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’ tttggagaggacacgctcgacggtacctATTACCCCTGTTATCCCTAaggatccgtcgaagta
  aacctctctcctgtgagctgccatggaTAATGGGACATAAGGGATcctaggcagctttcat

**T-DNA**
5’ tggcaggatatattttga//...aattcATTACCCCTGTTATCCCTAatgttaagttcgctctgta

**Junction 332**
5’ tttggagaggacacgctcgacggtacctATTACCCCTGTTATCCCTAatgttaagttcgctctgta

**Junction 410**
5’ tttggagaggacacgctcgacggtacctATTACCCCTGTTATCCCTAatgttaagttcgctctgta

**Junction 522**
5’ tttggagaggacacgctcgacggtacctATTACCCCTGTTATCCCTAatgttaagttcgctctgta
T-DNA insertion to DSBs is not limited to a single site

There are 80 Ascl restriction recognition sites in the *Arabidopsis* genome.
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*

- **ZFN-I**
- **ZFN-II**
- **ZFN-III**
- **ZFN-IV**

**plant chromosome**

**pro**

**target gene**

**ter**

**T-DNA**

make transgenic plant with induced expression of ZFNs

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.

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 \textit{in planta}.

### Triplet Recognition Helices

<table>
<thead>
<tr>
<th>DNA Recognition triplet helix</th>
<th>DNA Recognition triplet helix</th>
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<tbody>
<tr>
<td>AAA QRA NLRA</td>
<td>GAA QSSNLVR</td>
</tr>
<tr>
<td>AAC DSGNLVR</td>
<td>GAC DPGNLVR</td>
</tr>
<tr>
<td>AACG RGDNLKN</td>
<td>GAGk RSDNLVR</td>
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<tr>
<td>AAT TTGNLTV</td>
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<tr>
<td>ACA SPADLTR</td>
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<td>ACC DKKDLTR</td>
<td>GCC DCRDLAR</td>
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<td>ACT THLDLR</td>
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<td>GCC DPGHNLVR</td>
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<td>TAGk REDNLHT</td>
<td>TG3k RSDHLLT</td>
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TABLE ONE

Summary of the most optimal zinc finger domain sequences identified in this study for binding to 5'-CNN-3' target sites. The helices are for design M, helices derived by panning M, helices derived by site-directed mutagenesis or de novo design. The helices derived from panning M, helices derived by site-directed mutagenesis or de novo design.

<table>
<thead>
<tr>
<th>Target site 5’ → 3’</th>
<th>Finger-2 helix</th>
<th>Source</th>
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<tbody>
<tr>
<td>CAA</td>
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<tr>
<td>CTT</td>
<td>TTG-A-LTE</td>
<td>M</td>
<td>v</td>
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</tbody>
</table>

Birgit Dreier

\textit{JOURNAL OF BIOLOGICAL CHEMISTRY}

Andreas F. Kolb

\textit{TRENDS in Biotechnology}
tools for analyzing novel ZFN

in vitro digestion assay

T-DNA repair assay

transgene repair assay

whole plant DNA repair assay

pET28.SX-ZFN

pSAT-TS

Agel

pRCS-[ZFN-X][*GUS]

pRCS-[*GUS]

pRCS-[ZFN-X]

pRCS-[hsp.ZFN-X][*GUS]
Fast and simple assembly of ZFs backbone

SDO1

SDO2

SDO3

BBO1

BBO2

BBO3

ZF-I

ZF-II

ZF-III

Klenow reaction

PCR

pSAT (plant expression)

pET (E. coli expression)

XhoI

SpeI

p

NLS

FOK-I

t

p

FOK-I

t
Activity of FtsH2 ZFNs

'palindrome'-like sequences
Target Site Selection for AtFtsH2

At2g30950 FtsH2
4373 bp
FtsH2 ZFN1a digests a target site with 6 but not 8 bp spacer
FtsH2 ZFN1a with \((\text{Gly}_4\text{Ser})_2\) Linker

\[
\ldots \text{GAACCCGTTTTAATGCGCGGTTCACCGGG} \quad \text{GTTCACCGGG} \ldots \\
\ldots \text{CTTGGGCAAATTACGCGCCAAGTGGCCC} \ldots \\
\ldots \text{GAACCCGTTTTAATGCGCGGTTCACCGGG} \quad \text{GTTCACCGGG} \ldots \\
\ldots \text{CTTGGGCAAATTACGCGCCAAGTGGCCC} \ldots \\
\]

