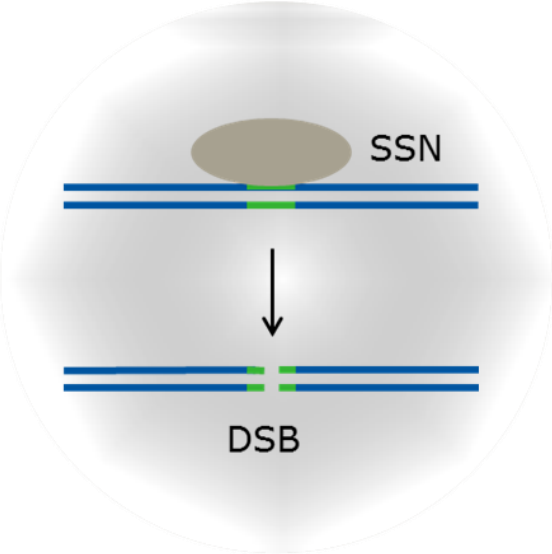
The use of novel editing techniques in (practical) breeding: possibilities and challenges

Richard GF Visser, Plant Breeding, WUR



Breeding (research) projects at Plant Breeding

- Food crops: potato, tomato, cabbage, lettuce, onion, apple, quinoa, button mushroom,...
- Bio-based crops: crambe, camelina, hemp, miscanthus...
- Ornamental crops: lily, tulip, rose...

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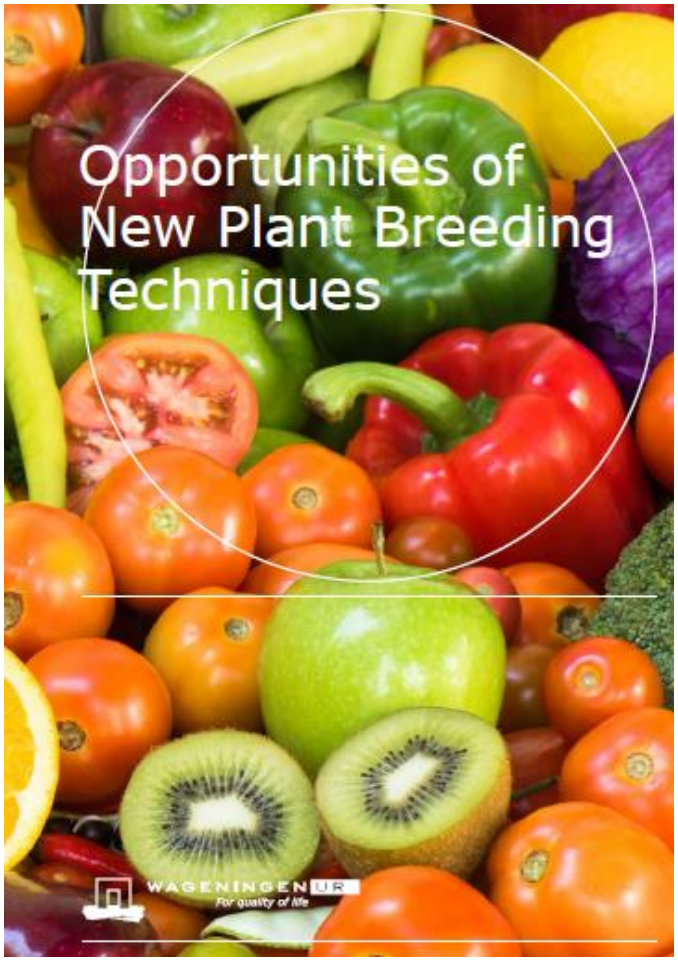
Improved breeding using New Plant Breeding Techniques

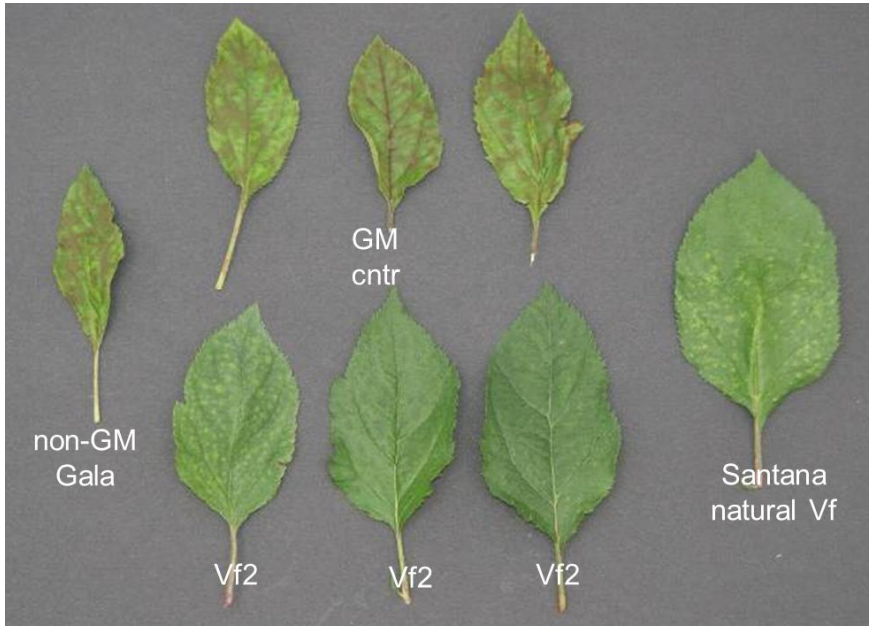
- Improved breeding or rather precision breeding
- Cisgenesis: genetic modification using only genes from the species itself
- Directed mutagenesis



http://edepot.wur.nl/357723

Cisgenic Gala apple

- Apple scab resistance (*Vf2*)
- Red flesh (*Myb10*)



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Directed mutagenesis at Plant Breeding

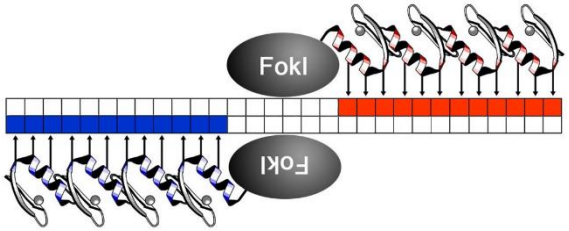
- In the past Zn fingers, Talens, now;
- CRISPR-Cas for mutagenesis in different crops:
  - -potato: R and S-genes, starch, carotenoids
  - -tomato: S-genes, taste attributes
  - -camelina, crambe: oil composition, anti-nutritional factors
  - -wheat: gluten
  - -chrysanthemum: haploid induction
  - -nicotiana: tests
- Many crops are polypoids
- Difficult using conventional mutation-induction techniques

