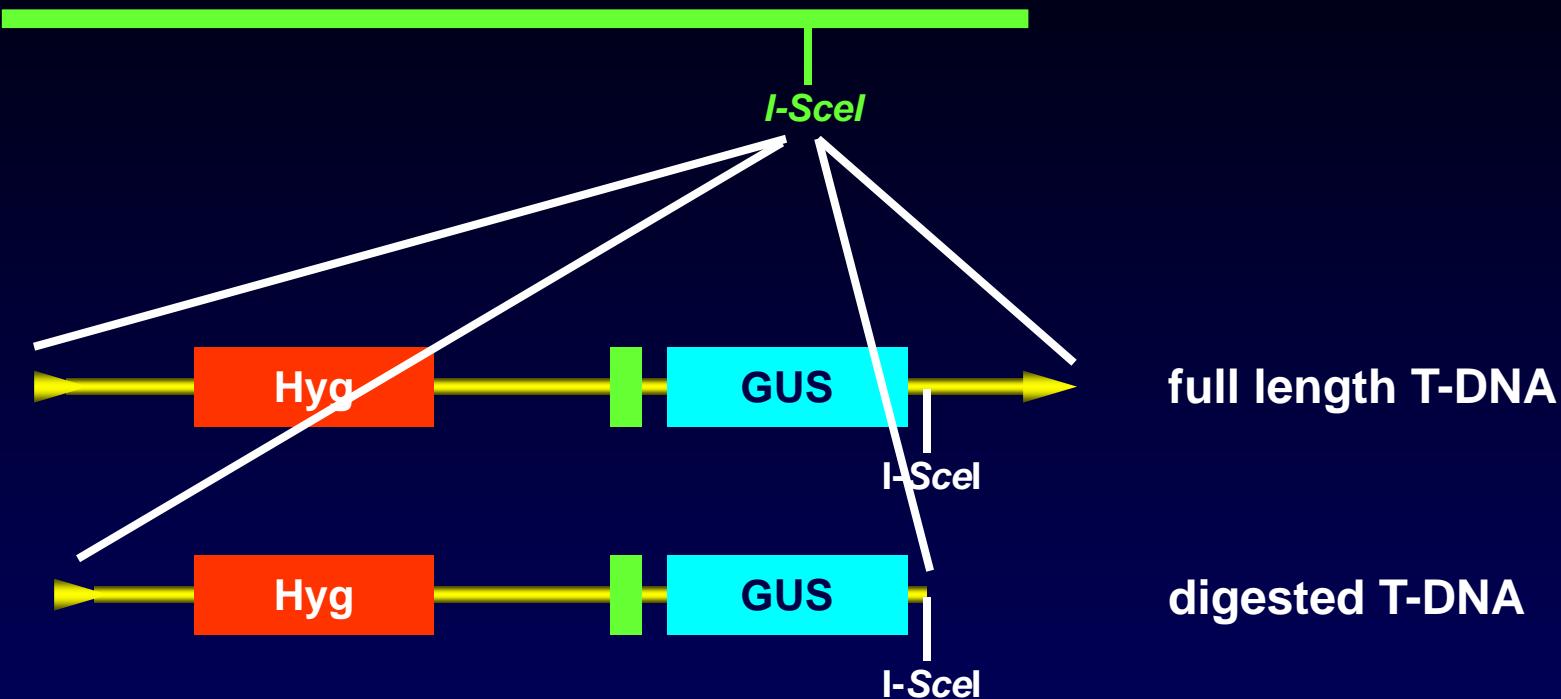
(Tzfira 2003 Plant Physiol)

Altering the plant’s DNA repair machinery leads to GT via HR

“High-frequency gene targeting in *Arabidopsis* plants expressing the yeast RAD54 gene.”

(Shaked, 2005, PNAS)

T-DNA molecules integrate as ds molecules into DSBs



Plant DNA

5' tttggagaggacacgctcgacggtacctATTACCTGTTATCCCTA~~ggatccgtcgaagta~~
aacaccttcctgtgcagctgccatggaa~~TAATGGGACAATAGGGAT~~ccttaggcagcttcat

T-DNA

5' tggcaggatataatttg...//...aattc~~ATTACCTGTTATCCCTA~~atgttacgtcctgta

Junction 332

5' tttggagaggacacgctcgacggtacctATTACCTGTTATCCCTA~~atgttacgtcctgta~~

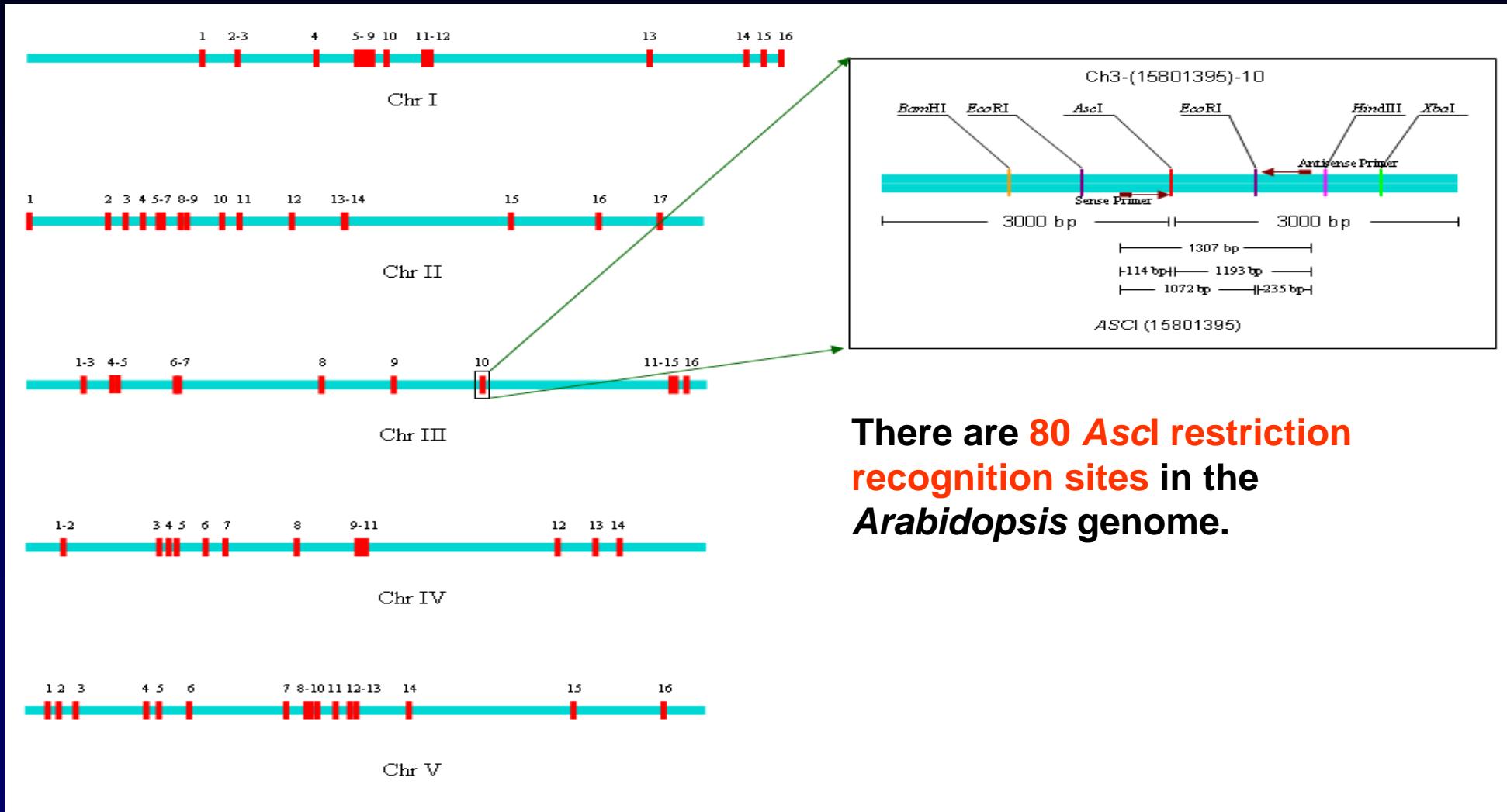
Junction 410

5' tttggagaggacacgctcgacggtacctATTACCTGTTATCCCTA~~atgttacgtcctgta~~

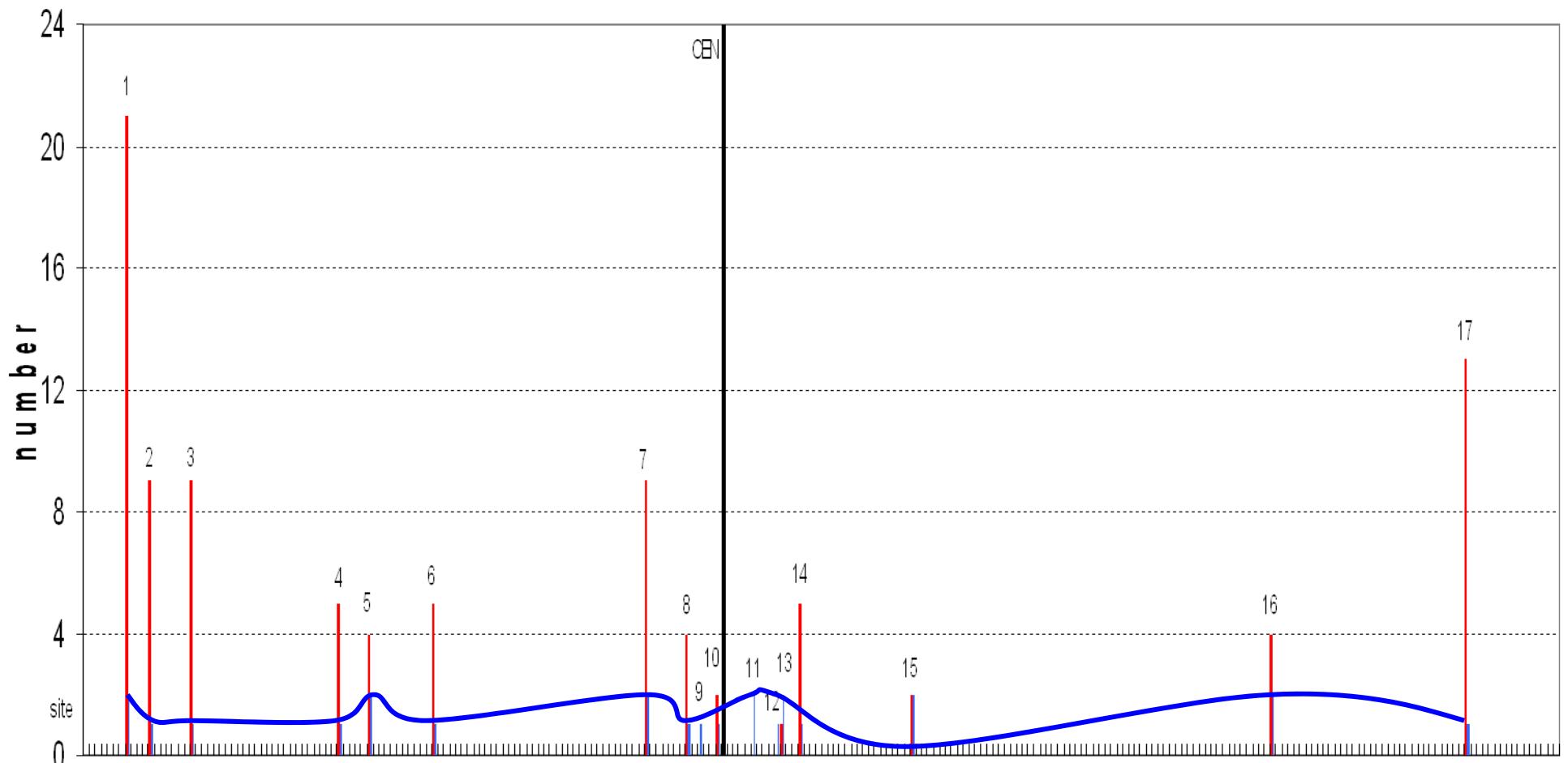
Junction 522

5' tttggagaggacacgctcgacggtacctATTACCTGTTATCCCTA~~atgttacgtcctgta~~

T-DNA insertion to DSBs is not limited to a single site



Ch 5, 3kb



Infection of *Arabidopsis* plants with *Ascl* expressing T-DNA resulted in integration into **all *Ascl* recognition sites**.

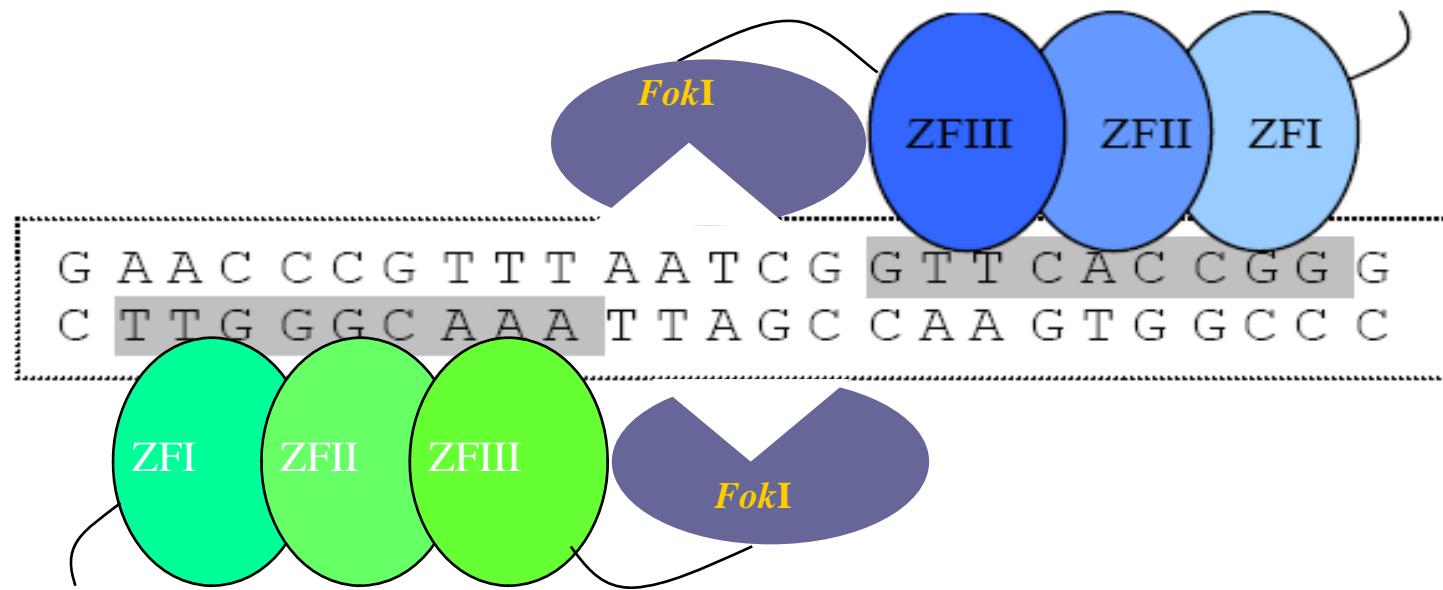
We analyzed approximately 940 insertions and found that ds T-DNA molecules can preferentially integrate into genomic DSBs.