ZF I  ZF II  ZF III

GGGGS
ZFN Pairs Digests Genomic FtsH2 Sequence

Uncut  Smal  Smal  Smal  PstI  PstI  PstI  Smal
ZFN1a  ZFN2  ZFN1a  ZFN3  ZFN2  ZFN3  ZFN4  ZFN1a
ZFN2  ZFN4

2 kb  4 kb  6 kb
6 kb  4 kb  6 kb  2 kb

6.4 kb

2.6 kb

FtsH2

110 bp
ZFN3

120 bp
ZFN1a

110 bp
ZFN4

non palindrome sequences
tools for analyzing newly assembled ZFN

- **in vitro digestion assay**
- **T-DNA repair assay**
- **transgene repair assay**
- **whole plant DNA repair assay**

**Plasmids**:
- pET28.SX-ZFN
- pSAT-TS
- pRCS-[ZFN-X][*GUS]
- pRCS-[*GUS]
- pRCS-[ZFN-X]
- pRCS-[hsp.ZFN-X][*GUS]

**Enzymes**:
- AgeI

**Markers**:
- KAN
- hsp
- GUS

**Features**:
- TGA

*ZFN: Zinc Finger Nuclease*
T-DNA repair assay

pRCS-[ZFN-X][*GUS]

- ZFN

+ ZFN (QQR)

+ ZFN (ZFN3)
tools for analyzing of newly assembled ZFN

- **in vitro digestion assay**
  - pET28.SX-ZFN
  - pSAT-TS

- T-DNA repair assay
  - pRCS-[ZFN-X][*GUS]
    - ZFN
    - GUS
    - *TGA*

- Transgene repair assay
  - pRCS-{hsp.ZFN-X}[GUS]
    - KAN
    - GUS
    - ZFN
    - hsp
    - *TGA*

- Whole plant DNA repair assay
  - pRCS-{hsp.ZFN-X}[*GUS]
whole plant DNA repair assay

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

KAN hsp ZFN GUS

ATG-TS-TGA-TS...

Ddel

ATGT TTCCCTCTCTGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCGAGGGGAAGAATTA
ATGT TTCCCTCCCTCGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCAGGGGAAGAATTA
ATGT TTCCCTCCCTCCTGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCTGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCTGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCTGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCTGAGGGGAAGAATTA
ATGT TTCCCTCCCTCCTGAGGGGAAGAATTA

ACGAAC .................. TGTAGA

~82 bp
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…. 
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
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.

The diagram shows a central acceptor plasmid (LB plasmid) and a donor plasmid (pPZP-RCS1/RCS2). There are additional satellite plasmids labeled SAT1 through SAT7, each with specific restriction enzyme sites (AscI, I-PpoI, I-SceI, I-CeuI, PI-PspI, PI-TliI). The donor satellite plasmids are marked with red X to indicate their removal or activation status.
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

Size-Exclusion Chromatography
The diagram shows a gene construct involving restriction sites and promoters.

1. **pSAT11-DsRed2-P**
   - **DsRed2-P**: Red promoter region.
   - **ZFN11** and **Agel**: Restriction sites.

2. **pSAT10-YFP-CHS**
   - **YFP-CHS**: Yellow fluorescent protein promoter region.
   - **ZFN10**, **Agel**, and **NotI**: Restriction sites.

3. **pRCS11**
   - **EcoRI**, **ZFN11**, **Kpnl**, and **HindIII**: Restriction sites.

The sequences highlighted indicate the regions of interest for genetic manipulation.
ZFN can also be used 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**

Below is a diagram illustrating the digestion of DNA using various restriction enzymes, including EcoRI, BglII, ZFN3/4, and ZF4. The diagram shows the expected fragment sizes for each digestion. The sequence data provided includes:

```
5'  ATC GAC GGC  AGATCTG CTA GAG GAG 
3'  TAG CTC CCG  TCTAGAC GAT CTC CTC 
```

The digestion products are indicated as follows:
- Uncut
- BglII
- ZFN3/4

The fragment sizes labeled are:
- 7k
- 5.5k
- 1.5k

The circular diagram highlights the expected digestion sites and fragment sizes, providing a visual representation of the DNA digestion process.
transgenic

Wild type
Possible broad range resistance to TYLCV

- 2-5rep
- Rec[FL]
- TYLCV15656RC
- FloridaAY530931R
- Puerto RicoAY134
- GeziraAY9044138RC
- [Cuba]AJ223505RC
- Culinianci Mexico0D
- SinaloaRC
- Malaga virus AF2
- Mild[Aichi]AB014
- Mild[Portugal]AF
- Mild[Shizuoka]AE
- Mild[Spain7297]A
- MildK76319RC
- Sardinia virusX6
- Sardini-[Spain]1
- Sardini-[Sicily]6
- SardiniL27708RC
- Consensus

