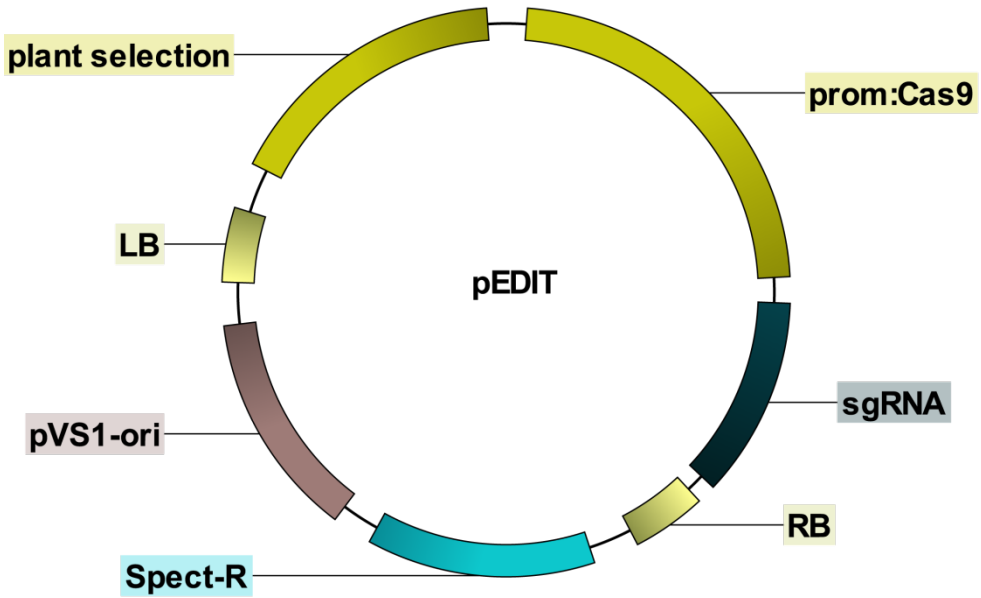
MARTIN-LUTHER-UNIVERSITÄT
HALLE-WITTENBERG

Chromosomal deletions using CRISPR-Cas9 gene editing toolkits

SEB Meeting Gothenburg
„New Breeding Technologies“
07.-08.07.2017
Johannes Stuttmann

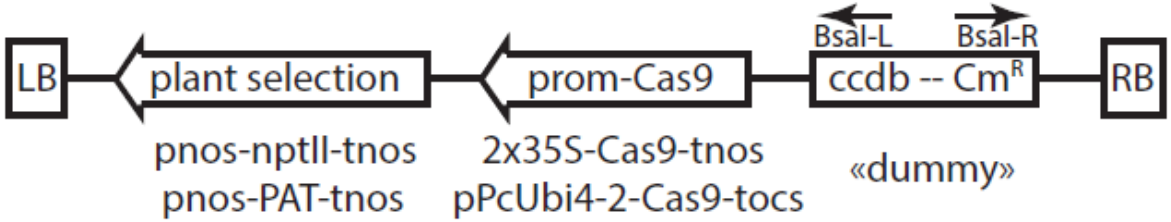
Our commitment to Cas9-based
RNA-guided nucleases:

- Generate mutations in genes of interest
- fairly common species
- for basic research purposes
- simple, reliable tools

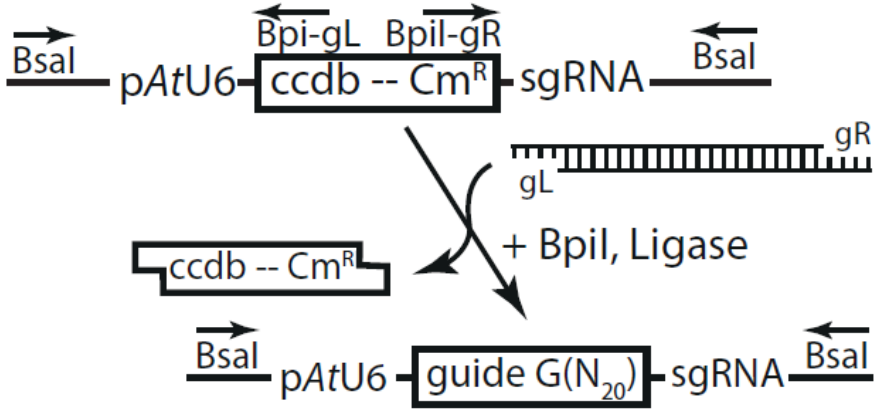


Dicot Genome Editing vectors for simple multiplexing

„recipient vectors“: pDGE1-4



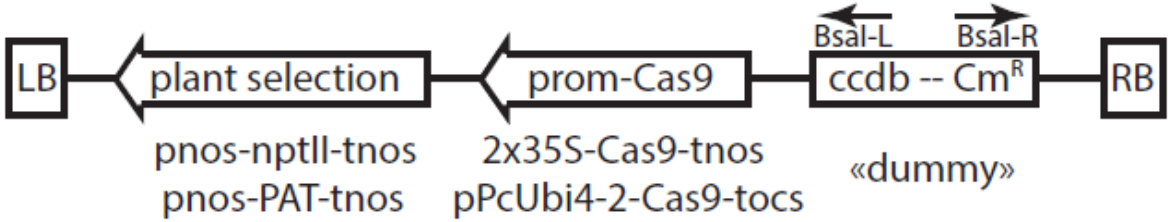
„sgRNA shuttle vectors“: pDGE5-15



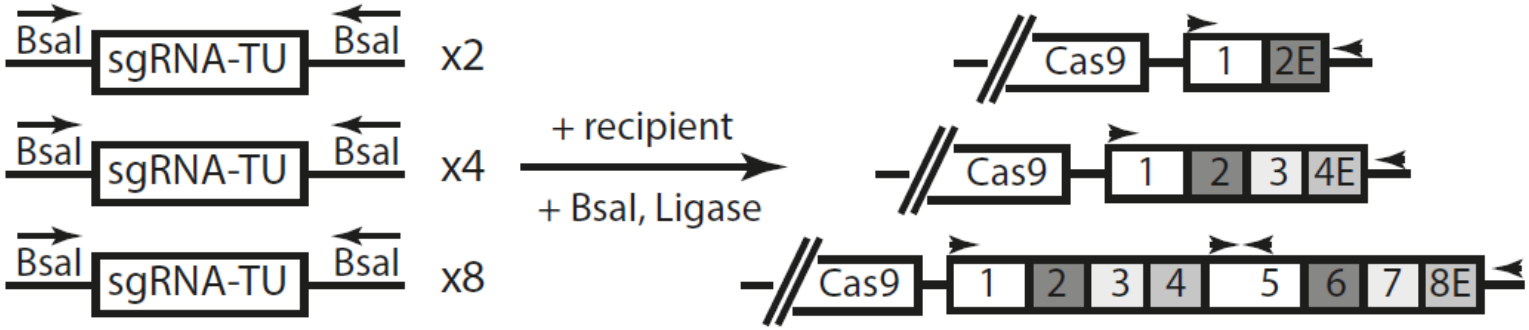
Ordon et al. 2016 Plant Journal

Dicot Genome Editing vectors for simple multiplexing

„recipient vectors“: pDGE1-4



Assembly of sgRNA array in recipient vector:



Ordon et al. 2016 Plant Journal

Dicot Genome Editing vectors for simple and rapid multiplexing

Day 1

- anneal oligos
- *Bpi*I cut/ligation
- Transformation
- direct inoculation of liquid cultures

Day 2

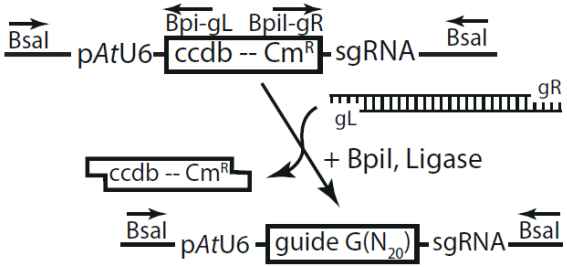
- miniprep
- *Bsa*I cut/ligation
- Transformation

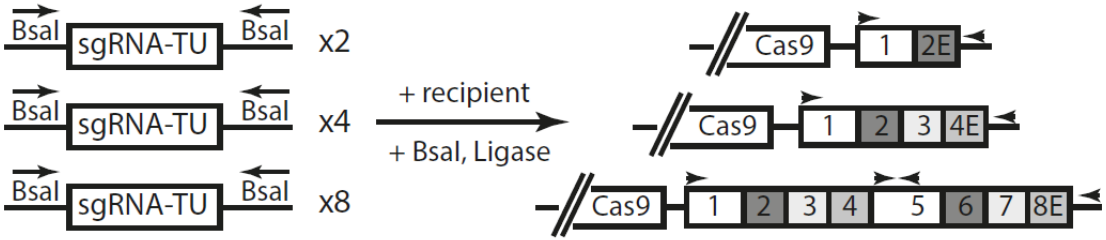
Day 3

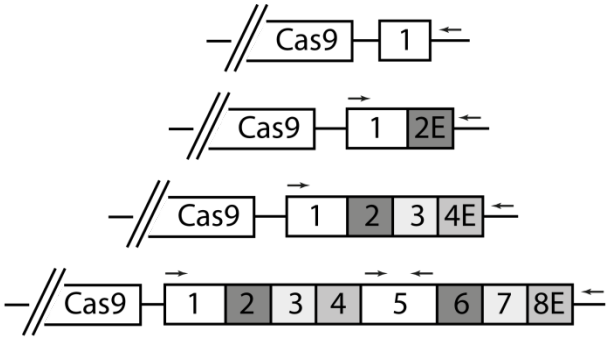
- start liquid cultures

Day 4

- miniprep, verification by digestion
- sequencing
- Agrobacterium transformation



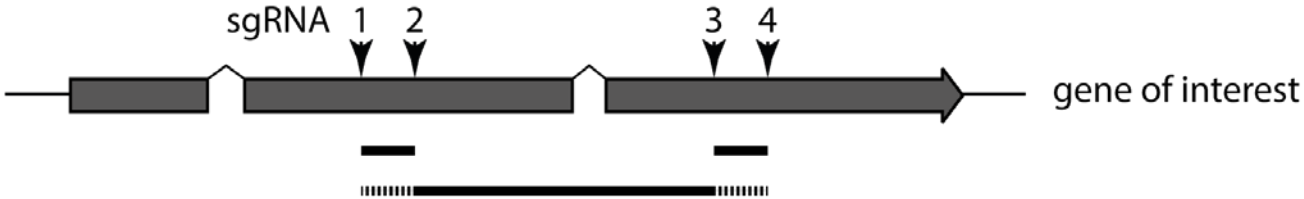




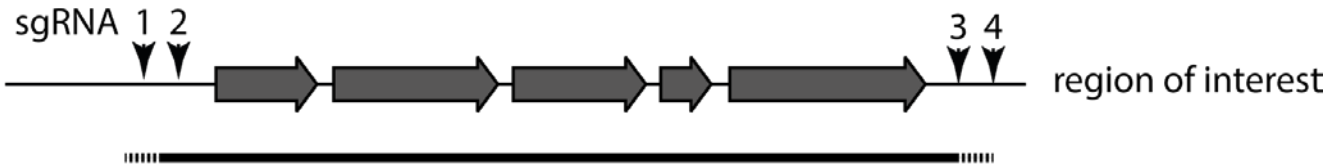
sequencing	oligos	plasmid prep
	5,8 +	2,5 + 1 = 10 €
	11,6 +	2,5 + 2 = 16 €
	23,2 +	2,5 + 4 = 30 €
	46,4 +	5 + 8 = 60 €

Generation of chromosomal deletions by paired nucleases

Two different strategies applied:

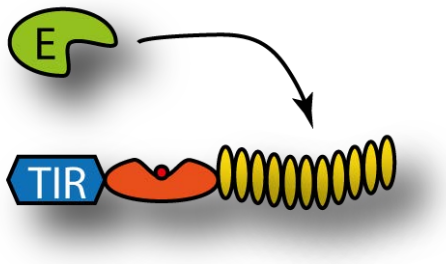


Nicotiana benthamiana:
EDS1, PAD4, SAG101s
Arabidopsis thaliana:
DM2c, DM2h



Arabidopsis thaliana:
EDS1a/EDS1b (5 kb)
DM2 cluster (120 kb)
DM2a-g (80 kb)

EDS1-based heterocomplexes: Required for TNL-mediated immunity



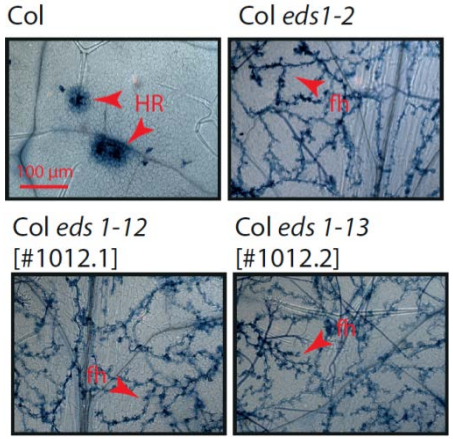
pathogen effector

Resistance protein (TNL)