Mutation breeding in polyploids is challenging

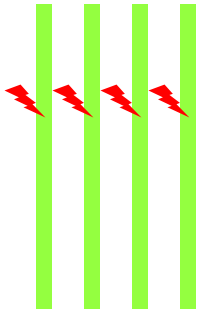
- Polyploids have multiple allelic versions of each gene eg:
  - Potato, tetraploid (4x)
  - Wheat, chrysanthemum, hexaploid (6x)
  - Strawberry, octoploid (8x)

- Knock-out mutation: all alleles have to be targeted

PBR & Dow AgroSciences ZFN project (2009/2010)

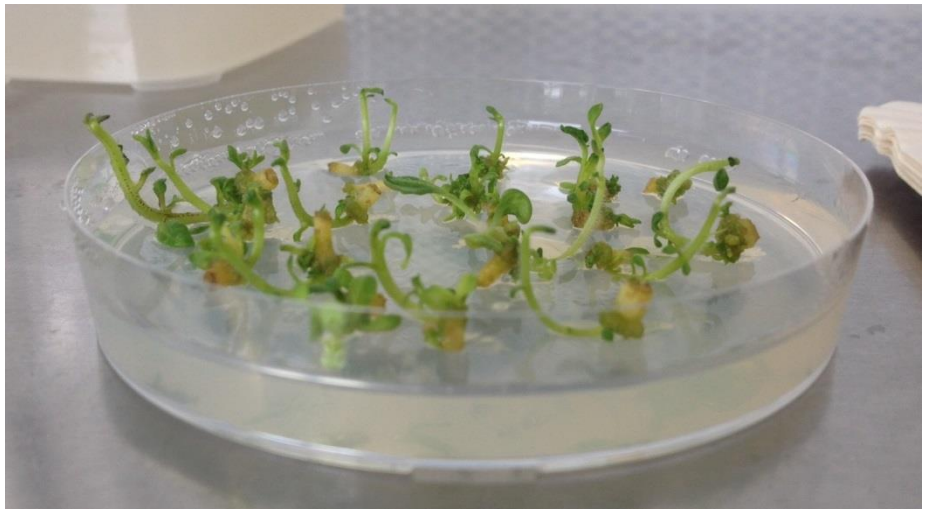


- Research question: can ZFNs induce multi-allelic mutations??
- Targeted mutagenesis in potato using ZFNs
- Target: *SbeII*-gene: starch branching enzyme
- For 'loss of *SbeII*-function' mutation, all 4 alleles have to be targeted



Transformation of potato cv 'Karnico'

- Potato (cv Karnico) is easy to transform
- Stable transformation with *SbeII*-ZFN pairs
  - Selection of ~100 putative transformants (Km^R-shoots)/construct
  - Integration of ZFN construct checked by PCR & sequencing



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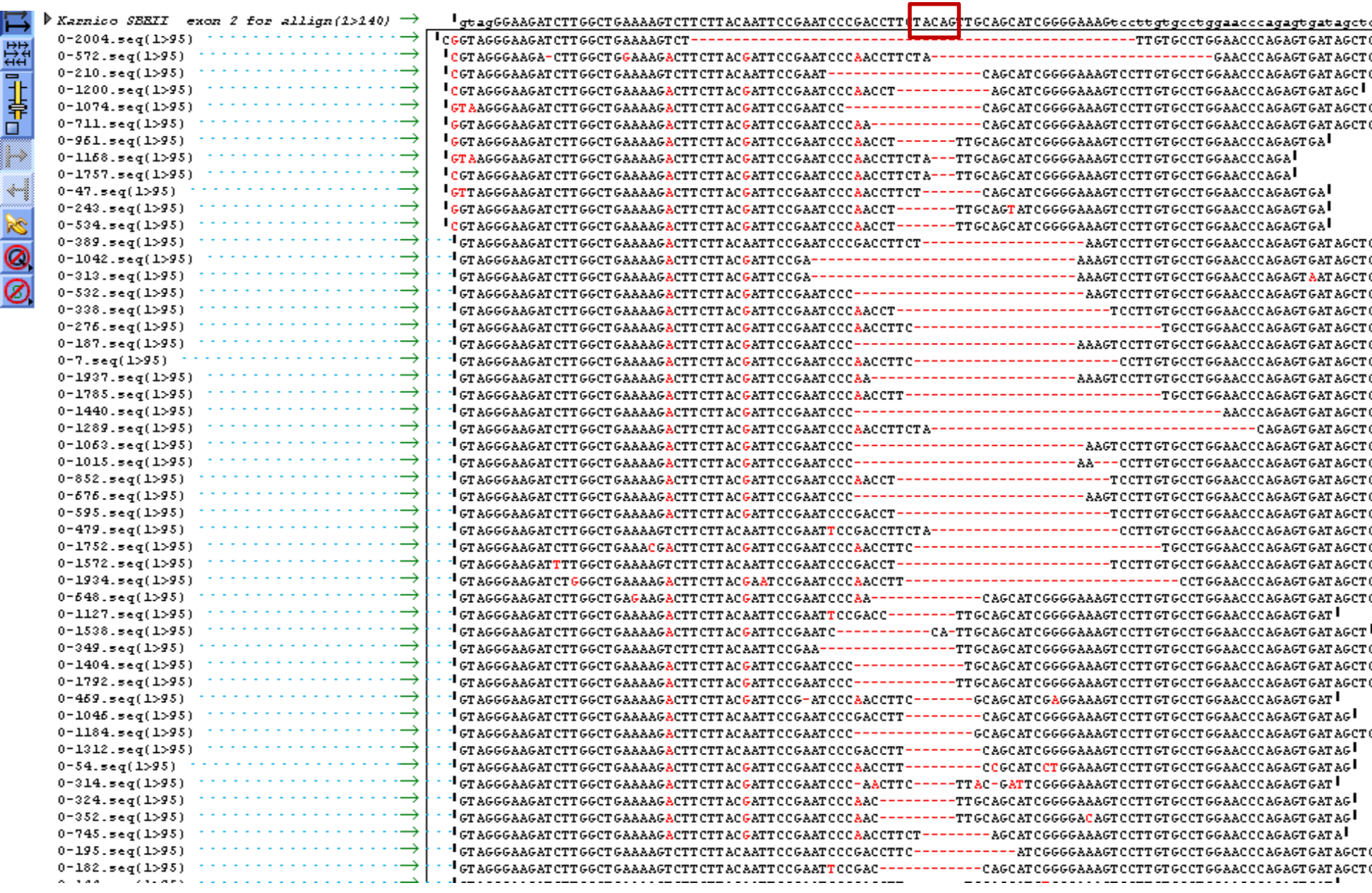
Mutation detection