T-DNA integration **is not really a random process**. The occurrence of DSBs is probably random.

The precise plant/T-DNA junctions indicates that T-DNA may integrate by a **simple DNA repair**.

But... engineering meganucleases for novel targets is tedious and nearly impossible.

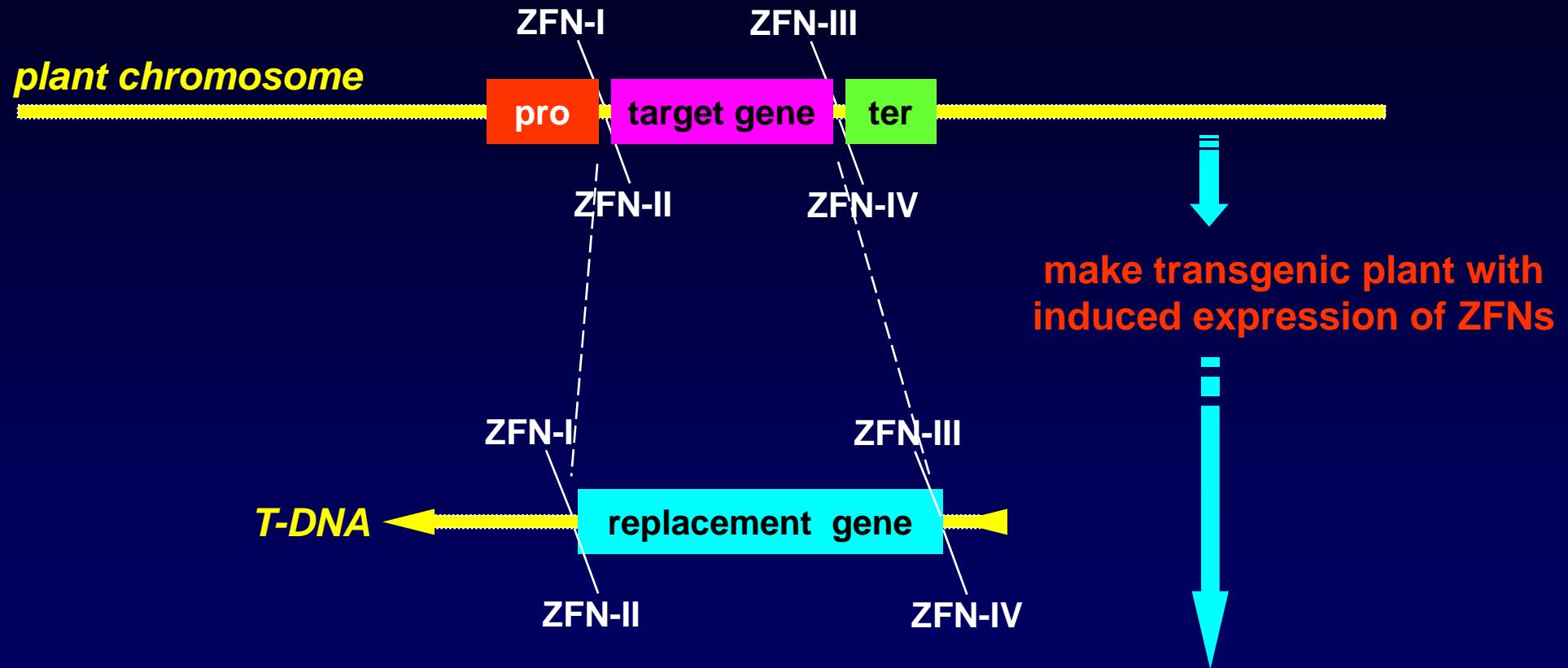
Zinc Finger Nucleases: a New Breed of Restriction Enzymes



ZFP + *FokI* Endonuclease Domain = ZFN

“general” strategy for gene replacement/deletion in plant cells:

I. Express 4 ZNF for specific gene digestion *in planta*



II. replace native gene with partial T-DNA molecule

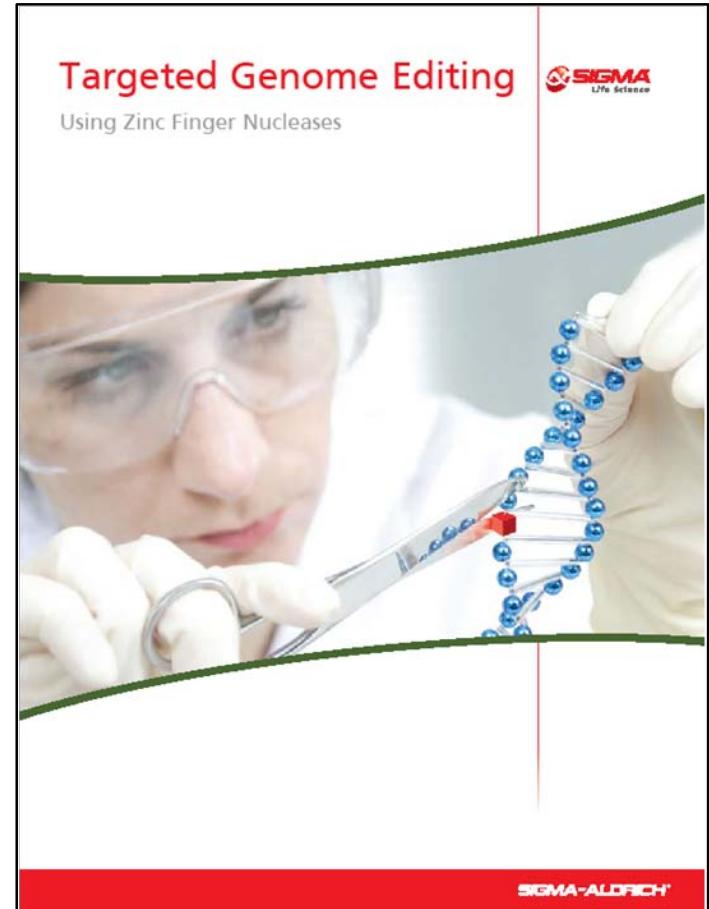
Challenges:

- I. Design 4 ZFN monomers for specific gene digestion *in planta*

From Sigma's web site...

"Coming in the Fall of 2008 :

Sigma is pleased to announce our strategic relationship with Sangamo Biosciences, a pioneer in the development of Zinc Finger Nuclease (ZFN) technology, to become the exclusive supplier of Zinc Finger Nuclease-based reagents and services to the research community."



Great news for animal science...

Validation and expression tools are directed toward animal and human cell line applications

ZFN-Mediated Targeted Genome Editing
Rapid Generation of Knock-out and Knock-in Cell Lines

Benefits:

- Rapid and permanent disruption of, or insertion into, any genomic loci
- Works in a variety of mammalian somatic cell types
- Highly specific
- One site single or bi-allelic edits
- Knockout or knock-in cell lines in as little as 2 months
- Edits occur in 5–20% of clone population
- No antibiotic selection required for screening

Applications inc:

- Target ID/validation
- Creation of gene knockouts in multiple cell lines
- Complete knockout of genes not amenable to RNAi
- Cell-based screens
- Creation of knock-in cell lines with fusion tags or promoters inserted into endogenous genes
- Biopharmaceutical production
- Creation of higher producing cell lines

ZFN-Mediated Targeted Genome Editing

Figure 2: ZFN-mediated genome editing takes place in the nucleus when a ZFN targeting the user's gene of interest is delivered into a permissive cell by transfection or electroporation. The ZFN cleaves the DNA at the target site, creating a double-stranded break. The cell then uses its own repair mechanisms to rejoin the broken ends, which can result in a targeted mutation or recombination.

For more information, email clinical@sigma-aldrich.com | ORDER: 800-323-9510 | TECHNICAL SERVICE: 800-513-9522

Targeted Genome Editing Using Engineered Zinc Finger Nucleases

Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks in user-specified locations. Zinc finger nucleases are important for oligo-specific megaredaction in that they are part of the natural DNA-repair processes, namely homologous recombination and nonhomologous end joining (NHEJ). Using Sigma's well-established and robust process, ZFNs are harnessed to generate precisely targeted genomic edits in cell lines with targeted gene deletions, insertions, or modifications.

Zinc Finger Nucleases: Highly-Specific Genomic Scissors

Figure 1: Each Zinc Finger Nucleus (ZFN) consists of two functional domains, and takes a total of about 10 weeks. a.) A DNA-binding domain is a chain of two fingers, each recognizing a unique hexamer sequence of DNA. b.) Two ZFNs are chosen to target different sites in the same gene with a combined specificity of c. 24 bp. a.) A DNA-cleaving domain comprised of the nucleic acid domain of the ZFN and DNA-cleaving domain are fused together; a highly-specific pair of ZFNs are created.

Phase 1: Generation of Target-Specific ZFN

a) In-Silico Design of ZFN Candidates (1 day)
b) ZFN Candidates Assembled in Lab (2-3 weeks)

Deliverable: 1 Validated ZFN
Phase 1 Timeline: 5-6 weeks

Figure 2: The process of creating a highly-specific ZFN consists of three steps, and takes a total of about 7-8 weeks. a.) Based on the target sequence, a member of the ZFN team will use software to design up to 8 ZFN candidate molecules. The algorithm, continuously improved by Sigma, will predict the best ZFN candidates based on their ability to bind to the target sequence and their potential to result in the highest cleavage activity. b.) ZFN candidates designed by the algorithm will be assembled in the lab. For each ZFN assembled, two-finger modules from the Sigma ZFN library are used to target the gene of interest. The DNA-binding domain of the ZFN must have DNA-cleaving specificity of >4-9 bp. c.) The final step of Phase 1 involves testing and validation of the ZFN. Sigma transfests the test ZFN into a proxy cell line (i.e. 293T for human and yeast) and waits to determine that the ZFN cleaves the genomic DNA target as intended.

Phase 2: Generation of Cell Line

a) Delivery of ZFN Pair into Cell (1 day)
b) ZFN-Mediated Editing Occurs (3 days)
c) Selection and Screening of Individual Clones (4 weeks)

Deliverable: 5-20% of Clones Have Desired Mutation
Phase 2 Timeline: 4-6 weeks

Examples of ZFN-Mediated Targeted Genome Editing

Application: ZFN-mediated Gene Knockout

Objective: To inactivate DmTR, a gene used as a negative marker for many reproduction applications

Results: Using ZFN-mediated gene deletion, new genetically distinct DmTR+ cell lines were generated. Each new cell line exhibited growth and functional properties consistent with the wild-type DmTR+ cell line. Interestingly, no gene disruption of DmTR was observed at a frequency of >1 % without the need for selection markers

Figure 3: Gel electrophoresis analysis of DmTR+ cell lines. The gel shows bands for WT, DmTR+, and DmTR- cell lines. The DmTR- cell line shows a single band at the expected size, while the DmTR+ cell line shows a doublet, indicating heterozygosity. The WT cell line shows a single band at the expected size.

Figure 4: Western blot for DmTR protein in wild-type (WT), DmTR+, and the newly generated DmTR- cell lines. The blot shows bands for DmTR and TIF1B as a loading control. The DmTR- cell line shows a single band at the expected size, while the DmTR+ cell line shows a doublet. The WT cell line shows a single band at the expected size.

Figure 5: Growth curve of DmTR+ cell lines. The graph plots relative cell density against culture period (days). The DmTR+ cell line (blue line) shows a growth rate significantly lower than the WT cell line (red line) and the DmTR- cell line (green line).

Figure 6: Western blot for DmTR protein in wild-type (WT), DmTR+, and the newly generated DmTR- cell lines. The blot shows bands for DmTR and TIF1B as a loading control. The DmTR- cell line shows a single band at the expected size, while the DmTR+ cell line shows a doublet. The WT cell line shows a single band at the expected size.

Figure 7: Growth and functional analysis of the DmTR- cell line. The graph plots relative cell density against culture period (days). The DmTR- cell line (blue line) shows a growth rate significantly lower than the WT cell line (red line) and the DmTR+ cell line (green line).