Nicotiana benthamiana:
EDS1, PAD4, SAG101s
Arabidopsis thaliana:
DM2c, DM2h

mutually exclusive heterodimers

Resistance

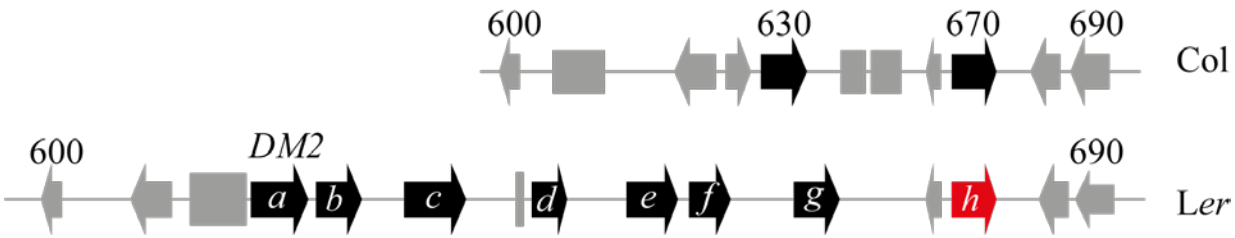


eds1-2: near isogenic line contains *DM2*^{Ler} cluster

Arabidopsis thaliana:
EDS1a/EDS1b (5 kb)
DM2 cluster (120 kb)
DM2a-g (80 kb)

Ordon et al. 2016 Plant Journal

The *DM2* cluster: A complex TNL *Resistance* gene locus



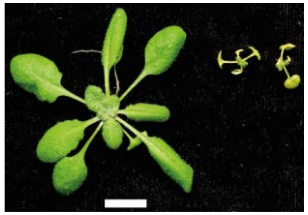
Nicotiana benthamiana:
EDS1, PAD4, SAG101s
Arabidopsis thaliana:
DM2c, DM2h

DM2^{Ler} cluster + genetic interactor:

SRF3$^{Kas/Kond}$

old3-1

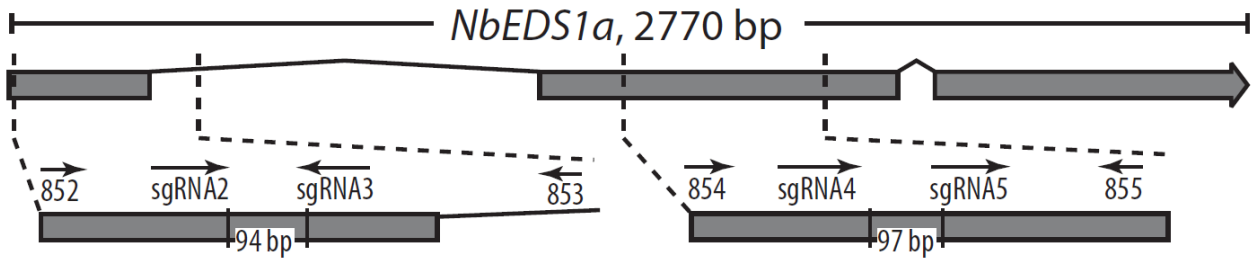
EDS1-YFP^{NLS}



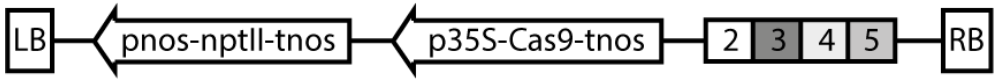
Arabidopsis thaliana:
EDS1a/EDS1b (5 kb)
DM2 cluster (120 kb)
DM2a-g (80 kb)

Alcazar at el. 2010 Nature Genetics
Tahir et al. 2013 Plant Journal
Stuttmann et al. 2016 PLoS Genetics

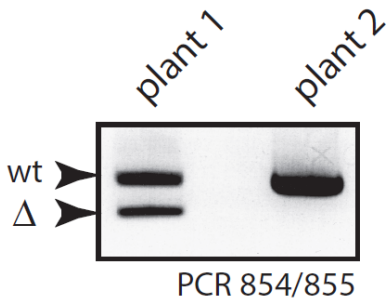
Generation of chromosomal deletions in *N.benthamiana: EDS1*

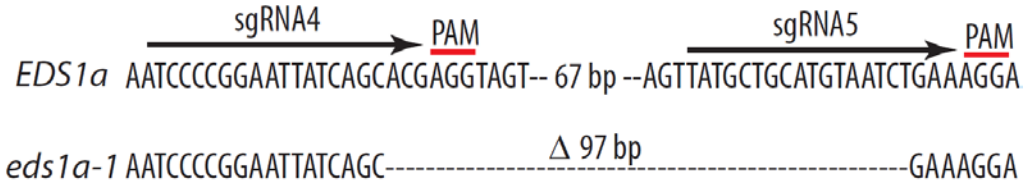


stable transformation



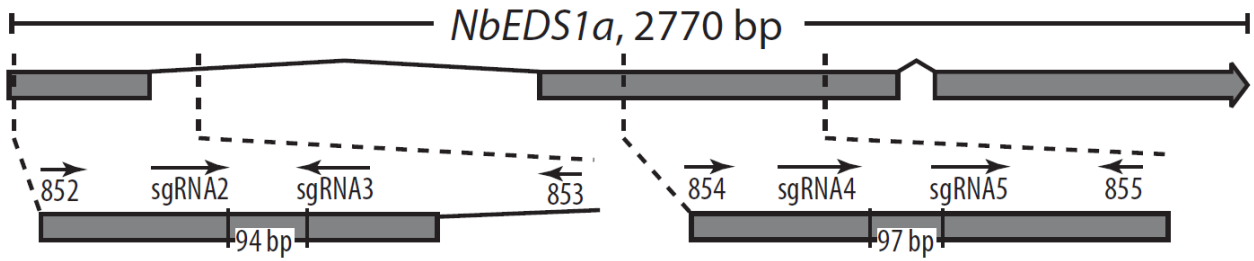
T_0 generation



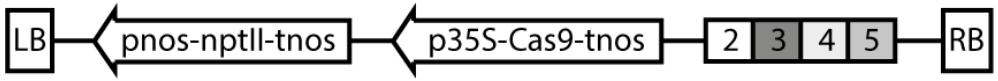


Ordon et al. 2016 Plant Journal

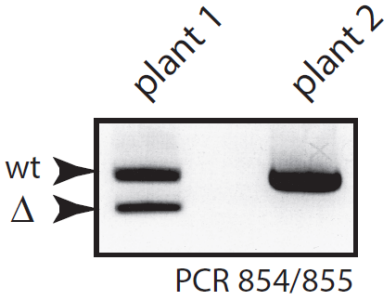
Generation of chromosomal deletions in *N.benthamiana: EDS1*