- Deep sequencing using Illumina Hiseq NGS
- PCR fragments with *SbeII* target site from 80 independent transgenic lines
- 100.000x coverage of each sample, from both sides

- Expected outcome for each independent plant:
  - mutation frequency
  - nature and size of mutation
  - extent of variation

Sample #86: p35S-SBEII-ex2; 727 del/2021 reads (36%)

Target: TACAG



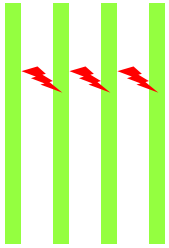
Results from deep sequencing

- 20% of ZFN-transgenic plants show deletions
- In these plants up to 40% of sequences have deletions
- No plants with 100% deletion

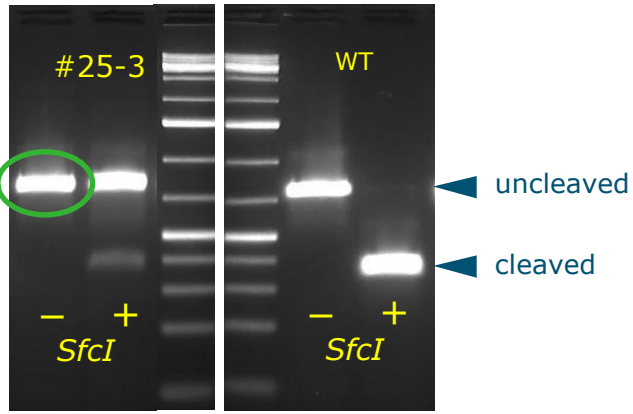
- Deletions found for all four *SbeII* alleles

- Large variability in deletion size, frequency within DNA from individuals: chimerism
- Get rid of chimerism via different approaches (gametes, in vitro regeneration, protoplasts)

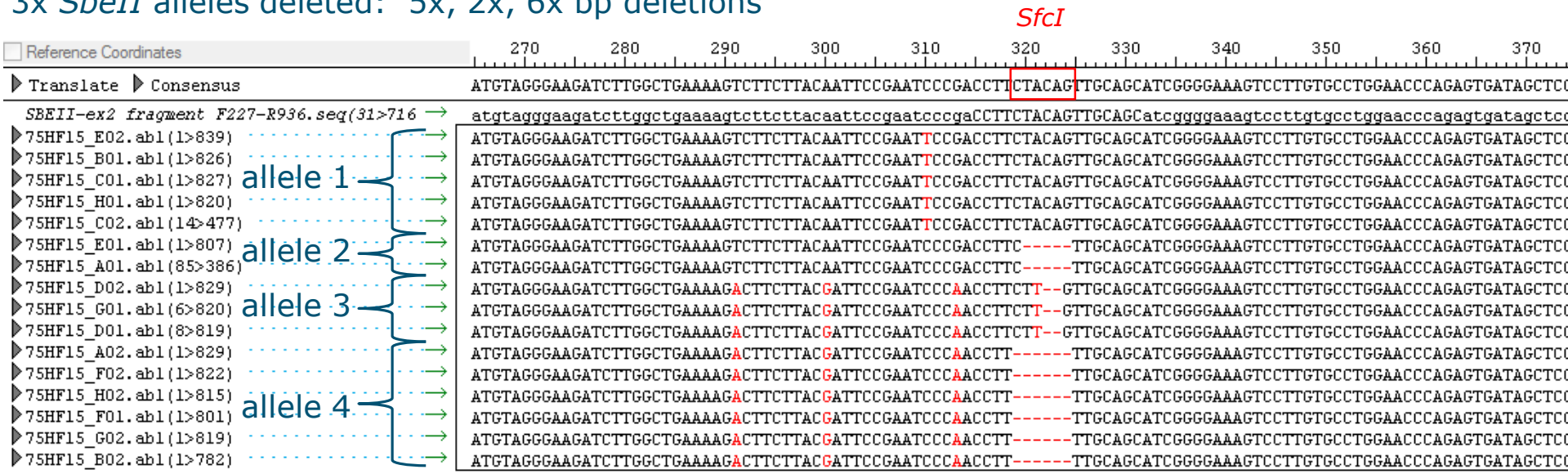
Sequence analysis

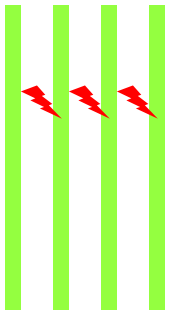


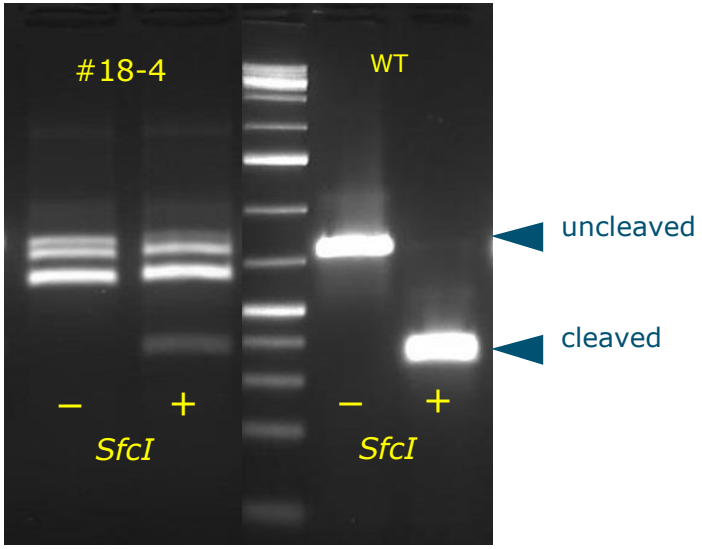
cloning + sequencing
of individual clones



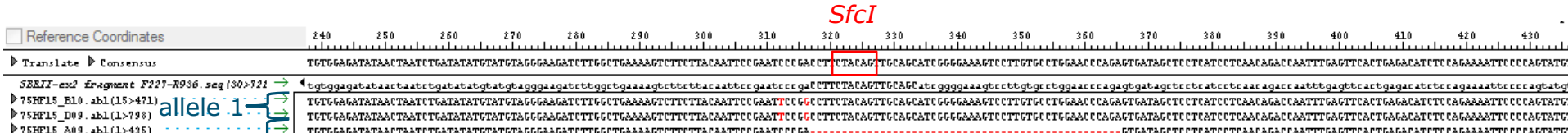
Regenerant #25-3:
3x *SbeII* alleles deleted: 5x, 2x, 6x bp deletions