<table>
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<tr>
<th>Sequence</th>
<th>Length</th>
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<tr>
<td>2-5rep</td>
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<tr>
<td>Rec[FL]</td>
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<td>FloridaAY530931R</td>
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**Consensus:**

```
gcttctgagtatcacgaagccacatagagccgtctagcttcgaagcttcacagtgt
```
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-ATGGGATGCA
G     T
C     C
A

### B

**Construct**

**Sequence**

| WT | TGCATCCAT-GTAAGT-ATGGGATGCA | 100% |
| 1  | TGCATCCAT-GTAAGT-ATGGGATGCT | 8%   |
| 2  | GGCATCCAT-GTAAGT-ATGGGATGCC | 97%  |
| 3  | TGCACTCCCT-GTAAGT-AGGGGATGCA | 2%   |
| 4  | TGCACTCCGT-GTAAGT-AGGGGATGCA | 98%  |
| 5  | TGCACTCCCT-GTAAGT-AAGGGGATGCA | 79%  |
| 6  | TGCACTCCAC-GTAAGT-GGGGATGCA | 100% |
| 7  | GGCACTCCCT-GTAAGT-AGGGGATGCA | 0%   |
| 8  | TGCACTCCCC-GTAAGT-GGGGATGCA | 0%   |
| 9  | AGCACTCCCT-GTAAGT-AGGGGATGCA | 97%  |
| 10 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
| 11 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 94%  |
| 12 | TGCACTCCAT-GTAAGT-GGGGGATGCA | 63%  |
| 13 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
| 14 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |

### C

**Construct**

**Sequence**

<table>
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<tr>
<th>Cutting</th>
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</thead>
<tbody>
<tr>
<td>Efficiency</td>
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</table>

| WT | TGCATCCAT-GTAAGT-ATGGGATGCA | 100% |
| 1  | TGCATCCAT-GTAAGT-ATGGGATGCT | 8%   |
| 2  | GGCATCCAT-GTAAGT-ATGGGATGCC | 97%  |
| 3  | TGCACTCCCT-GTAAGT-AGGGGATGCA | 2%   |
| 4  | TGCACTCCGT-GTAAGT-AGGGGATGCA | 98%  |
| 5  | TGCACTCCCT-GTAAGT-AAGGGGATGCA | 79%  |
| 6  | TGCACTCCAC-GTAAGT-GGGGATGCA | 100% |
| 7  | GGCACTCCCT-GTAAGT-AGGGGATGCA | 0%   |
| 8  | TGCACTCCCC-GTAAGT-GGGGATGCA | 0%   |
| 9  | AGCACTCCCT-GTAAGT-AGGGGATGCA | 97%  |
| 10 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
| 11 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 94%  |
| 12 | TGCACTCCAT-GTAAGT-GGGGGATGCA | 63%  |
| 13 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
| 14 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |

### D

- **Construct**
- **Sequence**
- **Cutting Efficiency**

![Modified Target Sites](image)

| WT | TGCATCCAT-GTAAGT-ATGGGATGCA | 100% |
| 1  | TGCATCCAT-GTAAGT-ATGGGATGCT | 8%   |
| 2  | GGCATCCAT-GTAAGT-ATGGGATGCC | 97%  |
| 3  | TGCACTCCCT-GTAAGT-AGGGGATGCA | 2%   |
| 4  | TGCACTCCGT-GTAAGT-AGGGGATGCA | 98%  |
| 5  | TGCACTCCCT-GTAAGT-AAGGGGATGCA | 79%  |
| 6  | TGCACTCCAC-GTAAGT-GGGGATGCA | 100% |
| 7  | GGCACTCCCT-GTAAGT-AGGGGATGCA | 0%   |
| 8  | TGCACTCCCC-GTAAGT-GGGGATGCA | 0%   |
| 9  | AGCACTCCCT-GTAAGT-AGGGGATGCA | 97%  |
| 10 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
| 11 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 94%  |
| 12 | TGCACTCCAT-GTAAGT-GGGGGATGCA | 63%  |
| 13 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
| 14 | TGCACTCCAT-GTAAGT-AGGGGATGCA | 0%   |
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 acceptence
Personal:

Andriy Tovkach, (Ph.D. student)
Avner Levi (Postdoc)
Vardit Zeevi (Tech)

Collaborator:

Avi Levi (Weizmann institute, Israel)

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