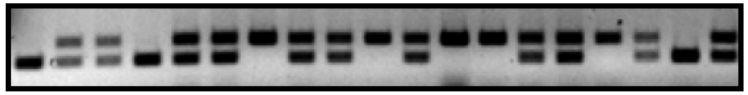
stable transformation



T_0 generation



T_1 generation

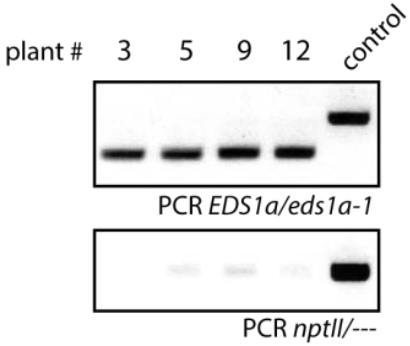


PCR 854/855

wildtype	17
heterozygous	28
Nbeds1a-1	15

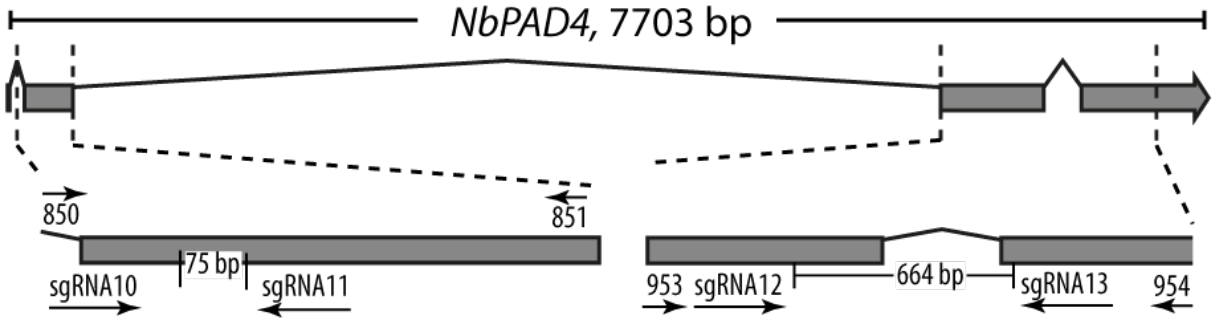
$Chi^2 = 0,4$

$[H_0 = 1:2:1]$

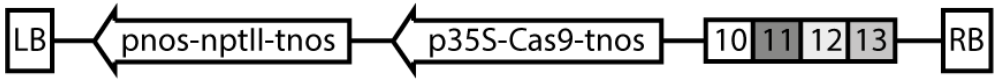


Ordon et al. 2016 Plant Journal

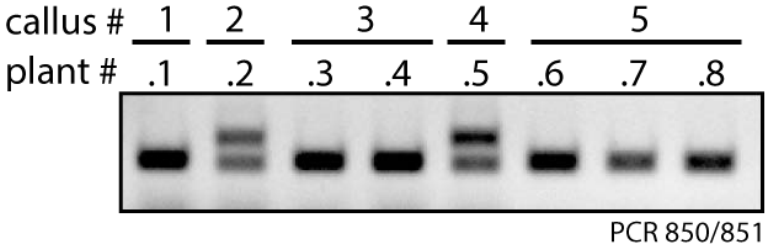
Generation of chromosomal deletions in *N.benthamiana: PAD4*



stable transformation

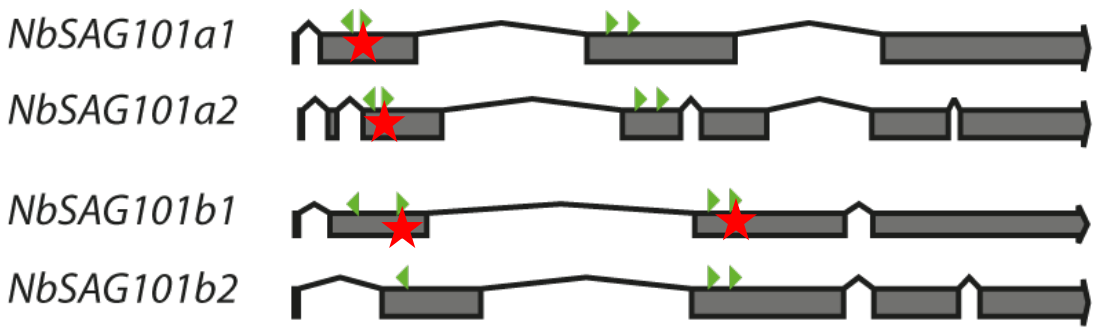


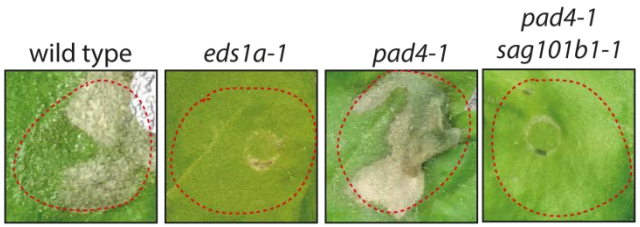
T_0 generation



Ordon et al. 2016 Plant Journal

Generation of chromosomal deletions in *N.benthamiana: SAG101s*



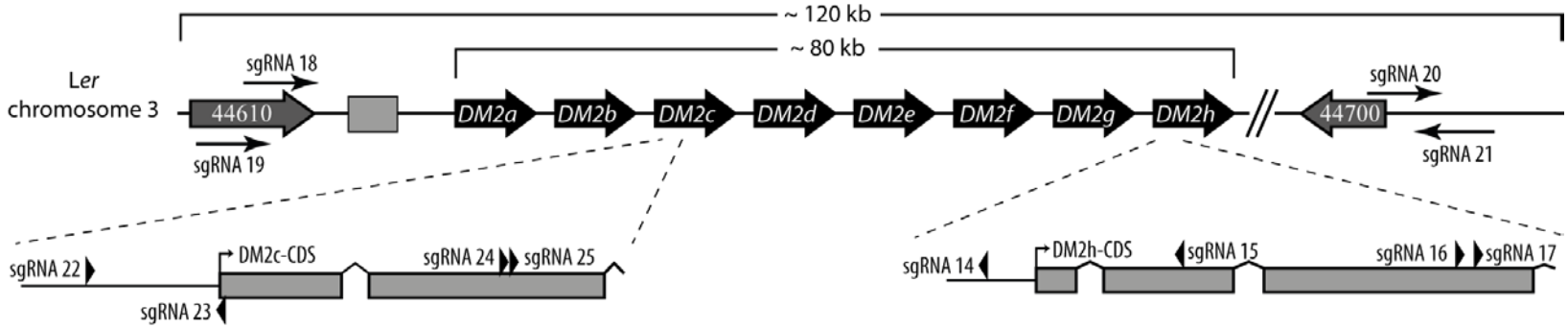


stable transformation

Overall:

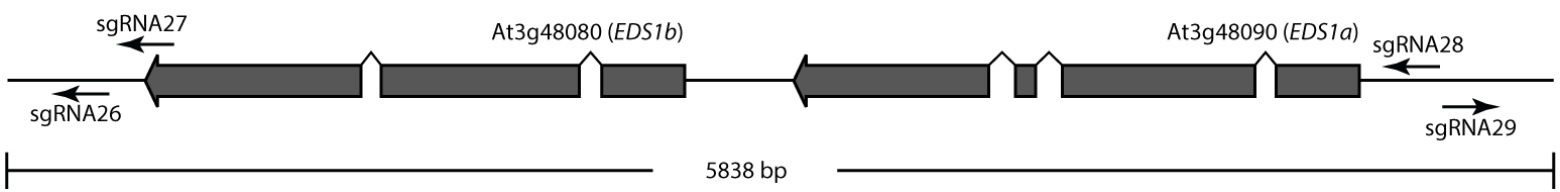
- Cas9-mediated knock-out highly efficient in *N. benthamiana* (35S:Cas9; p*At*U6:sgRNA)
- Point mutations are most frequent
- Small deletions arise at high frequencies; homozygous in T_0
- Deletions between outer target sides were not detected (neither transiently nor stable)
- High variations in sgRNA efficiencies; transient results apparently indicative for results upon stable transformation

Generation of chromosomal deletions in *Arabidopsis thaliana*



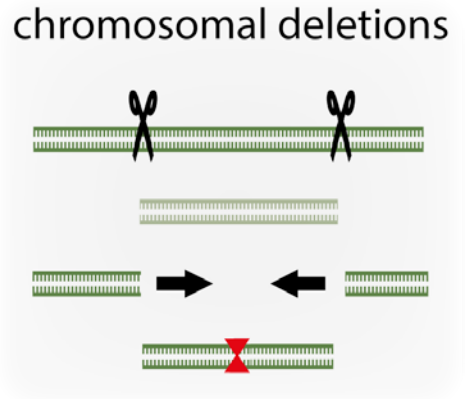
dm2h (point mutations) -> 10 %
dm2c (Δ 40 nt) -> 10 %
dm2 (Δ 120 kb) -> 0,5 % (heterozygous)

eds1a/b (Δ 6 kb) -> 0,5 % (homozygous)

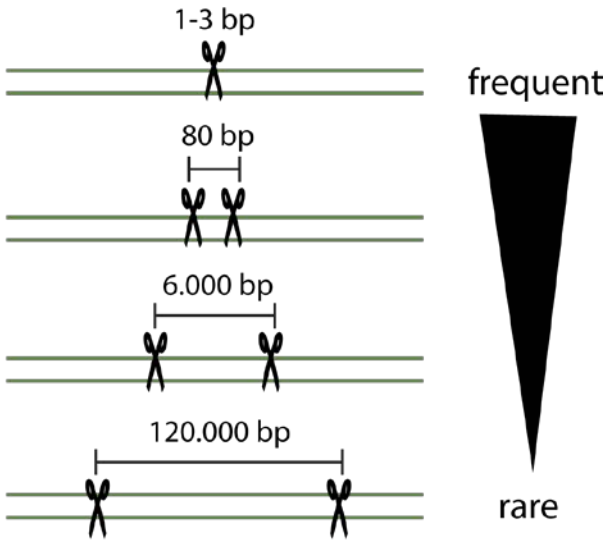


Ordon et al. 2016 Plant Journal

Generation of chromosomal deletions via Cas9-based nucleases:



- Precise ligation of chromosome ends
- No toxicity; no signs of structural changes
- Addressing several target sites by multi-plexing increases chances to obtain desired deletions
- Elimination of gene families or non-coding DNA

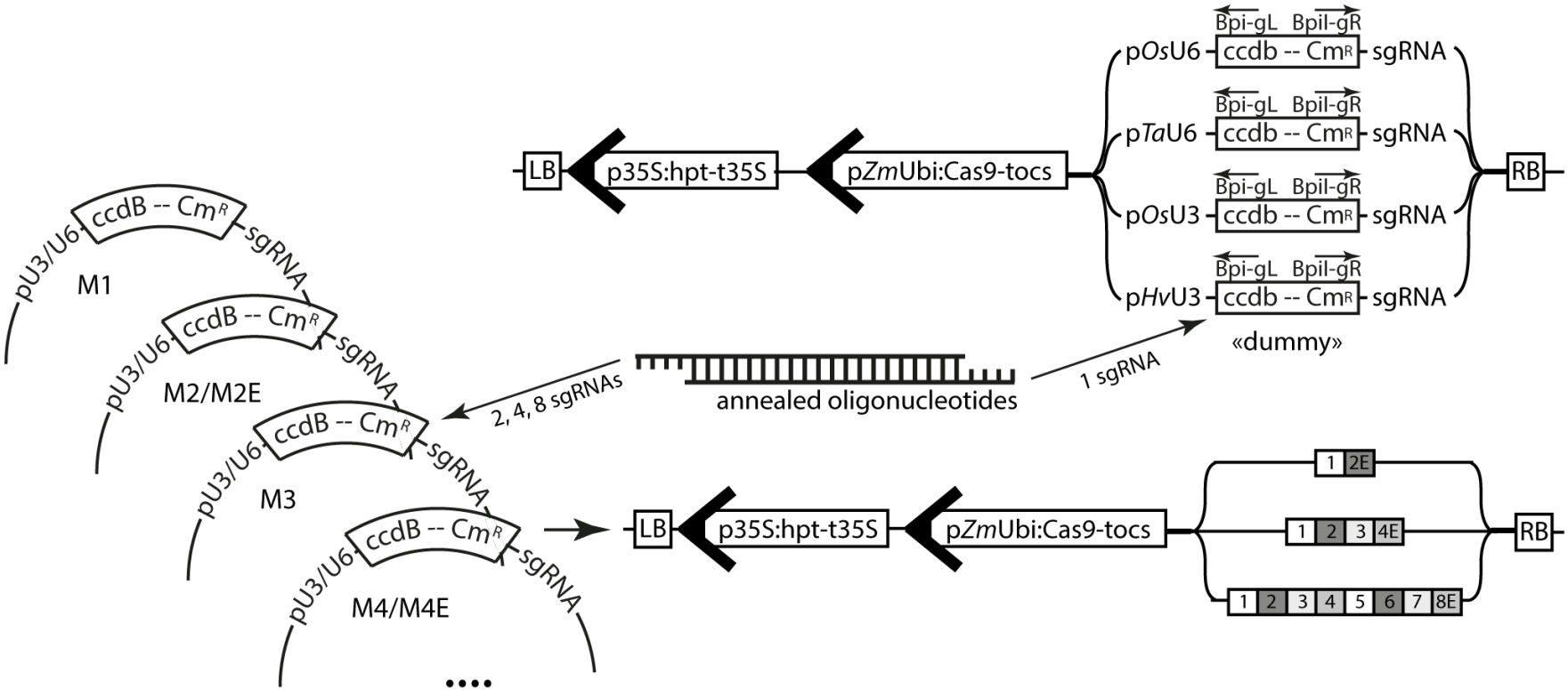


→ Potentially inconvenient detection

→ Straightforward screening, SSLP markers for subsequent genetic analyses

May require some screening (and efficient sgRNAs)