Regenerant #18-4:
3x *SbeII* alleles with large deletions (49x, 145x, 141x bp deletion)



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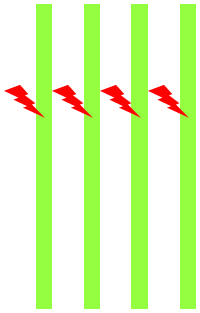
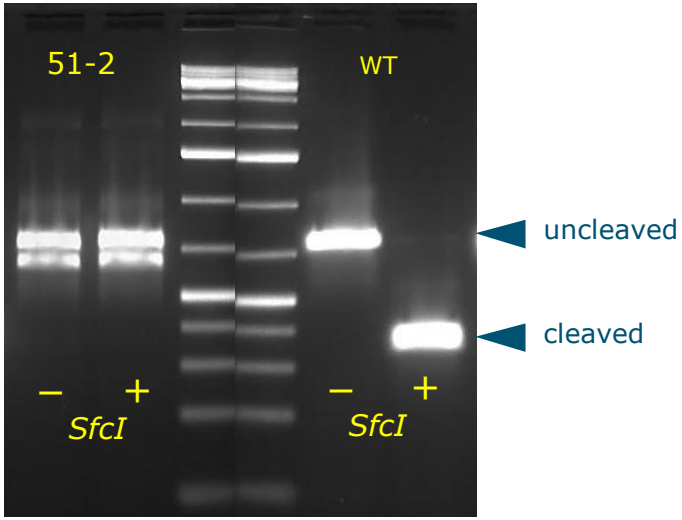
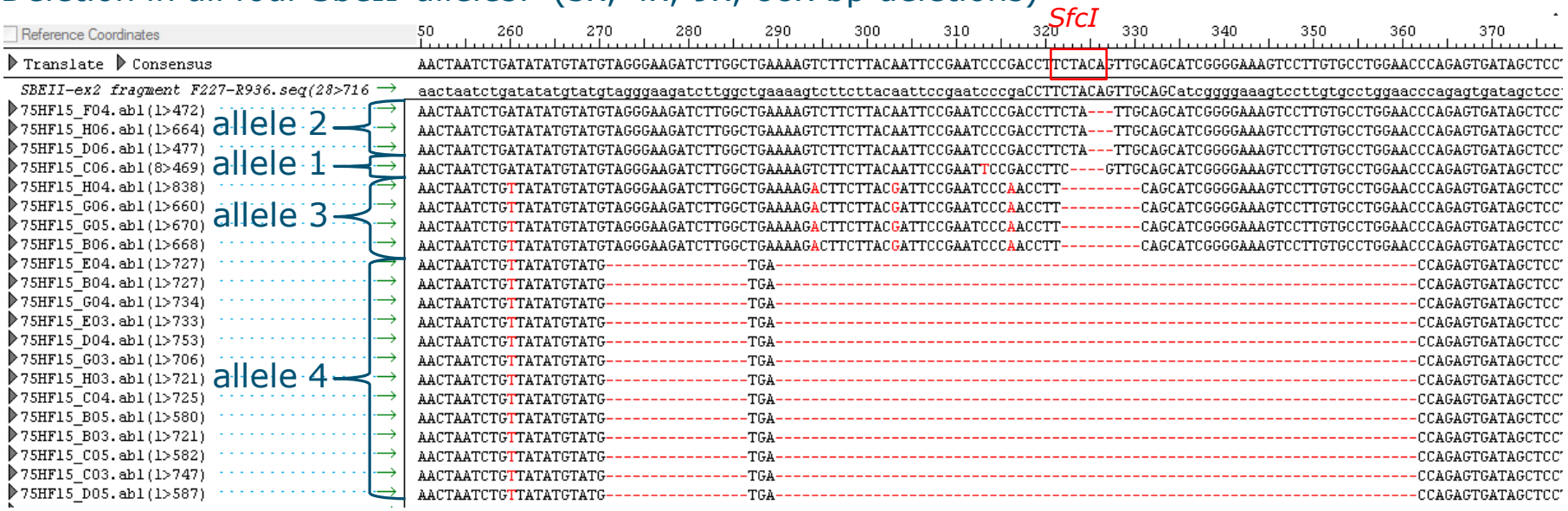
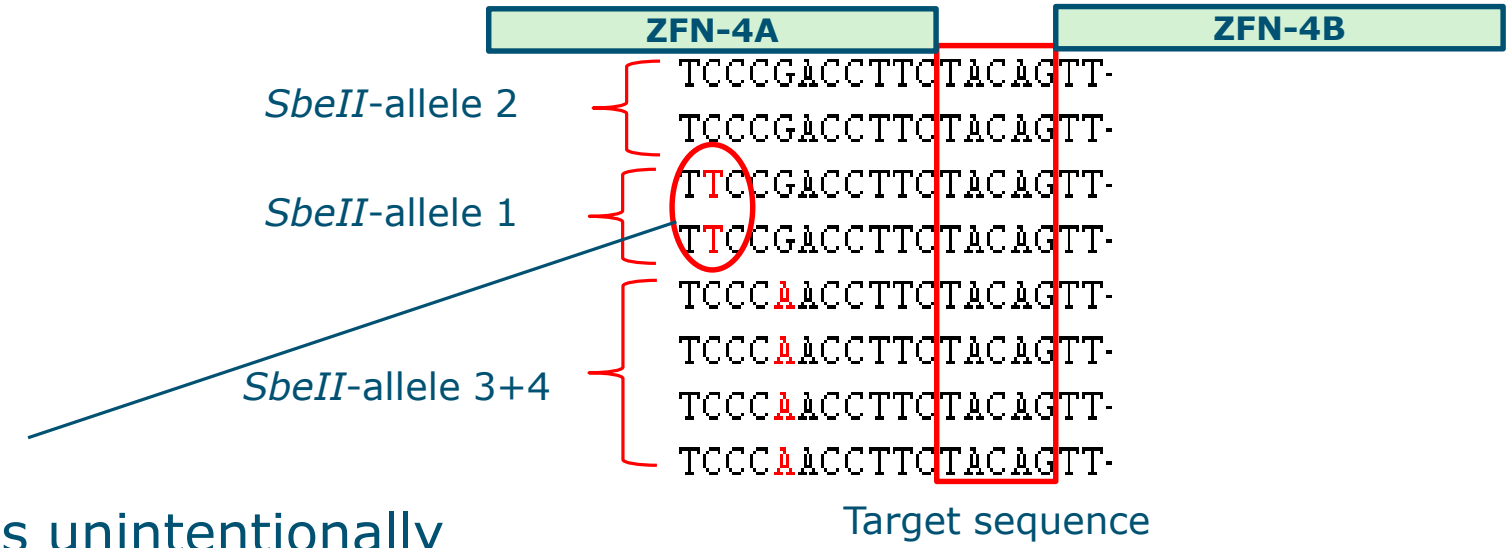