Targeted Genome Editing Using Zinc Finger Nucleases

SIGMA Life Sciences

Targeted Genome Editing
Using Zinc Finger Nucleases

Sigma's Genome by Design Service Process and Deliverables

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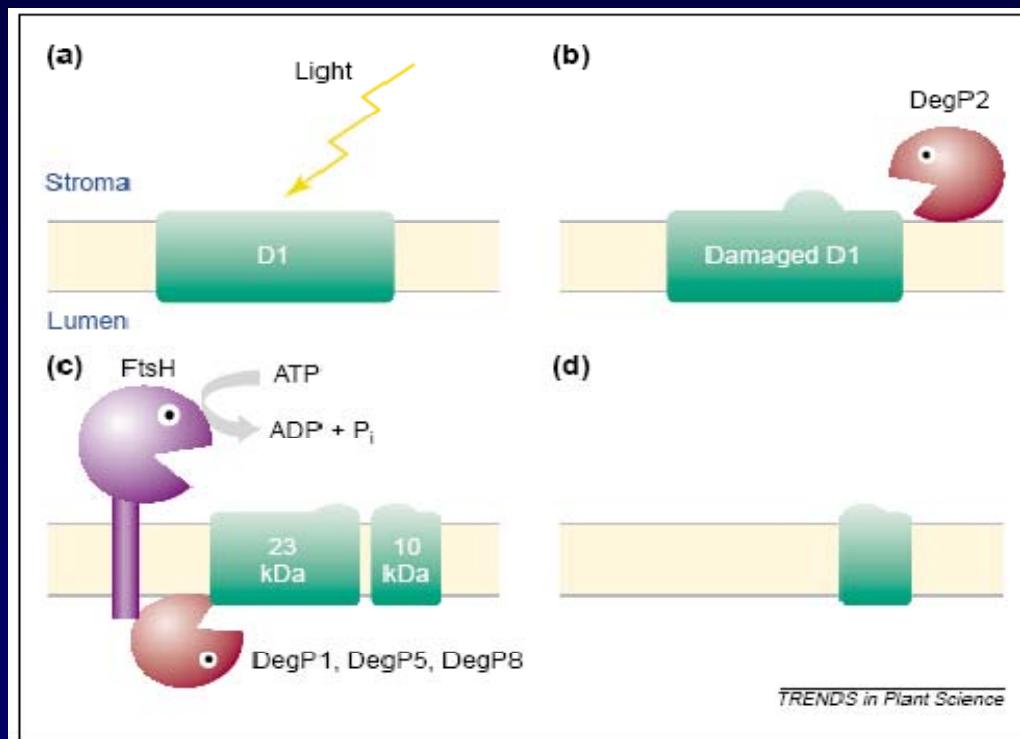
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Dedicated tools are still missing for plant research and biotechnology.

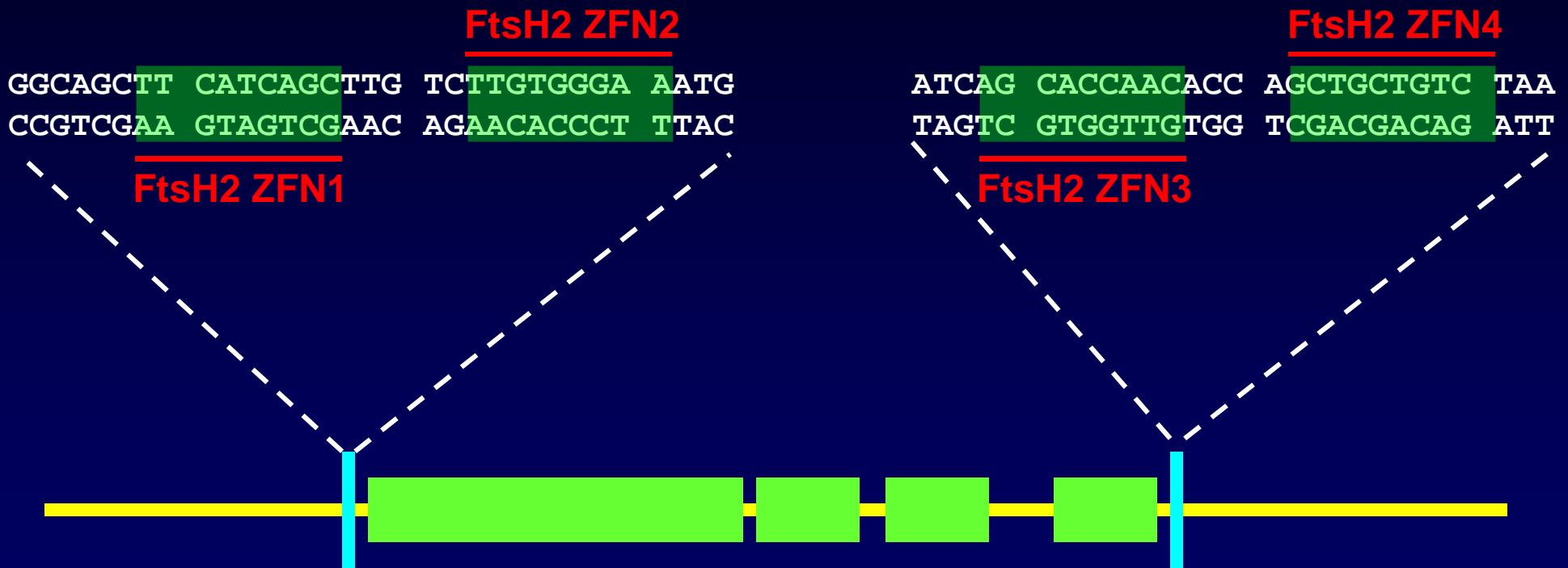
FtsH2 – At2g30950



- Encodes an ATP-dependent metalloprotease
- Involved in the turnover of the photosystem II D1 protein
- Mutants develop white leaf variegation phenotype



Target Site Selection for AtFtsH2



At2g30950 FtsH2

4373 bp

I. Design 4 ZFN monomers for specific gene digestion *in planta*

Triplet Recognition Helices

DNA triplet	Recognition helix	DNA triplet	Recognition helix
	-1123456		-1123456
AAA	QRANLRA	GAA	QSSNLVR
AAC	DSGNLRV	GAC	DPGNLVR
AAGk	RKDNLKN	GAGk	RSDNLVR
AAT	TTGNLTV	GAT	TSGNLVR
ACA	SPADLTR	GCA	QSGDLRR
ACC	DKKDLTR	GCC	DCRDLAR
ACGk	RTDTLRD	GCGk	RSDDLVR
ACT	THLDLIR	GCT	TSGELVR
AGA	QLAHLRA	GGA	QRAHLER
AGC	N/A	GGC	DPGHLVR
AGGk	RSDHLTN	GGGk	RSDKLVR
AGT	HRTTLTN	GGT	TSGHLVR
ATA	QKSSLIA	GTA	QSSSLVR
ATC	N/A	GTC	DPGALVR
ATGk	RRDELNV	GTGk	RSDELVR
ATT	HKNALQN	GTT	TSGSLVR
TAGk	REDNLHT	TGGk	R3DHLLT

TABLE ONE

Summary of the most optimal zinc finger domain sequences identified in this study for binding to 5'-CNN-3' subsites

The helices that bound to the 5'-CNN-3' target sites are listed. P, helices derived from panning; M, helices derived by site-directed mutagenesis or *de novo* design.

Target site 5' → 3'	Finger-2 helix	Source	Fig. 2 panel
CAA	QSG-N-LTE	M	l
CAC	SKK-A-LTE	M	m
CAG	RAD-N-LTE	M	n
CAT	TSG-N-LTE	M	o
CCA	TSH-S-LTE	M	p
CCC	SKK-H-LAE	P	b
CCG	RND-T-LTE	M	q
CCT	TKN-S-LTE	M	r
CGA	QSG-H-LTE	M	s
CGC	HTG-H-LLE	P	f
CGG	RSD-K-LTE	M	t
CGT	SRR-T-CRA	P	h
CTA	QNS-T-LTE	M	u
CTC	None		
CTG	RND-A-LTE	P	k
CTT	TTG-A-LTE	M	v

Birgit Dreier¹ JOURNAL OF BIOLOGICAL CHEMISTRY

tools for analyzing novel ZFN

in vitro digestion assay

pET28.SX-ZFN

ZFN

pSAT-TS

Agel

**

T-DNA repair assay

pRCS-[ZFN-X][*GUS]

ZFN

GUS

TGA

transgene repair assay

pRCS-[*GUS]

KAN

GUS

TGA

pRCS-[ZFN-X]

ZFN

whole plant DNA repair
assay

pRCS-[*hsp*.ZFN-X][*GUS]