Tools for genome editing in monocotyledonous plants

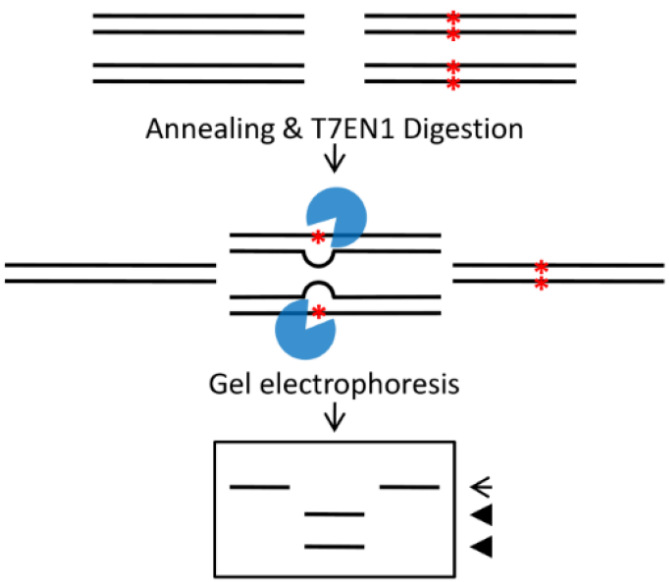


Kumar et al. in preparation

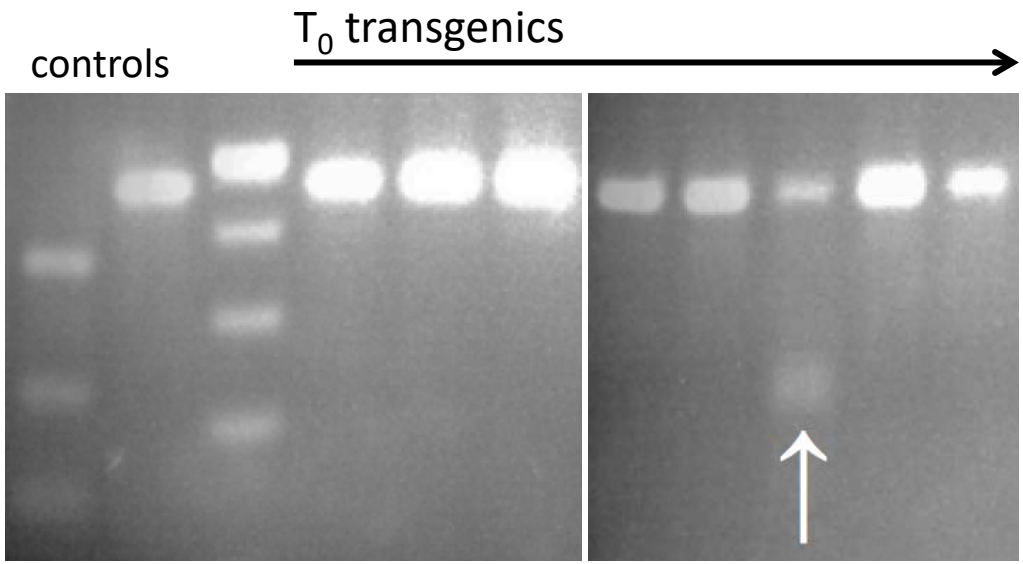
First barley genome editing attempts: Not as desired!

- p*Zm*Ubi:Cas9
- p*Os*U6:sgRNA; 2 sgRNAs
- Hygromycin marker
- Vector backbone not previously tested in barley transformation

Poor selection;
many false-positives



Screening 34 T_0 plants by T7E:



Miriam Lenk, Corina Vlot
Helmholtz Centre Munich

Shen et al. 2013 Cell Research

Implementation of *Hv*U3 promoter: Improved efficiencies

- p*Zm*Ubi:Cas9
- p*Hv*U3:sgRNA; 1 sgRNA
- pLH6000 backbone incl. Hygromycin marker

Genotyping from calli [PCR + direct sequencing]:

Target **PAM**

```
                    ACTTCGGGTGCACCCGCGCGCGG
Callus-1(WT)    GTGCAGGAACTTCGGGTGCACCCGCGCGCGGTCGAAGTCCCCTGTCTCCA
Callus-2        GTGCAGGAACTTCGGGTGCACCC--GCGCGGTCGAAGTCCCCTGTCTCCA
Callus-3        ---------------------CCGCGCGCGGTCGAAGTCCCCTGTCTCCA
Callus-4        ---------------------CCCCGCGCGGTCGAAGTCCCCTGTCTCCA
Callus-5        ---------------------CCCCGCGCGGTCGAAGTCCCCTGTCTCCA
Callus-6(WT)    GTGCAGGAACTTCGGGTGCACCCGCGCGCGGTCGAAGTCCCCTGTCTCCA
Callus-8        -------------------------GCGCGGTCGAAGTCCCCTGTCTCCA
Callus-9        GTGCAGGAACTTCGGGGGCCCCC--GCGCGGTCGAAGTCCCCTGTCTCCA
Callus-10       -----------------------CCGCGCGGTCGAAGTCCCCTGTCTCCA
```

Kumar et al. in preparation

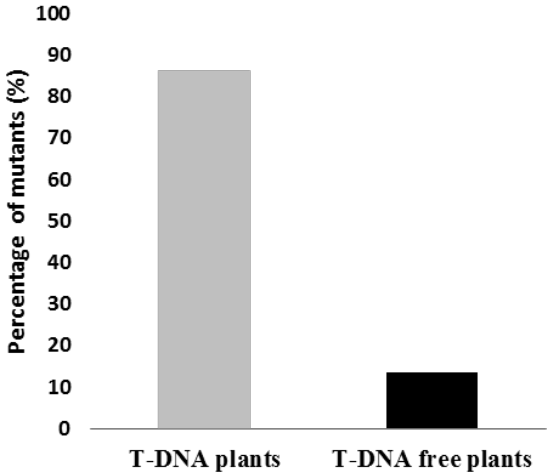
Implementation of *Hv*U3 promoter: Improved efficiencies

- p*Zm*Ubi:Cas9
- p*Hv*U3:sgRNA; 1 sgRNA
- pLH6000 backbone incl. Hygromycin marker