Regenerant #51-2:
Deletion in all four *SbeII*-alleles! (3x, 4x, 9x, 88x bp deletions)



Allele-specificity of ZFN

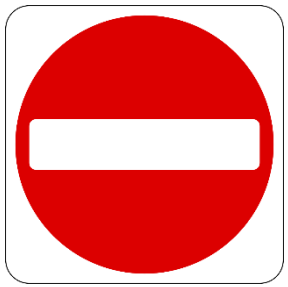


This SNP was unintentionally missed in the ZFN design: allele 1 less frequently targeted

	allele 2	allele 1	allele 3+4
Expected deletion frequency	25%	25%	50%
Observed plant #30	28%	6%	66%

Conclusions

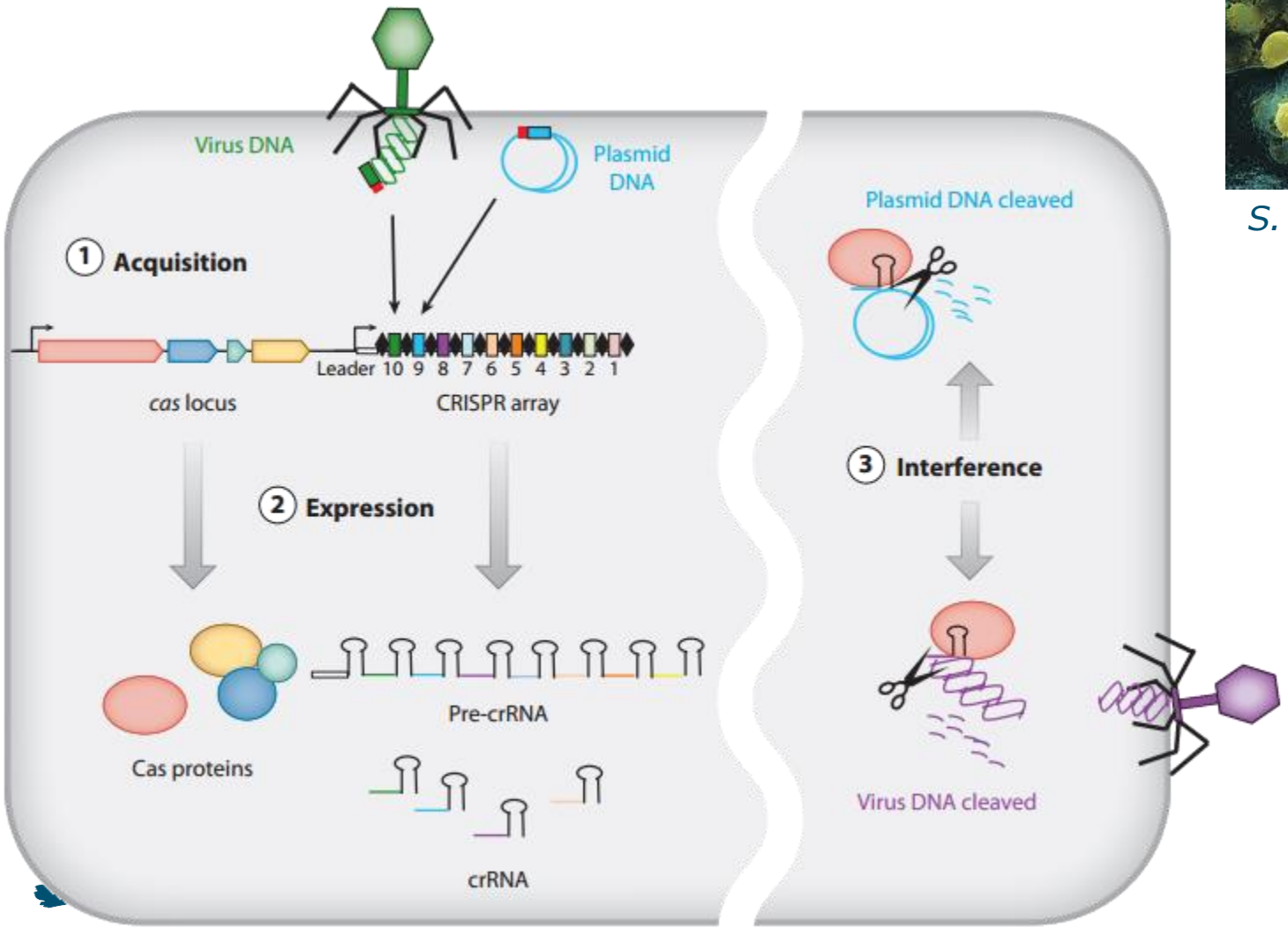
- ZFNs very useful for mutagenesis in polyploid species: multi-allelic deletions are possible
- Transformation process used results in regenerants chimeric for mutations:
  - secondary regeneration is good option to fix mutation
  - But all in all



  - too low chance of obtaining desired results,
  - too expensive,
  - too slow and thus → CRISPR/Cas9

CRISPR/Cas9: Adaptive immune system

S. Pyogenes M1 GAS



Resistance against powdery mildew in tomato using knock down of S genes

The *S* gene *Powdery Mildew Resistance 4* (*PMR4;* encodes a callose synthase. Silencing of the gene with the highest level of homology by RNAi was previously reported to result in resistance to powdery mildew in tomato (Huibers et al., 2013).