KAN

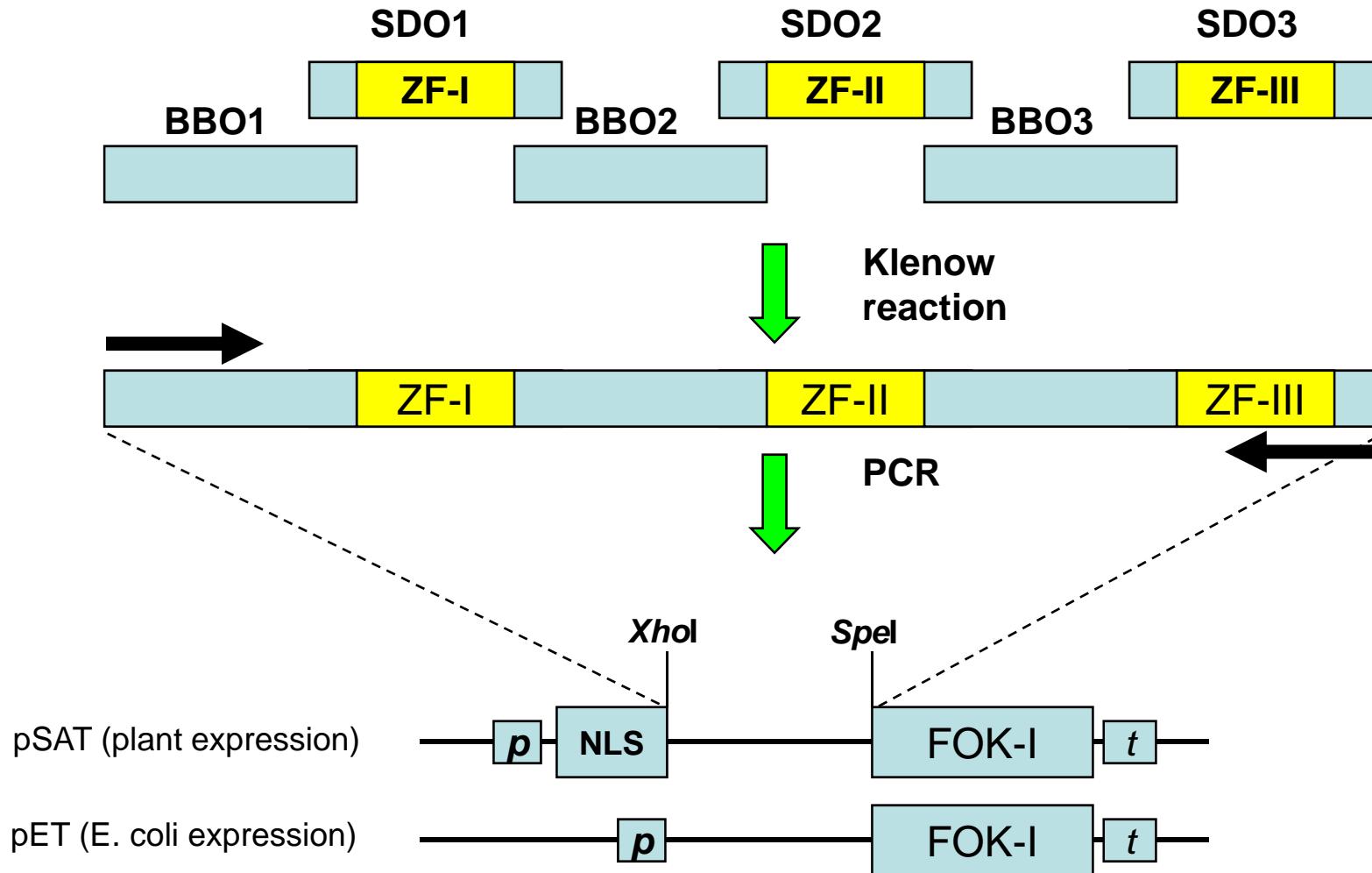
hsp

ZFN

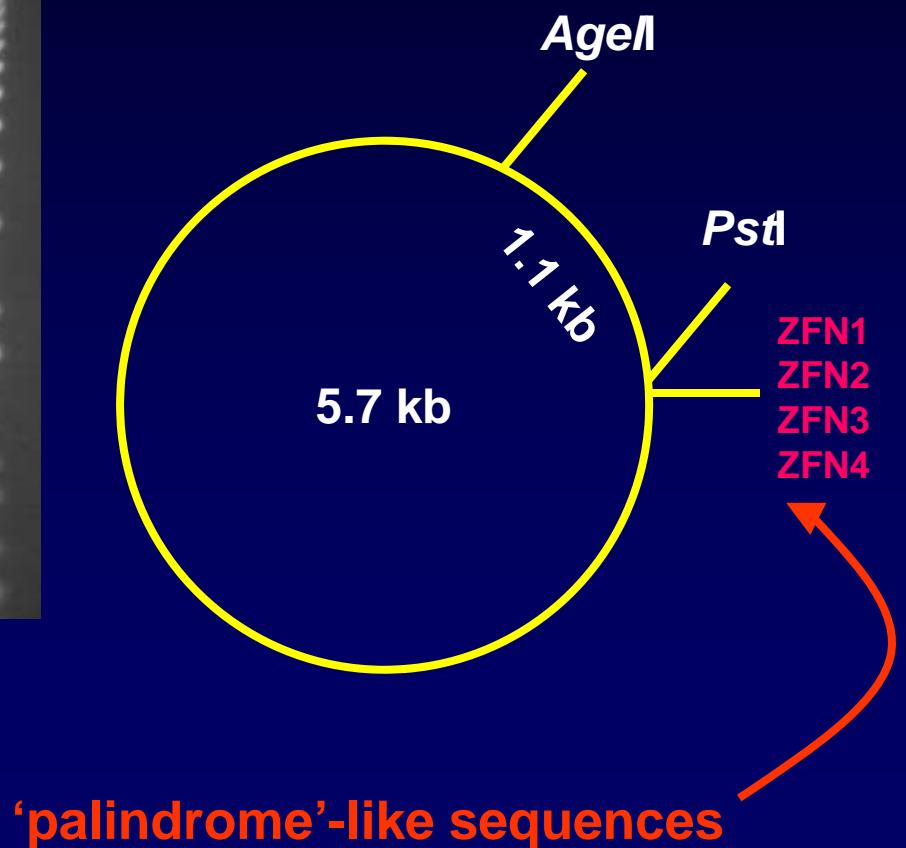
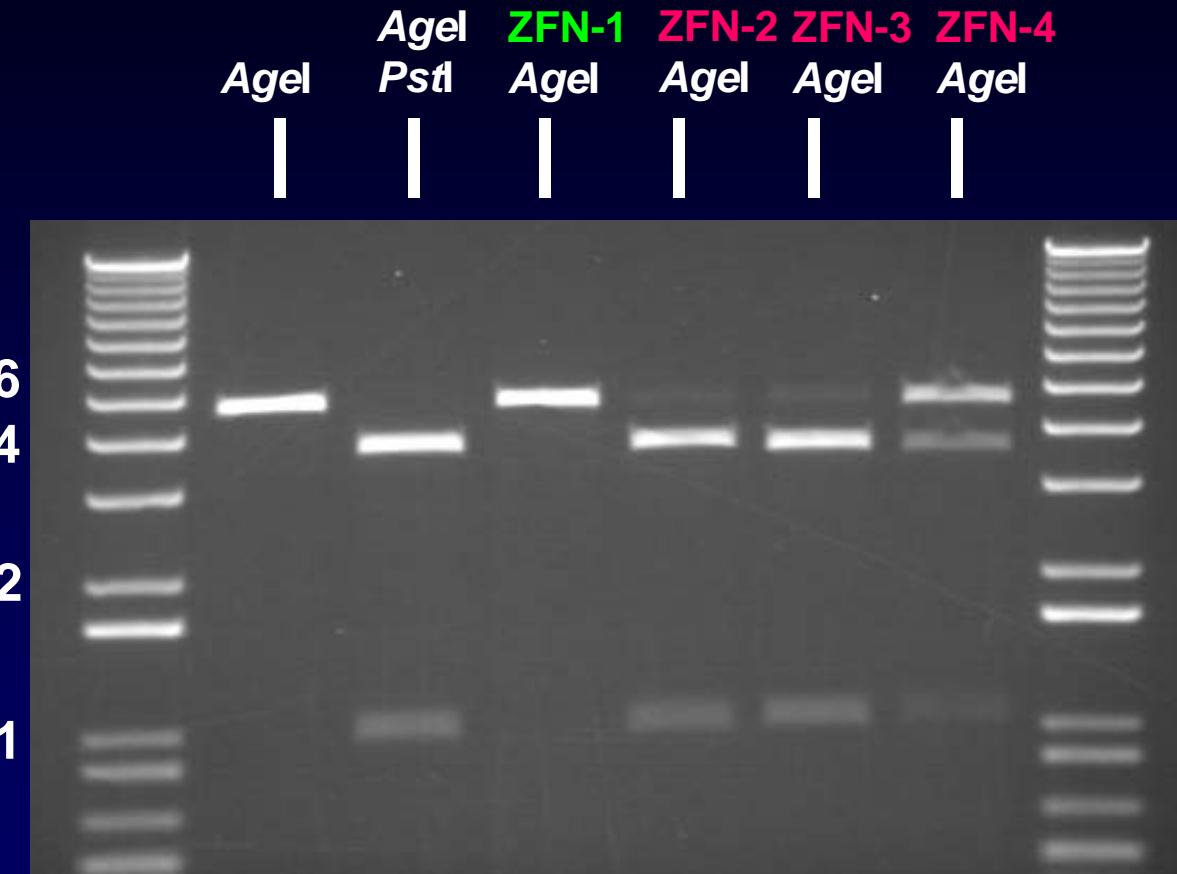
GUS

TGA

Fast and simple assembly of ZFs backbone



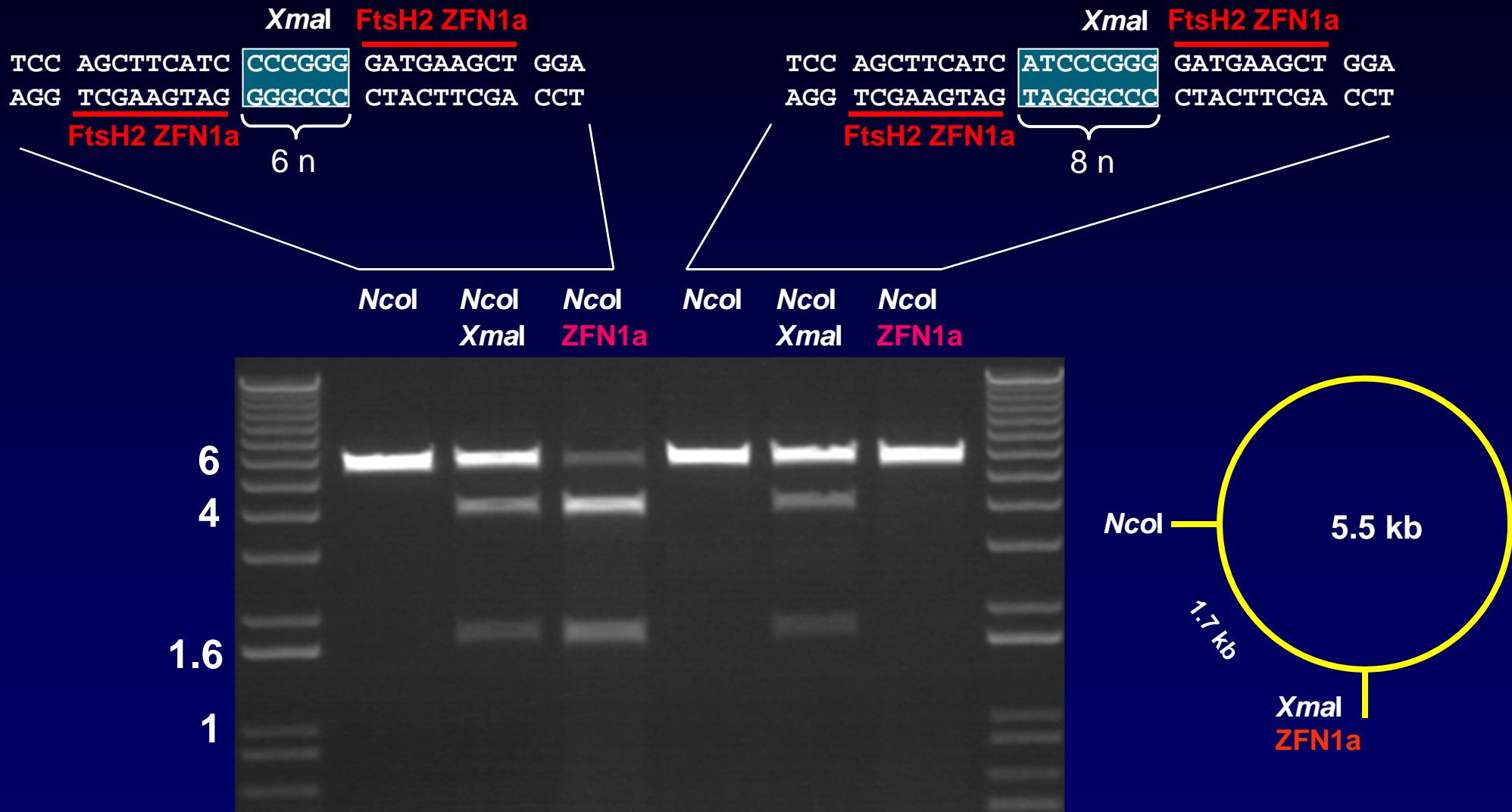
Activity of FtsH2 ZFNs



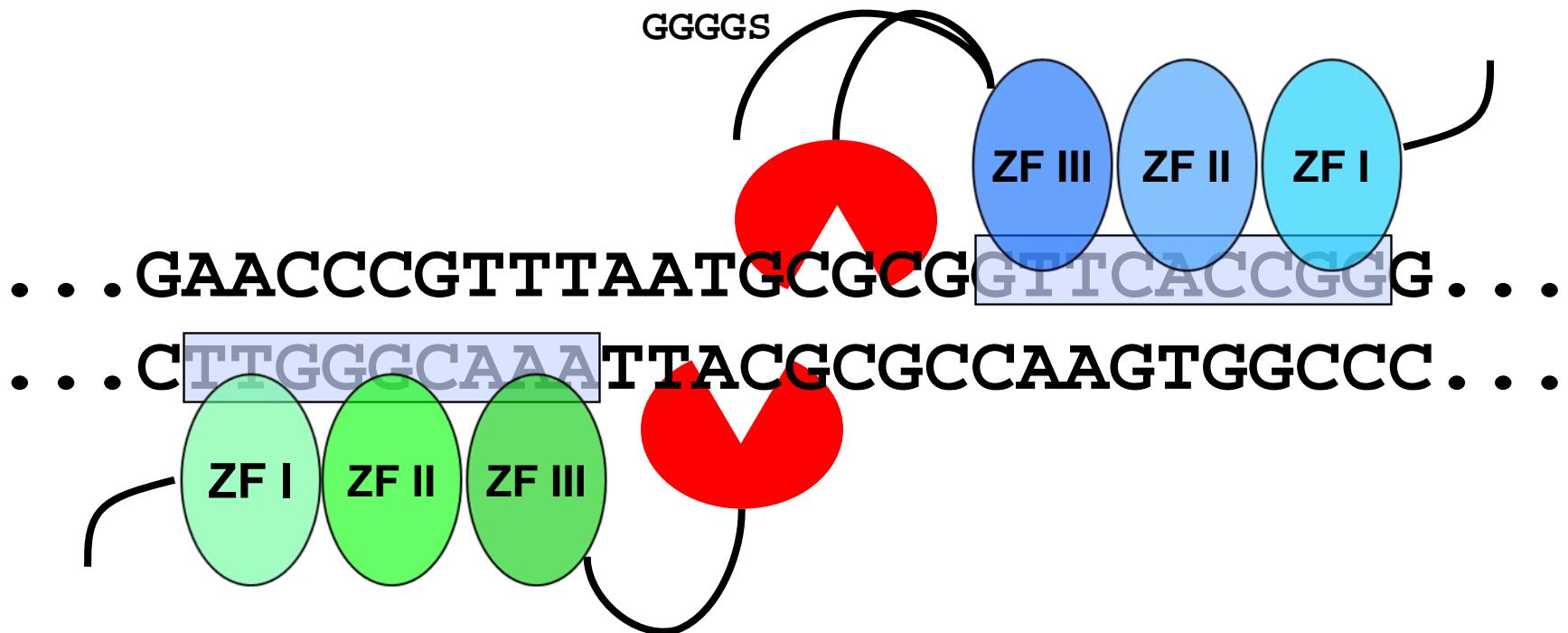
Target Site Selection for AtFtsH2



FtsH2 ZFN1a digests a target site with 6 but not 8 bp spacer



FtsH2 ZFN1a with (Gly₄Ser)₂ Linker



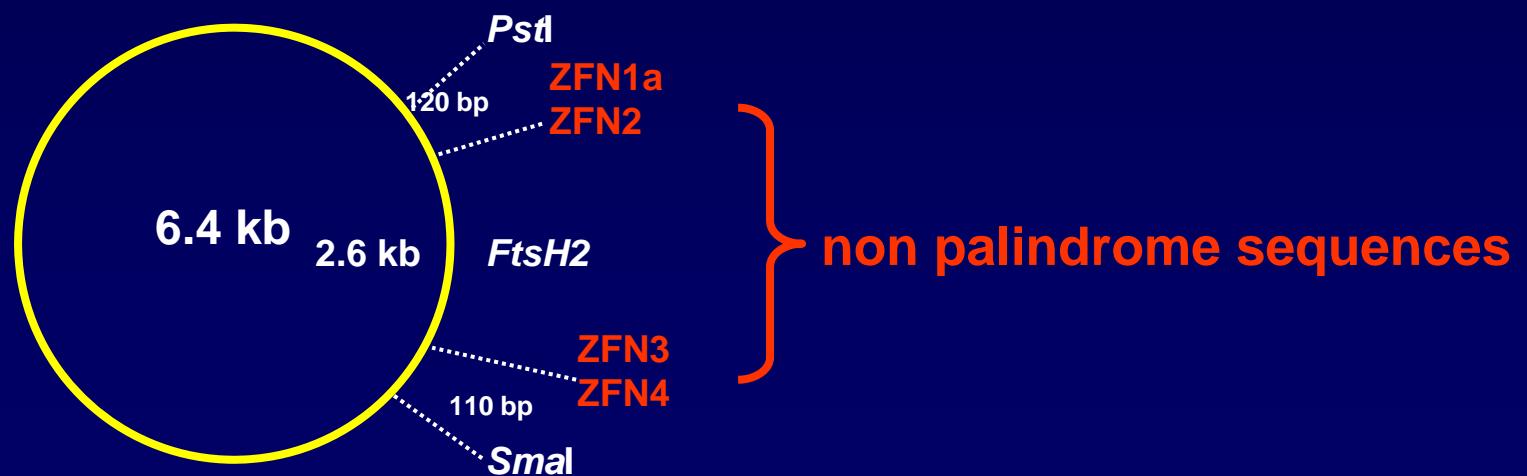
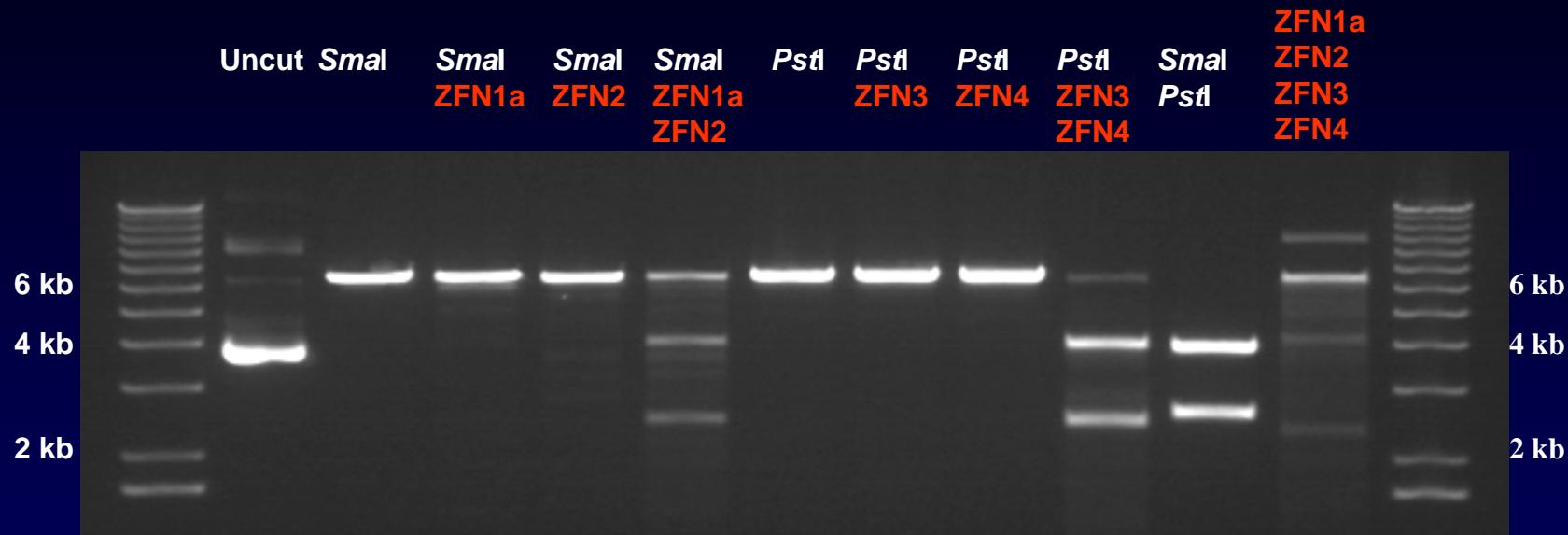
...GAACCCGTTAA