Recovery of homozygous, transgene-free lines in T_1 generation

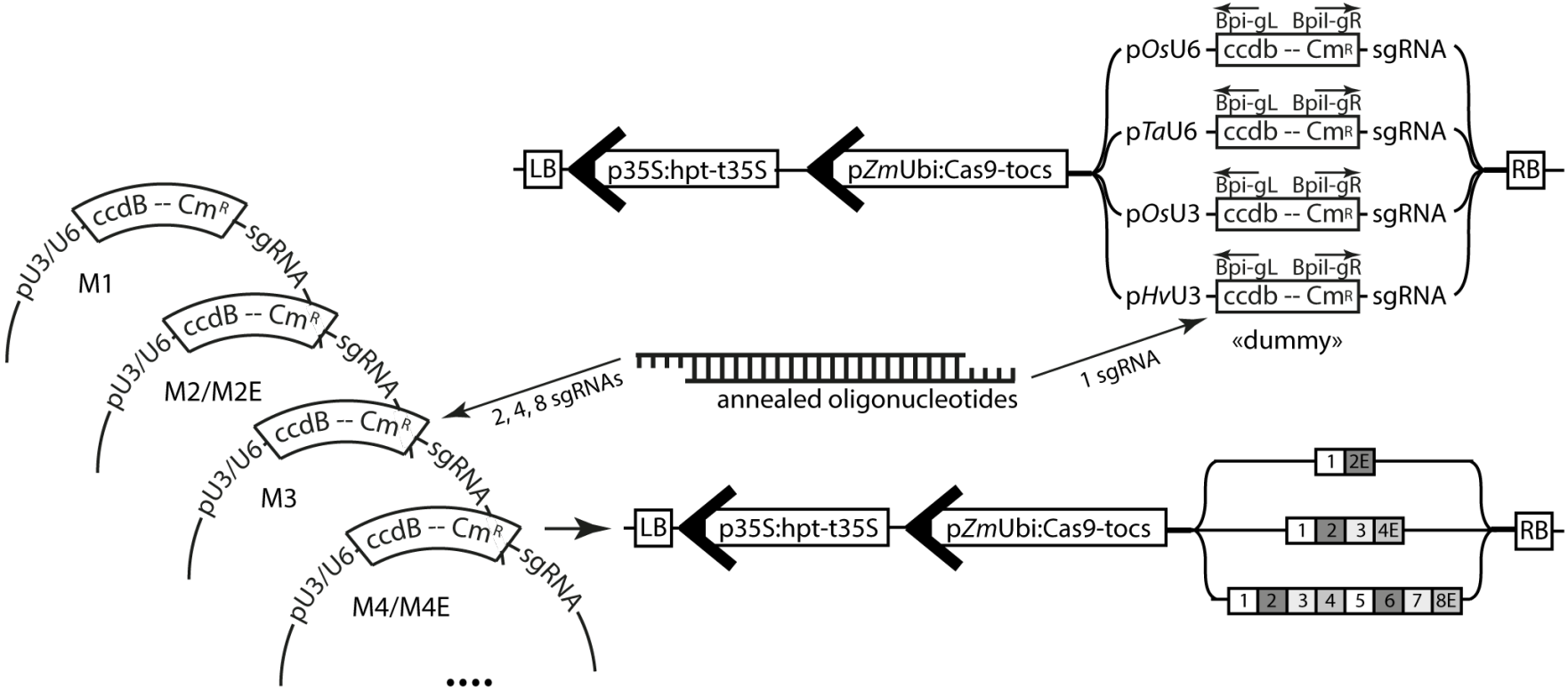
Target **PAM**

```
GGTGTGCAGGAACTTCGGGTGCACCCGCGCGCGGTCGAAGTCCCCTGTCTCCA    WT
GGTGTGCAGGAACTTCGGGTGCACCC-CGCGCGGTCGAAGTCCCCTGTCTCCA    -1
GGTGTGCAGGAACTTCGGGTGCACCCGCGCG--GTCGAAGTCCCCTGTCTCCA    -2
GGTGTGCAGGAACTTCGGGTGCAC----GCGCGGTCGAAGTCCCCTGTCTCCA    -4
GTGTGCAGGAACTTCGGGTGCACCCGCTGCGCGGTCGAAGTCCCCTGTCTCCA    +1
```



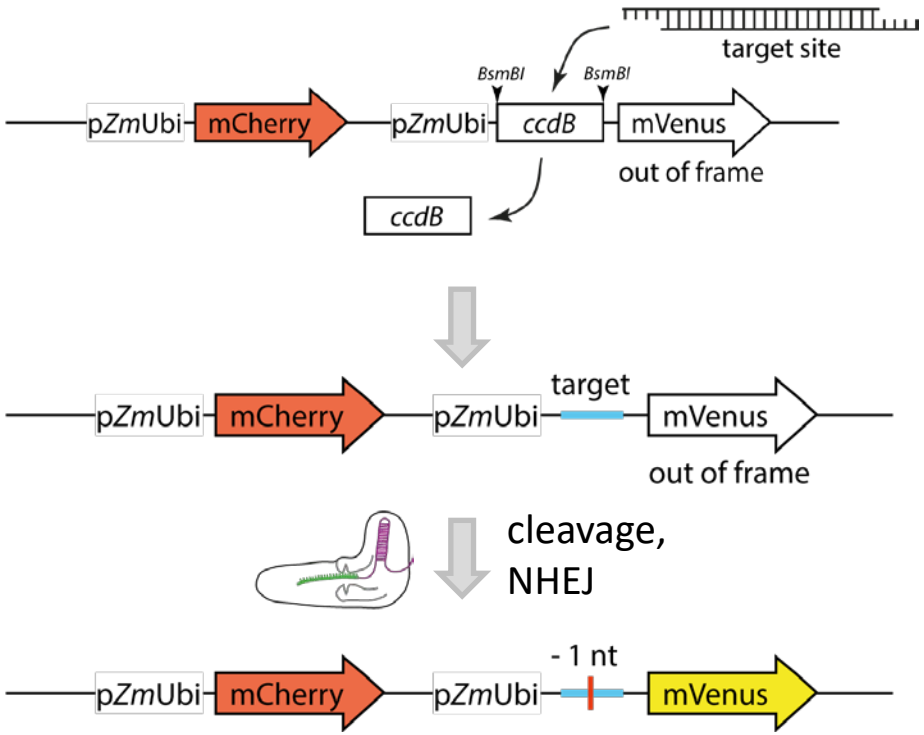
Kumar et al. in preparation

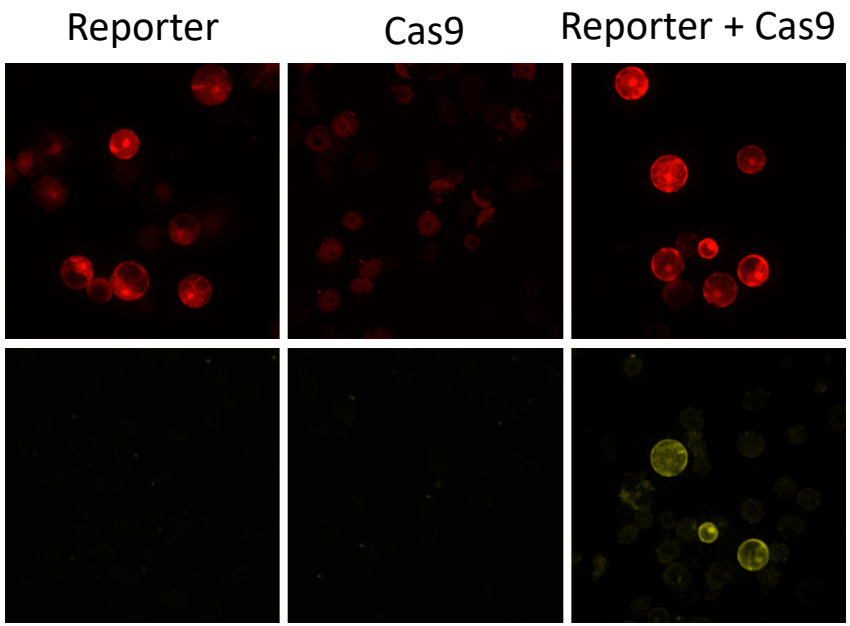
- Convenient system for application in monocots
- *Hv*U3 promoters appears to increase efficiency
- Choice of sgRNA / target site remains an important variable