Now proof of concept with CRISPR/Cas9

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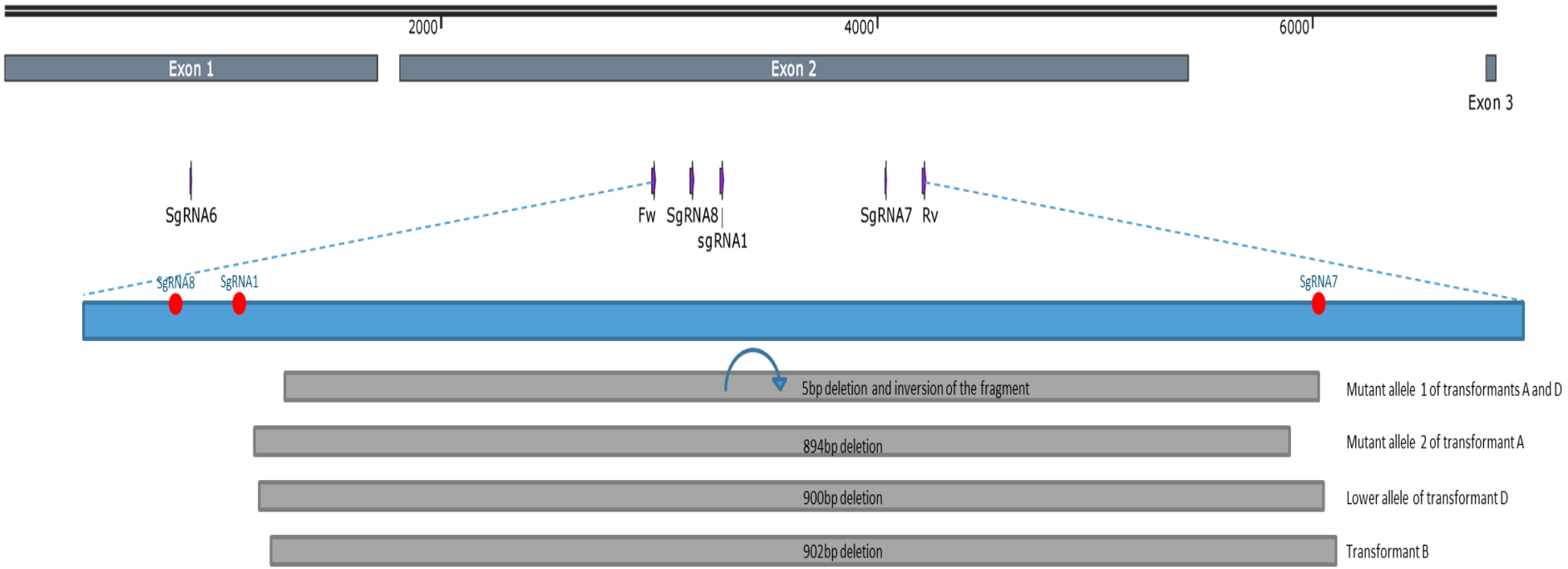
Mutation events in the T1 *pmr4* CRISPR/Cas9 mutant lines.



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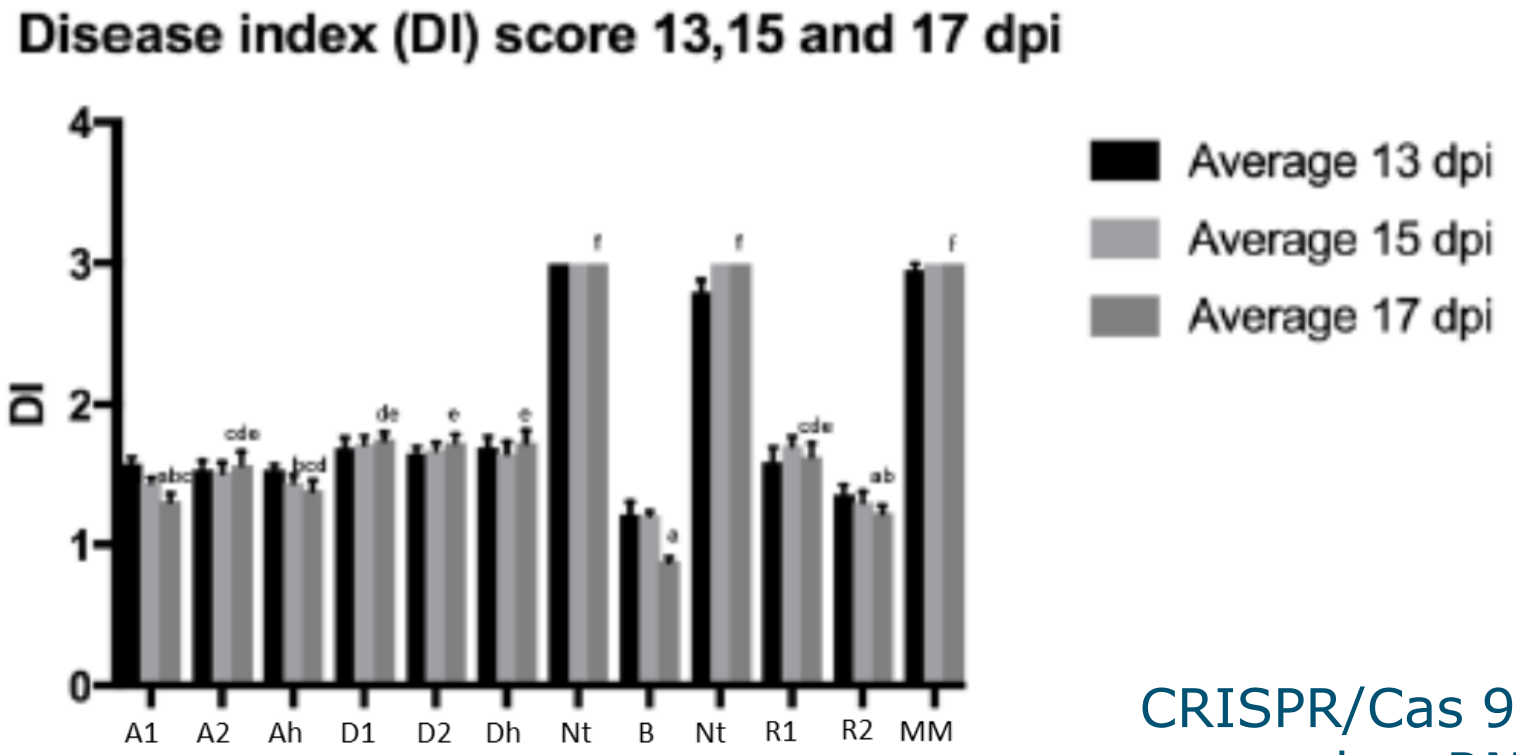
Pathogen characteristics on different lines

		Per Appresorium		Per Primary Haustorium			Hyphae per Infection Unit				
			Primary		Secondary						
Genotype	Primary Ap per IU	Primary HR	HS	Secondary Hyphae	HR	Secondary Hs	1	2	3	4	5
Moneymaker	100	22	80	100	10	85	20	8	30	42	0
Transformant A	88	81.8	11.3	80	20	60	10	10	0	0	0
Transformant B	96	91.6	47.9	95.6	52.1	47.8	16	16	26	2	0
Transformant D	92	84.7	36.9	94.1	29.4	47.05	16	16	12	4	0

Disease resistance test of several lines



CRISPR/Cas 9 works as good as RNAi

Targeted mutagenesis in Chrysanthemum

- "DIYers play God at the kitchen table"

  .... but not with Chrysanthemum.....