...CTTGGGC**AAA**TTAC

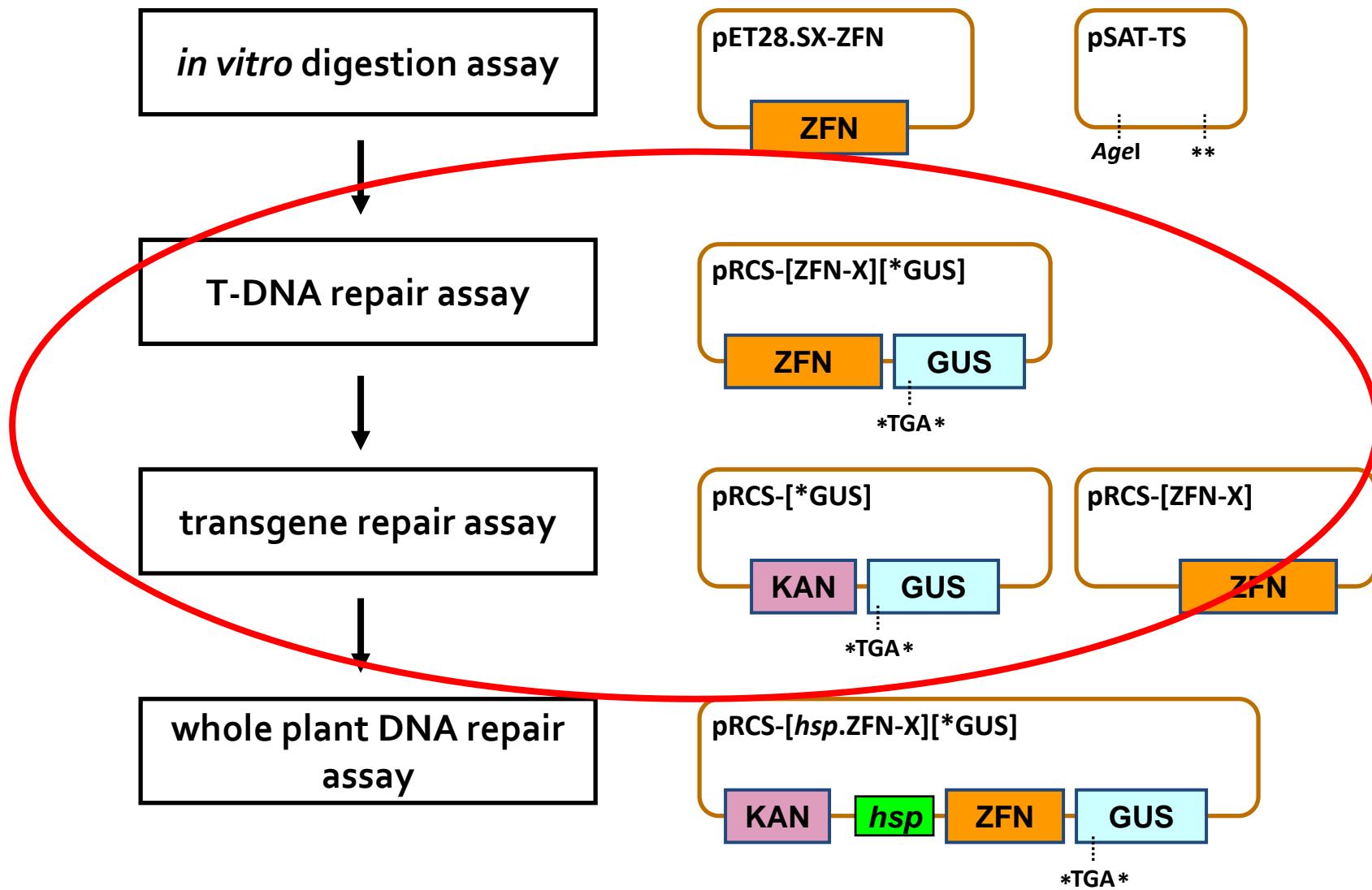
TGCGCG**GTTCAACCGG**G...

CGGCCAAGTGGCCC...

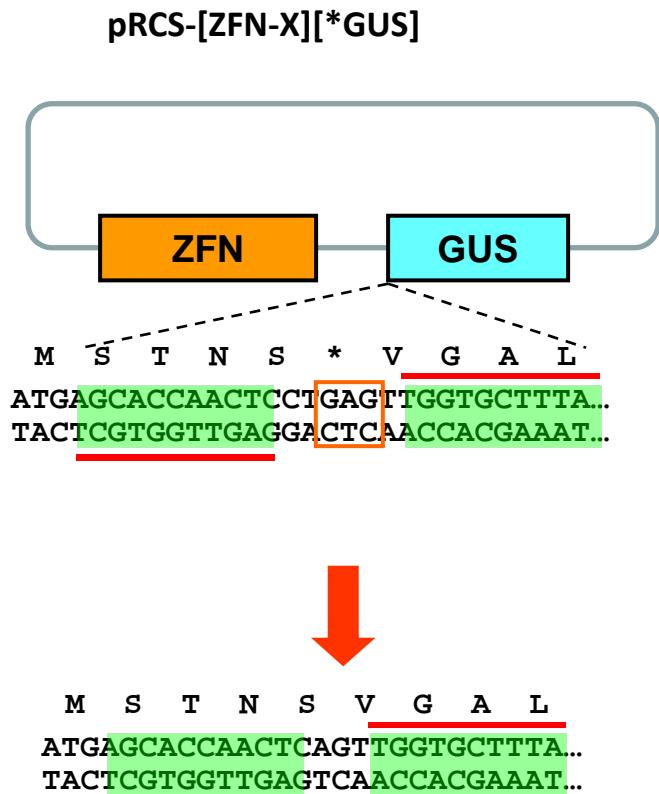
ZFN Pairs Digests Genomic *FtsH2* Sequence



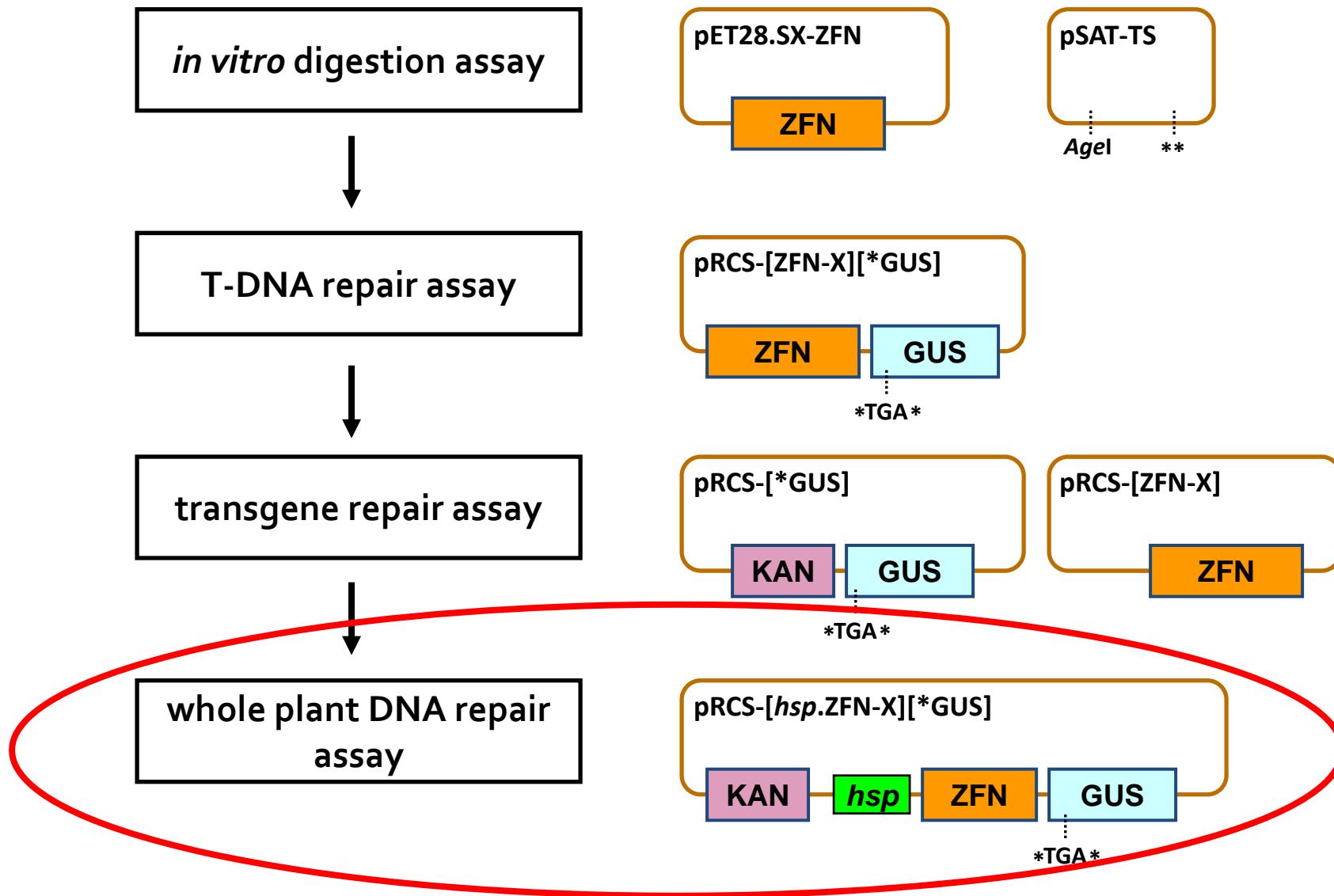
tools for analyzing of newly assembled ZFN



T-DNA repair assay

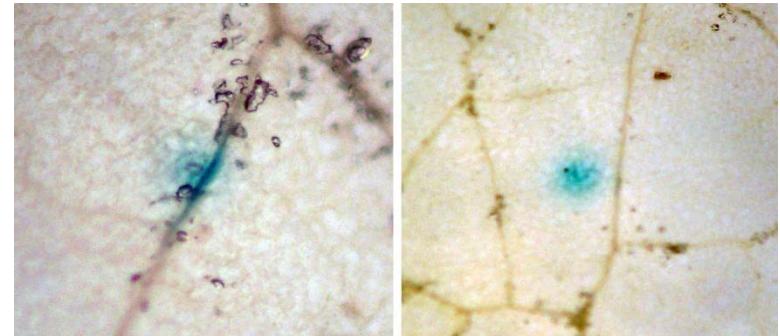
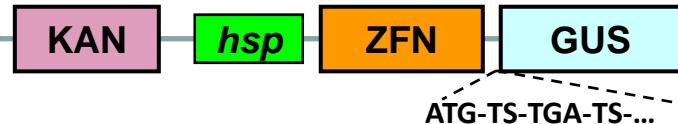


tools for analyzing of newly assembled ZFN



whole plant DNA repair assay

pRCS-[*hsp.ZFN-X*][*GUS]



Ddel

ATGTTCTTCCCCCTCCTGAGGGGAAAGAATT
ATGTTCTTCCCCCTCCCGAGGGGAAGAATT
ATGTTCTTCCCCCTCCAGAGGGGAAGAATT
ATGTTCTTCCCCCTCCTGGGGGGAAGAATT
ATGTTCTTCCCCCTCTTGAGGGGAAGAATT
ATGTTCTTCCCCCTCCTGAGGGGAAGAATT
ATGTTCTTCCCCCTCCTG--GGGAAGAATT
ATGTTCTTCCC-----GAGGGGAAGAATT
ACGAAC.....^{-82 bp}.....TGTAGA

Challenges:

- I. Design 4 ZFN monomers for specific gene digestion *in planta***
- II. Validate ZFN monomers activity in model plants**
- III. Validate ZFN monomers activity in target plants**
- IV. Deliver 4 ZFN to target plants**
- V. Combine ZFN expression with second transformation cycle**
- VI. Develop methods for regenerating and selecting mutant lines**

III. Validate ZFN monomers activity in target plants

Research conducted by Sangamo and several universities during the past 5-6 years resulted in significant progress in validating and using ZFN in various target species:

human cell lines

(gene correction, HIV-1 resistance, degradation of mutated human mitochondrial DNA, targeted gene edition)

Zebra fish

(gene inactivation)

Drosophila

(targeting endogenous gene)

C. elegans

(targeting endogenous gene)

Chinese hamster

(targeting artificial gene)

III. Validate ZFN monomers activity in target plants

Similar effort should be made in plants if we wish to harness the power of ZFN for genome modification in crop plants, forest trees and other scientifically and biotechnologically important species.

Arabidopsis – mutagenesis of artificial sequence

Tobacco – inducing homologous recombination of transgene

canola – targeting of undisclosed native gene (DOW)

3-4 years before significant progress will be made with crop plants....