Kumar et al. in preparation

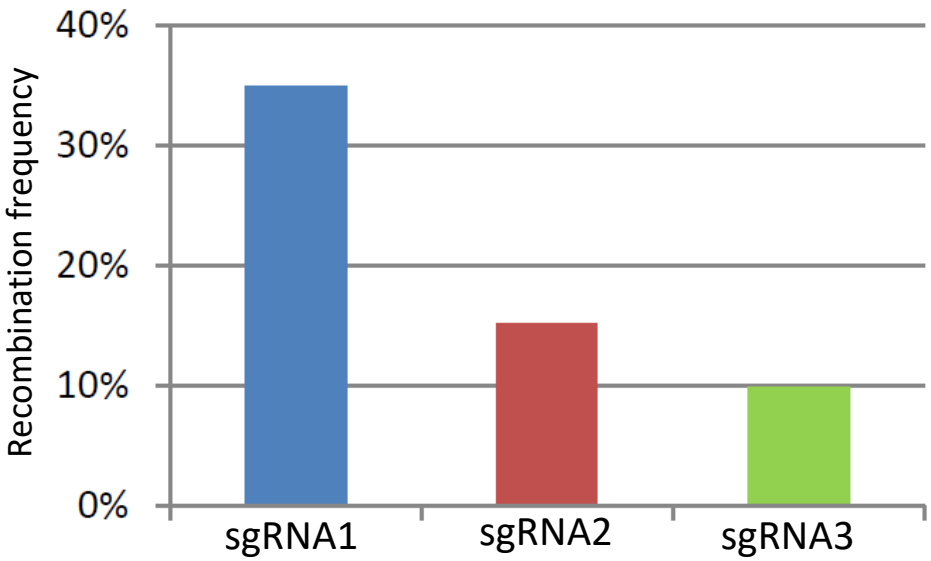
Adaptable fluorescence-based recombination reporters for sgRNA evaluation

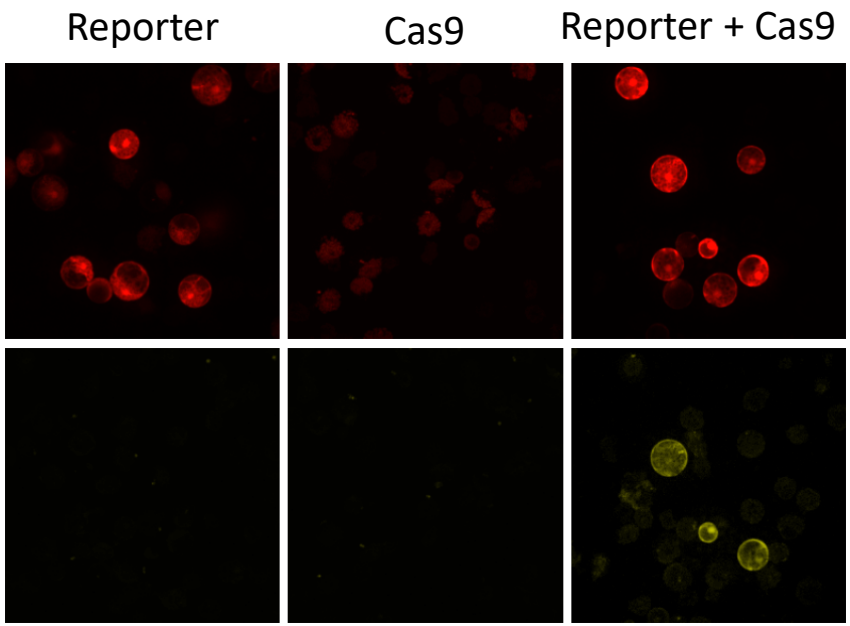




Miriam Lenk, Corina Vlot
Helmholtz Centre Munich

Adaptable fluorescence-based recombination reporters for sgRNA evaluation

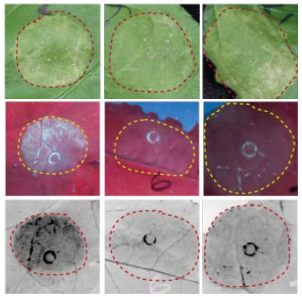


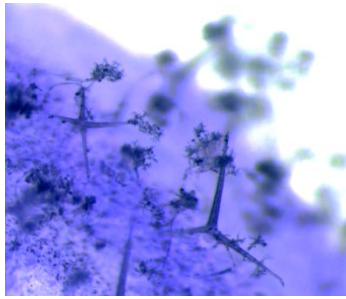


- Differences in sgRNA efficiency are reproducibly measured
- sgRNA selection is one major variable – reporters might allow rapid evaluation
- Are reporter-measured efficiencies meaningful in respect to genomic targets?

Miriam Lenk, Corina Vlot
Helmholtz Centre Munich

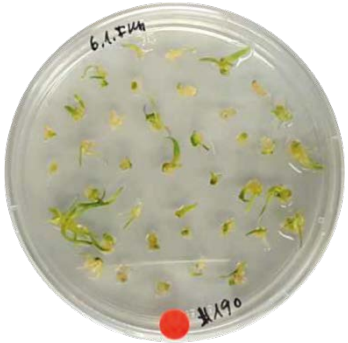
University of Halle:
Johannes Gantner (PhD)
Jana Ordon (MSc)
Theresa Ilse (MSc)
Carola Kretschmer (Tech)

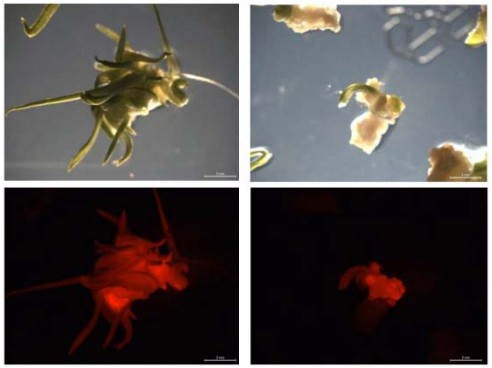




University of Gießen:
Neelendra Kumar
Jafargholi Imani
Karl-Heinz Kogel

JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN





Helmholtz Centre Munich:
Corina Vlot
Miriam Lenk

HelmholtzZentrum münchen
German Research Center for Environmental Health