- Request from ornamental breeding companies:
  - haploid inducer (haploid = halved genome size)
  - target mutagenesis of CENH3 in Chrysanthemum

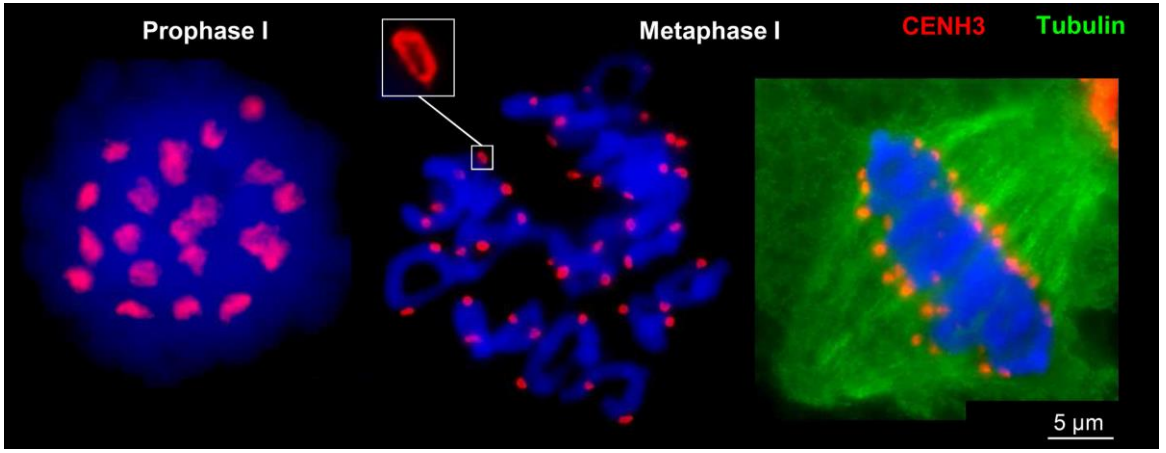
- Fundamental research project supported by 7 breeding companies + Dutch government (Min. Economic Affairs)

Applications of (di)haploids

- Reduced genome helpful for:
  - QTL-mapping
  - Other genetic mapping and genomic studies
  - Hybrid breeding (reverse breeding)
  - In tetraploids: crossing of dihaploids with diploid wild relatives
  - etc.

Ravi and Chen (2010): haploid induction by CenH3-modification

- Centromere-specific histone H3 variant
  - Centromeric histone protein
  - Present in active centromeres, binds directly to DNA
  - Chromosome segregation in mitosis and meiosis



Schubert et al (2016) Front. Plant Sci. 7:28.

CRISPR-Cas induced modifications of chrysanthemum CmCenH3-NTT

- Protocol:
  - design gRNAs targeting CmCenH3-NTT
  - construct CRISPR-Cas-transformation vector
  - introduce in plant by transformation
  - screen for mutations