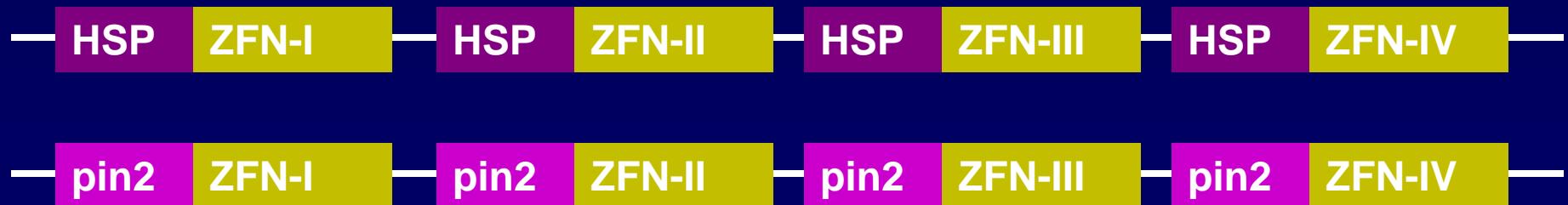
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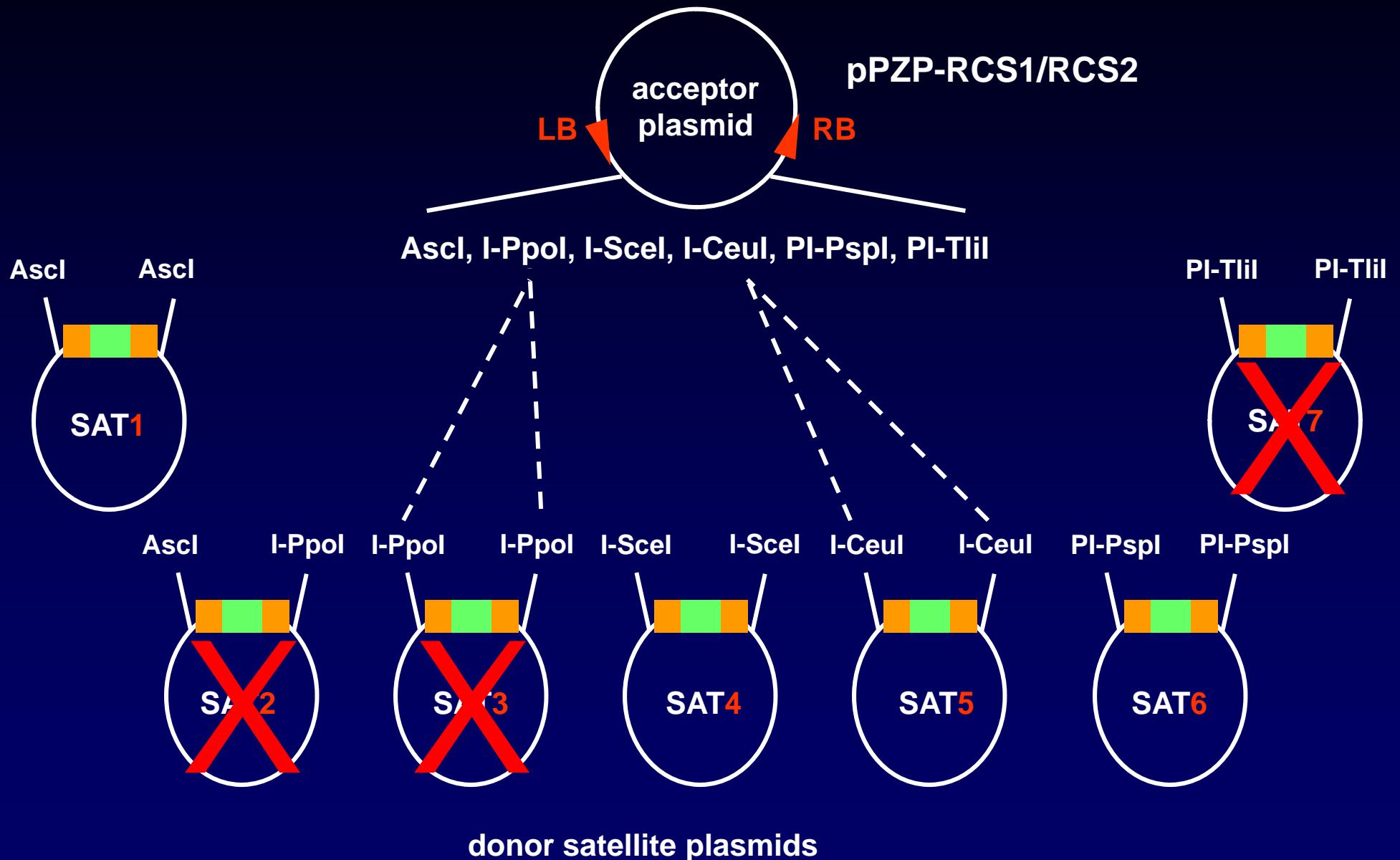
Multi gene delivery to plant cells is still challenging....

Expression of 4 ZFNs under Heat Shock (HS) or wound inducible (pin2) Promoters

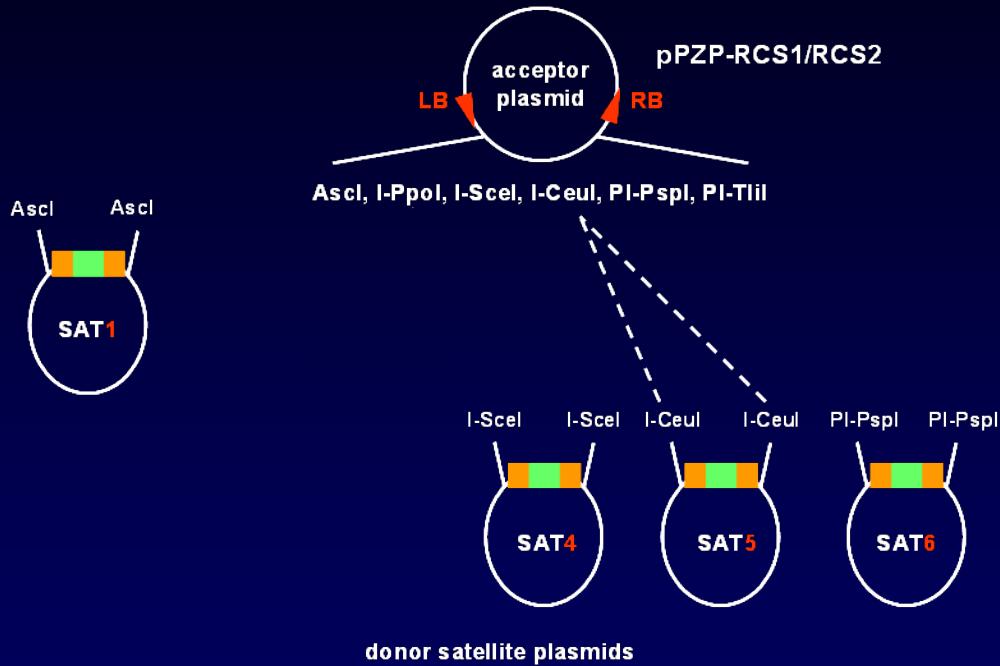
- Introduce ZFNs under HSP and/or pin2
- Expose seedlings to 42°C or wounding
- Combined with *Agrobacterium* infection to generate insertions/substitutions events



Several expression cassettes can be mounted onto a single plasmid

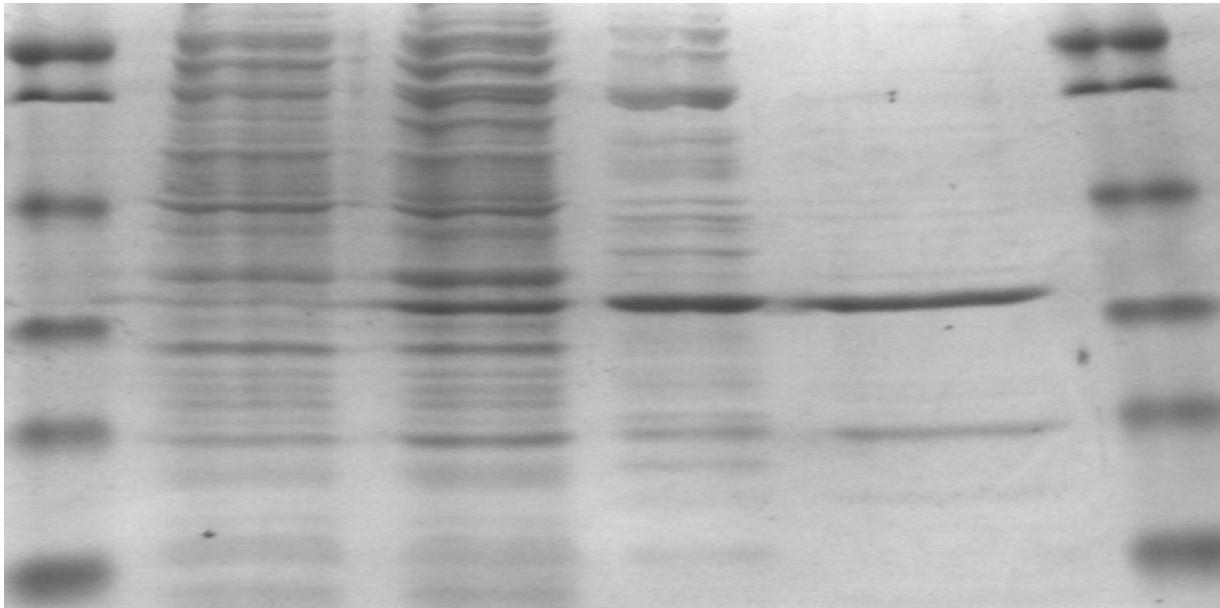


only four (useful !!) rare cutting enzymes are commercially available today



Can we use ZFNs for construction of plant transformation vectors ?

Purification of ZFN-IV Enzyme



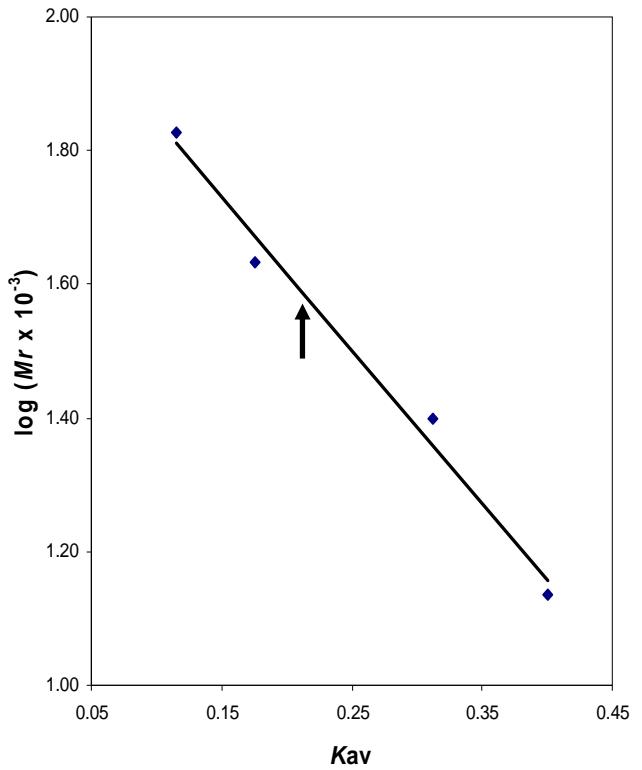
-IPTG

+IPTG

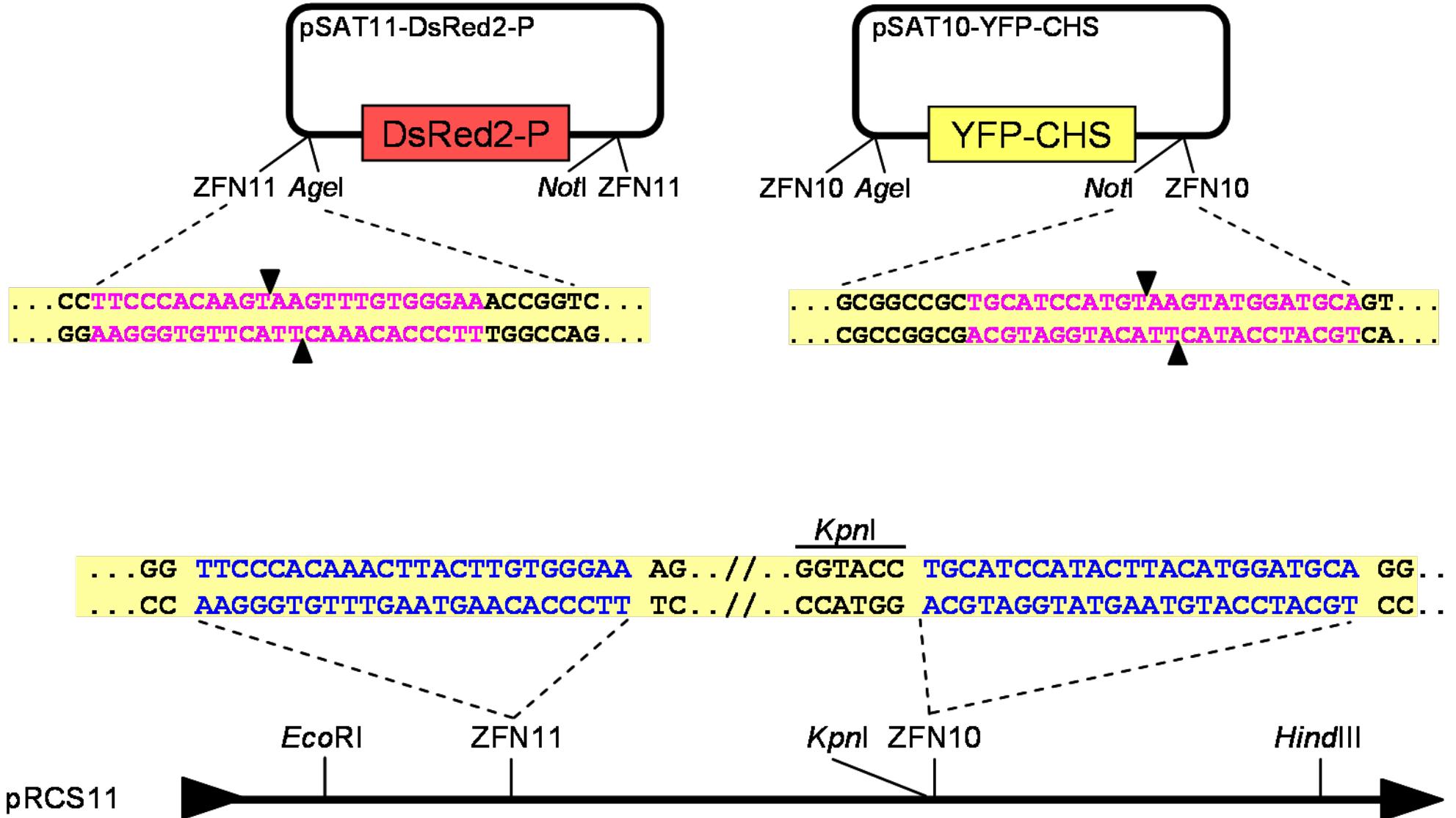
Lysate

NiNTA

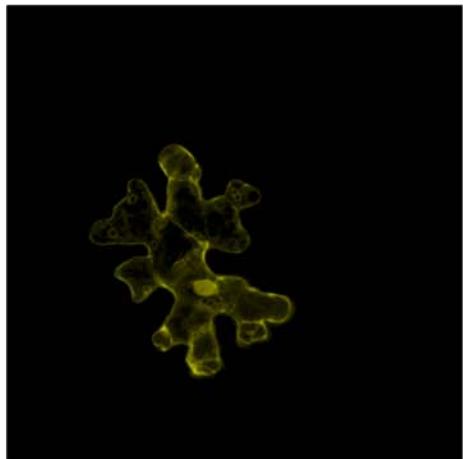
GF



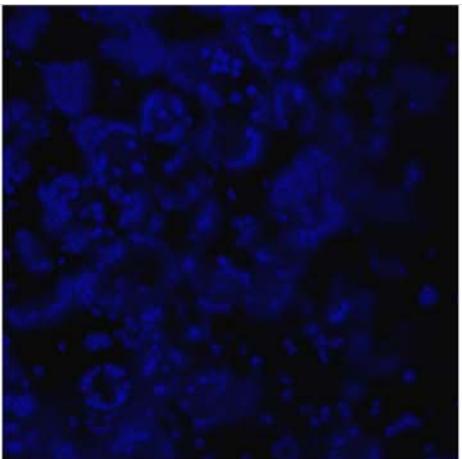
Size-Exclusion Chromatography



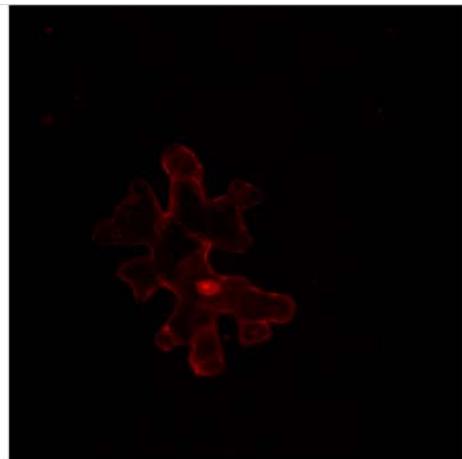
YFP-CHS



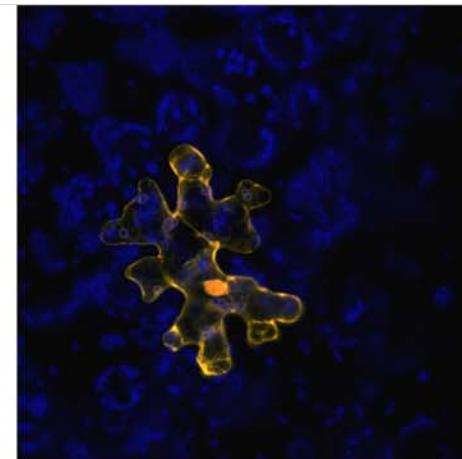
chloroplasts



DsRed2-P

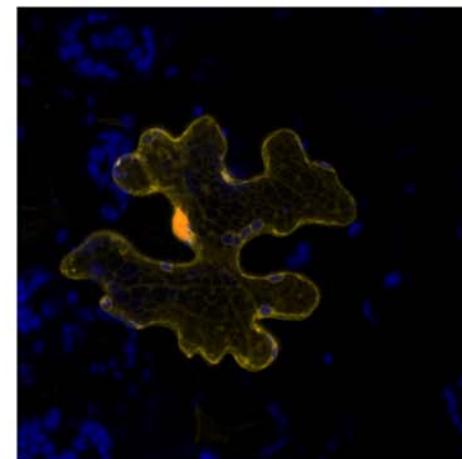
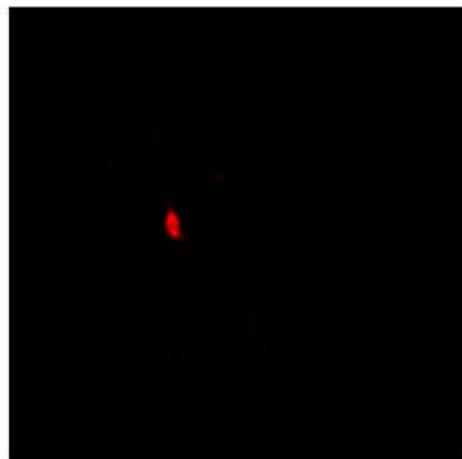
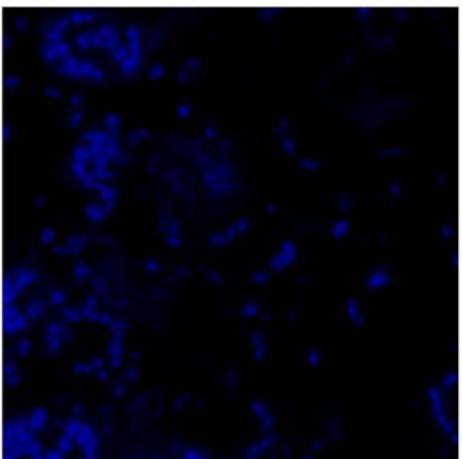
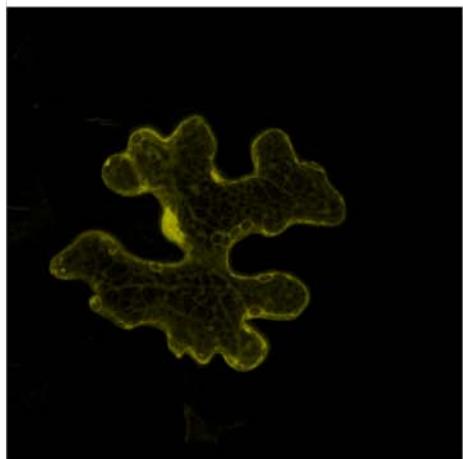


merge



- N

+ N



ZFN11 *NotI* *AgeI* ZFN11

```
... GG TTCCCACAA ACTTAC TTGTGGGAA GCGGCCGC...//...ACCGGT TTCCCACAA ACTTAC TTGTGGGAA AG...
... CC AAGGGTGT TGAATG AACACCCTT CGCCGGCG...//...TGGCCA AAGGGTGT TGAATG AACACCCTT TC...
```

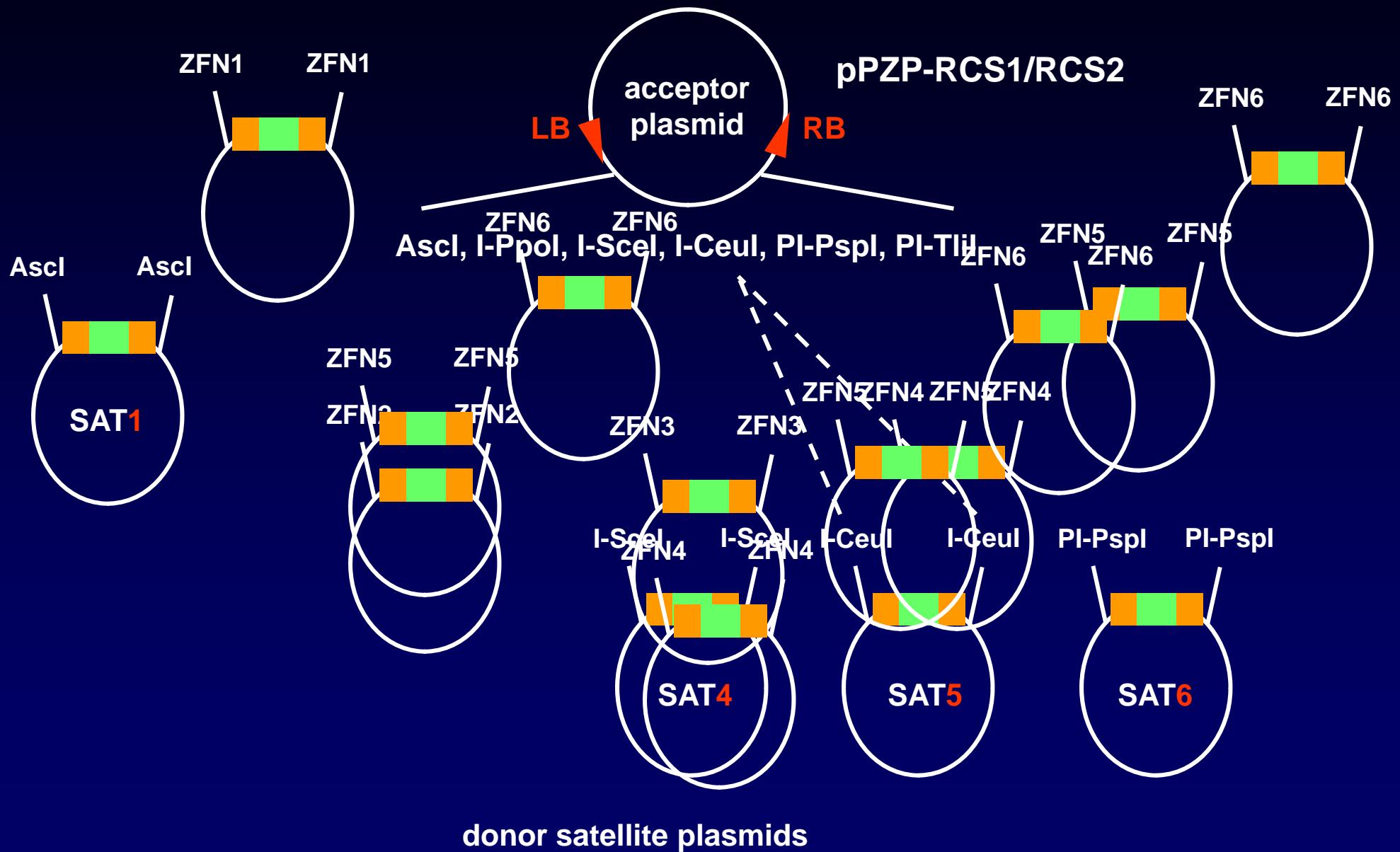
DsRed2-P expression cassette

KpnI ZFN10 *NotI* *AgeI* ZFN10

```
C GGTACC TGCATCCAT ACTTAC ATGGATGCA GCGGCCGC...//...ACCGGT TGCATCCAT ACTTAC ATGGATGCA GG...
C CCATGG ACGTAGGTA TGAATG TACCTACGT CGCCGGCG...//...TGGCCA ACGTAGGTA TGAATG TACCTACGT CC...
```

YFP-CHS expression cassette

ZFN can also be use for cloning of expression cassettes



Challenges:

- I. Design 4 ZFN monomers for specific gene digestion *in planta***
- II. Validate ZFN monomers activity in model plants**
- III. Validate ZFN monomers activity in target plants**
- IV. Deliver 4 ZFN to target plants**
- V. Combine ZFN expression with second transformation cycle**
- VI. Develop methods for regenerating and selecting mutant lines**

Protocols need to be developed for various species and different transformation methods....

Zinc-finger-nuclease-mediated resistance to plant viruses

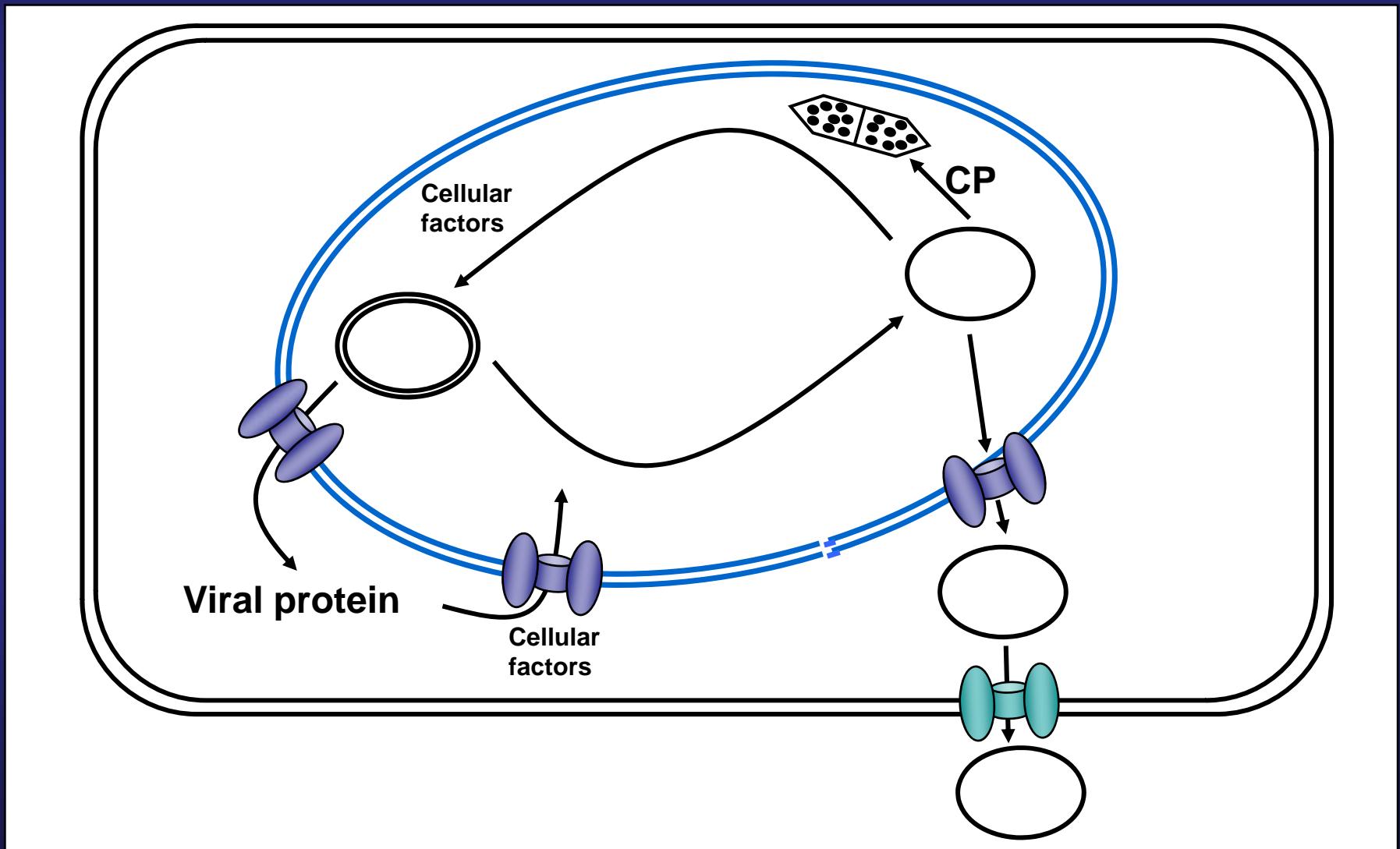
TYLCV infects tomato



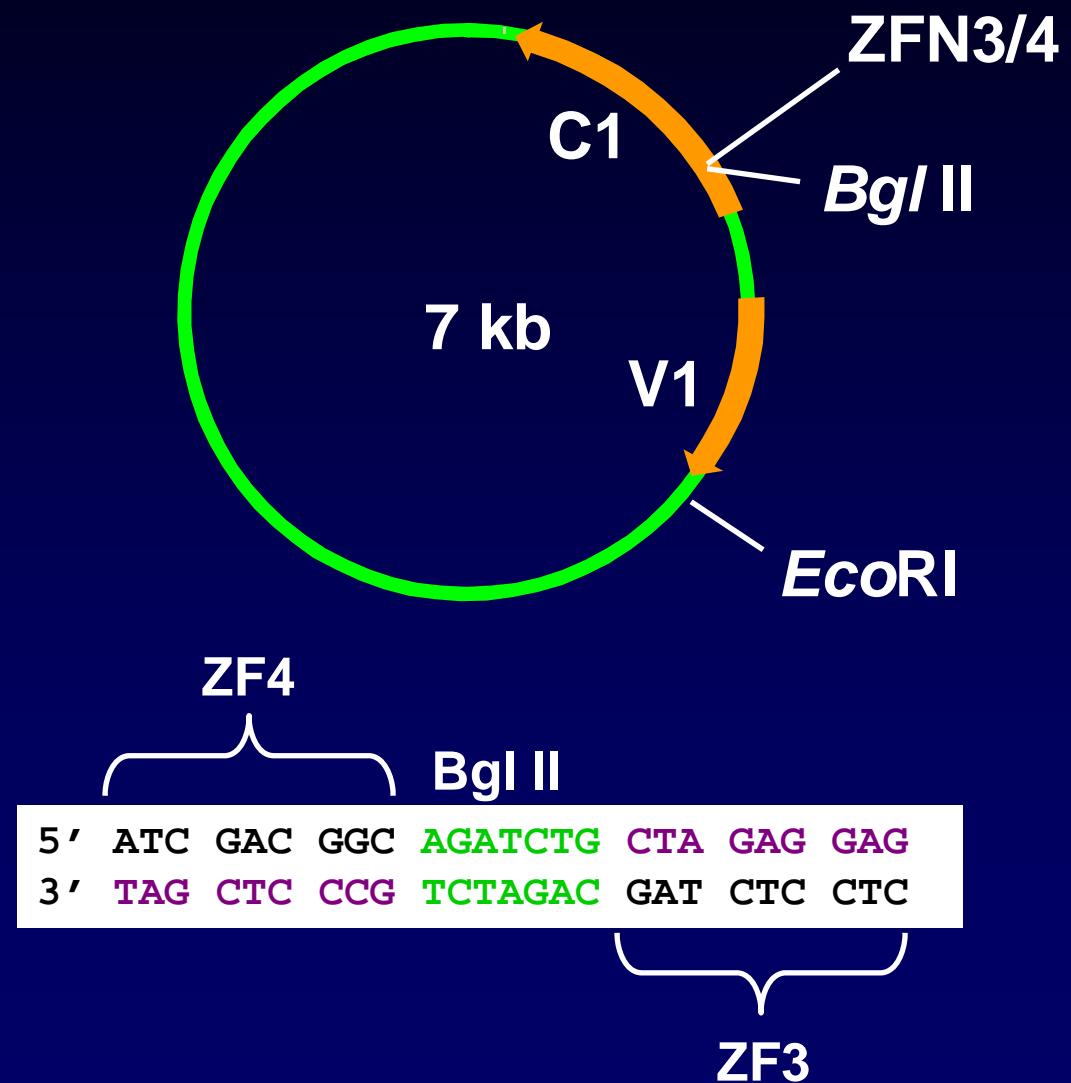
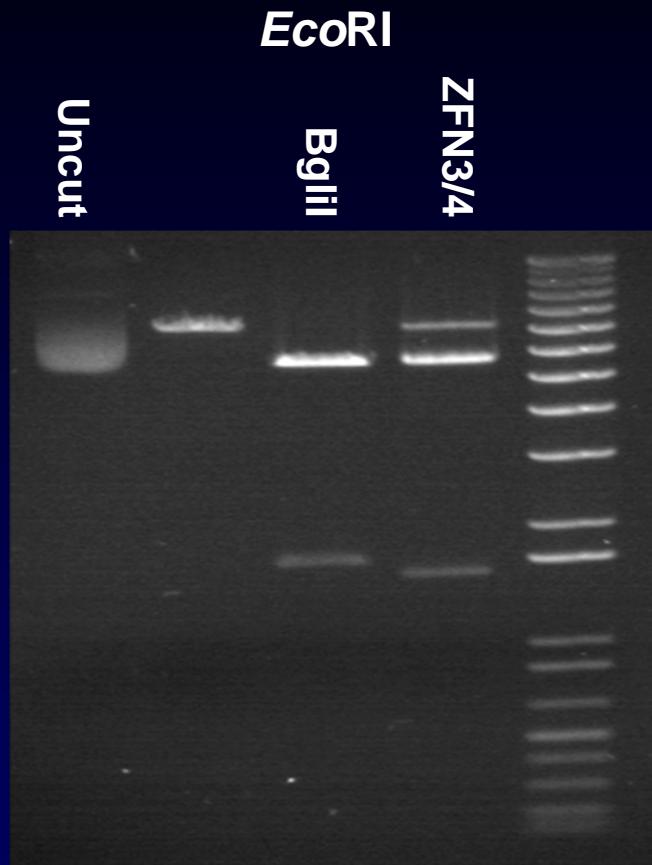
BDMV infects Bean plant



Geminivirus DNA replication cycle and movement from cell-to-cell



in-vitro digestion of BDMV- DNA B clone



transgenic



Wild type



Possible broad range resistance to TYLCV

2-5rep	CGGTTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	334
Rep[FL]	CGGTTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	258
TYLCVX15656RC	CGGTTCTTCGACTGGTATCCCCAAACAGGTCAAGCACATT	253
FloridaAY530931R	CGGTTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	419
Puerto_RicoAY134	CGATTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	419
GeziraAY044138RC	CGATTCTTCGACCTGGTATCCCCAAqCAGGgCAGCACATT	422
[Cuba]AJ223505RC	CGGTTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	412
Culiacan_MexicoD	CGGTTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	419
SinaloaRC	CGGTTCTTCGACCTGGTATCCCCAAACAGGTCAAGCACATT	419
Malaga_virus_AF2	CGATTCTTCGACCTGGTATCCCCAAqCAGGgCAGCACATT	422
Mild[Aichi]AB014	CGATTCTTCGACCTGGTATCCCCAAqCAGGgCAGCACATT	259
Mild[Portugal]AF	CGATTCTTCGACCTGGTATCCCCAAqCAGGgCAGCACATT	422
Mild[ShizuokuaAB]	CGATTCTTCGACCTGGTATCCCCAAqCAGGgCAGCACATT	259
Mild[Spain7297]A	CGATTCTTCGACCTGGTATCCCCAAqCAGGgCAGCACATT	422
MildX76319RC	CGATTCTTCGACCTGGTATCCCCAqCAGGgCAGCACATT	259
Sardinia_virusX6	CGATTCTTCGACCTGGTATCCCCAAAcCAGGTCAAGCACATT	415
Sardini-[Spain1]	aGATTCTTCGAtCTGGTATCCCCAAAcCAGGaTCAGCACATT	417
Sardinia-[Sicily]	aGATTCTTCGAtCTGGTATCCCCAAAcCAGGaTCAGCACATT	415
SardiniaL27708RC	aGATTCTTCGAtCTGGTATCCCCAAAcCAGGaTCAGCACATT	417
Consensus	g ttcttcgaa tggtatccccaa ag cagcacatt	
2-5rep	TCCATCCAAACATTCAAGGCAGCTAACAGATGT	374
Rep[FL]	TCCATCCAAACATTCAAGGCAGCTAACAGATGT	298
TYLCVX15656RC	TCCATCCGAAACATTCAAGGCAGCTAACAGATGT	293
FloridaAY530931R	TCCATCCAAACATTCAAGGCAGCTAACAGATGT	459
Puerto_RicoAY134	TCCATCCAAACATTCAAGGCAGCTAACAGATGT	459
GeziraAY044138RC	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGAcGT	462
[Cuba]AJ223505RC	TCCATCCAAACATTCAAGGCAGCTAACAGATGT	452
Culiacan_MexicoD	TCCATCCGAAACATTCAAGGCAGCTAACAGATGT	459
SinaloaRC	TCCATCCGAAACATTCAAGGCAGCTAACAGATGT	459
Malaga_virus_AF2	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGATGT	462
Mild[Aichi]AB014	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGAcGT	299
Mild[Portugal]AF	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGAcGT	462
Mild[ShizuokuaAB]	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGAcGT	299
Mild[Spain7297]A	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGAcGT	462
MildX76319RC	TCCATCCAAACATTCAAGGAGCTAAatcCagttCAGAcGT	299
Sardinia_virusX6	TCCATCCGAAACATTCAAGGAGCTAAatcCagttCAGAcGT	455
Sardini-[Spain1]	TCCATCCGAAACATTCAAGGAGCTAAatcCagttCAGAcGT	457
Sardinia-[Sicily]	TCCATCCGAAACATTCAAGGAGCTAAatcCagttCAGAcGT	455
SardiniaL27708RC	TCCATCCGAAACATTCAAGGAGCTAAatcCagttCAGAcGT	457
Consensus	tccatcc aacattcagg agctaa c qa gt]	

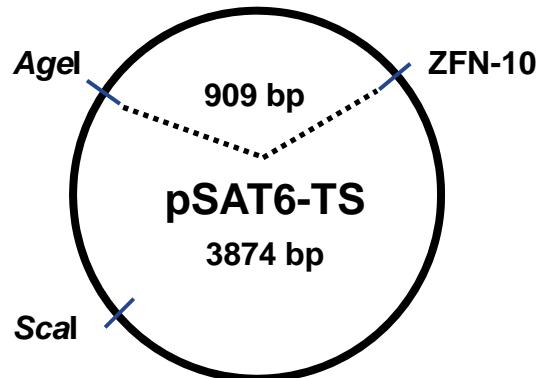
Risks and limitations:

I. Mutagenesis of off target sites

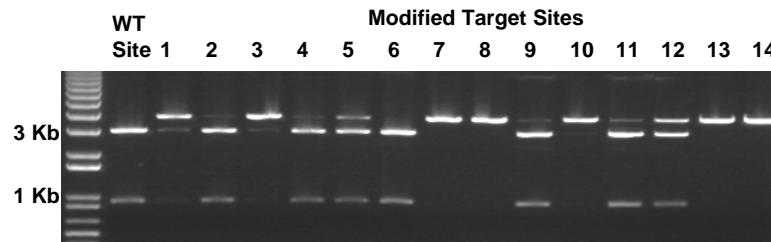
A

RH1 - **GCA** Q S G D L R R
RH2 - **GAT** T S G N L V R
RH3 - **ATG** R R D E L N V

TGCATCCAT-GTAAGT-ATGGATGCA
GG T
C C
A

B**C**

Construct	Sequence	Cutting Efficiency
WT	TGCATCCAT-GTAAGT-ATGGATGCA	100%
1	A GCATCCAT-GTAAGT-ATGGAT G T	8%
2	G GCATCCAT-GTAAGT-ATGGAT G CC	97%
3	TGCATCC T -GTAAGT- A GGGATGCA	2%
4	TGCATCC G T-GTAAGT- A CGGATGCA	98%
5	TGCATCC T T-GTAAGT- A AGGATGCA	79%
6	TGCATCC A C-GTAAGT- G TGGATGCA	100%
7	G GCATCC T T-GTAAGT- A GGGAT G CC	0%
8	TGCATCC CC -GTAAGT- G GGGATGCA	0%
9	A GCATCC T C-GTAAGT- G AGGAT G CT	97%
10	TGCATCCAT-GTAAGT-ATG C ATGCA	0%
11	TGCATCCAT-GTAAGT-ATGG C TGCA	94%
12	TGCATCCAT-GTAAGT-ATGGAC G CA	63%
13	TGCATCCAT-GTAAGT-ATG C C T GCA	0%
14	TGCATCCAT-GTAAGT-ATG C C C GCA	0%

D

Risks and limitations:

- I. Mutagenesis of off target sites – PCR analysis of ‘known’ sites**
- II. Toxicity – new ZFN architecture**
- III. Transgene removal – breeding out ZFN cassettes (advantage of using a single transgene)**
- IV. Targeting complex genomes – multi copy genes, gene clusters**
- V. Limited access to the technology**
- VI. Public acceptance**

Collaborator:

Personal:

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Avner Levi (Postdoc)

Vardit Zeevi (Tech)

Avi Levi (Weizmann institute, Israel)

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