no WGS available

two paralogs: CenH3A + CenH3B

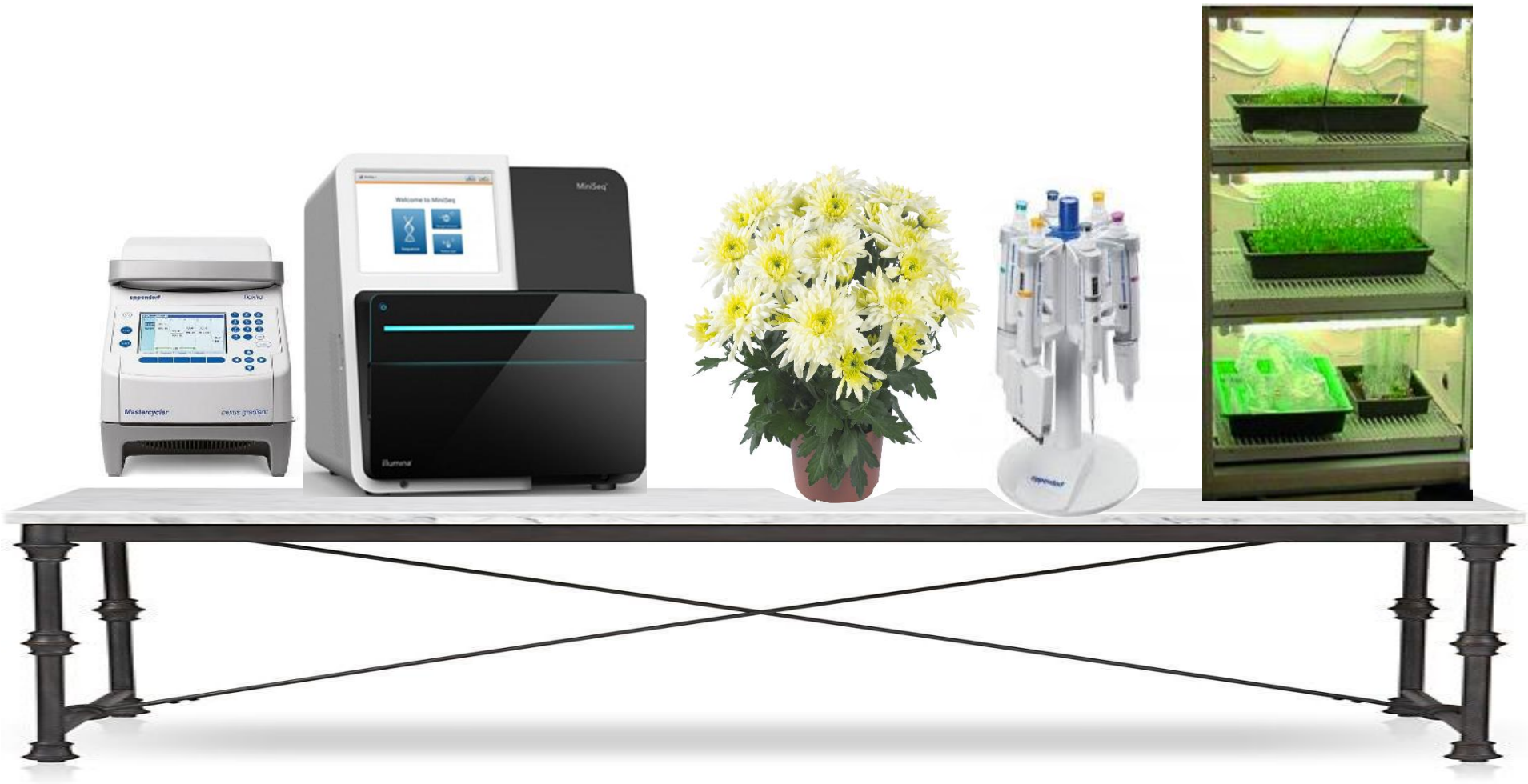
hexaploid! 2x6=12 copies

400bp cds contains 6kb introns

pCaMV35 is not very active in chrysanthemum

strong genotype-effect on success of transformation

Chrysanthemum-CRISPR-Cas on kitchen table



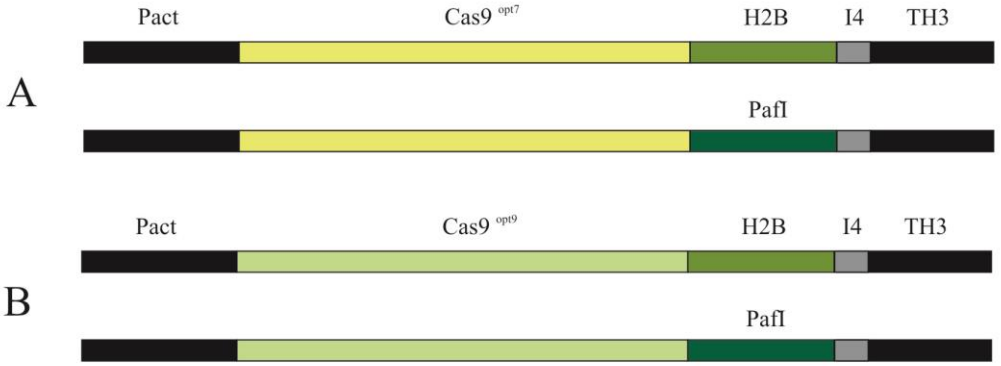

CRISPR
experiments
in
mushroom

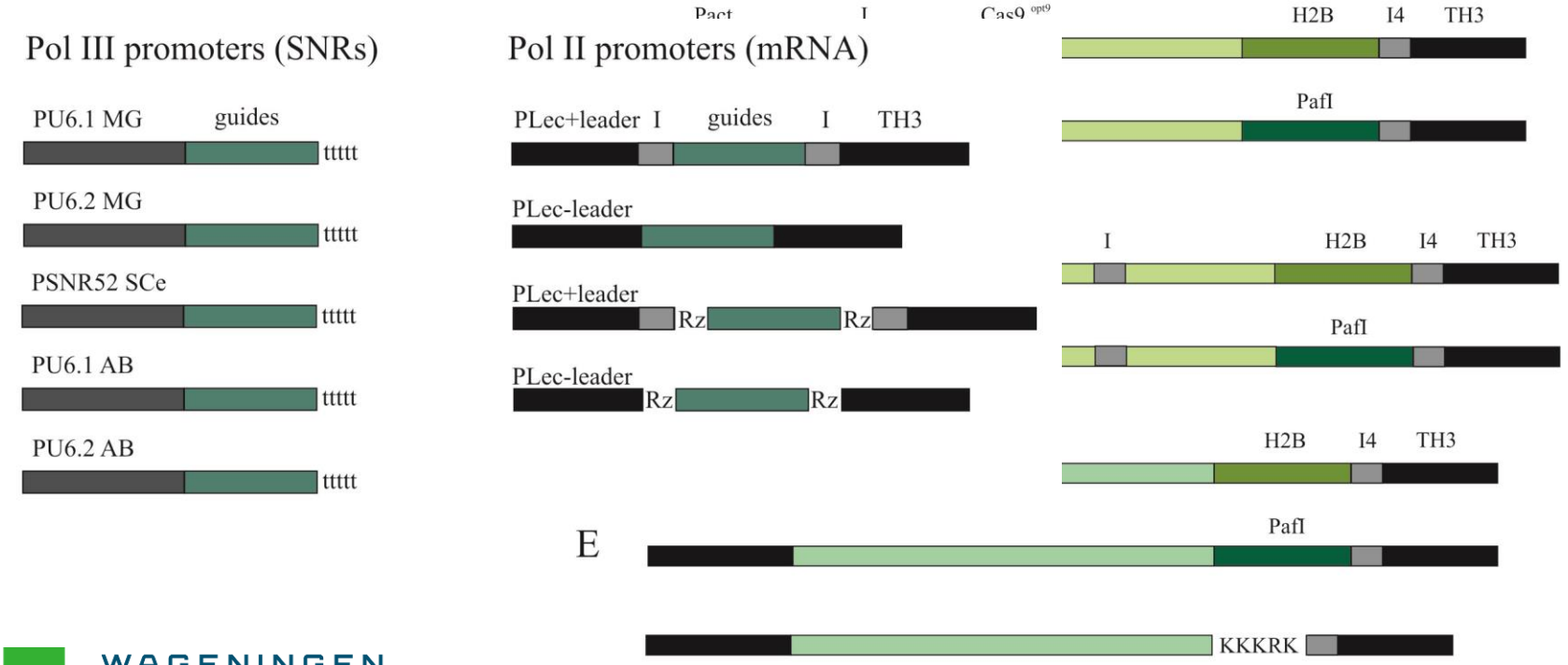
Use to study role of genes involved in

- Homologous recombination
- Development spores (MSH4)
- Vegetative incompatibility
- Strain stabiltity
- Resistance/susceptibility to diseases
- Substrate degradation
- Fruiting body formation
- .....

.....and one of the first officially approved CRISPR/Cas products in the USA (patent protected)

Many different constructs made and tested with different targeting signals and promotors





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Cas9 opt:

- Codon optimized for *A. bisporus*
- Cryptic introns removed
- Constructs containing introns '(5' and 3')

5' SS:

G | **G T** G/A A G T

Internal SS:

C/T G/A C **T** G/A A C/T

3' SS:

C/T **A G**

45-60 bp

8-10 % >GC exon

- Based on knowledge obtained *S.commune*
- Examples: hAAT
  Antibodies
  MnPeroxidase
  CnVS
  GCB
  GnTI & II
  Laccase
  Mannosidase

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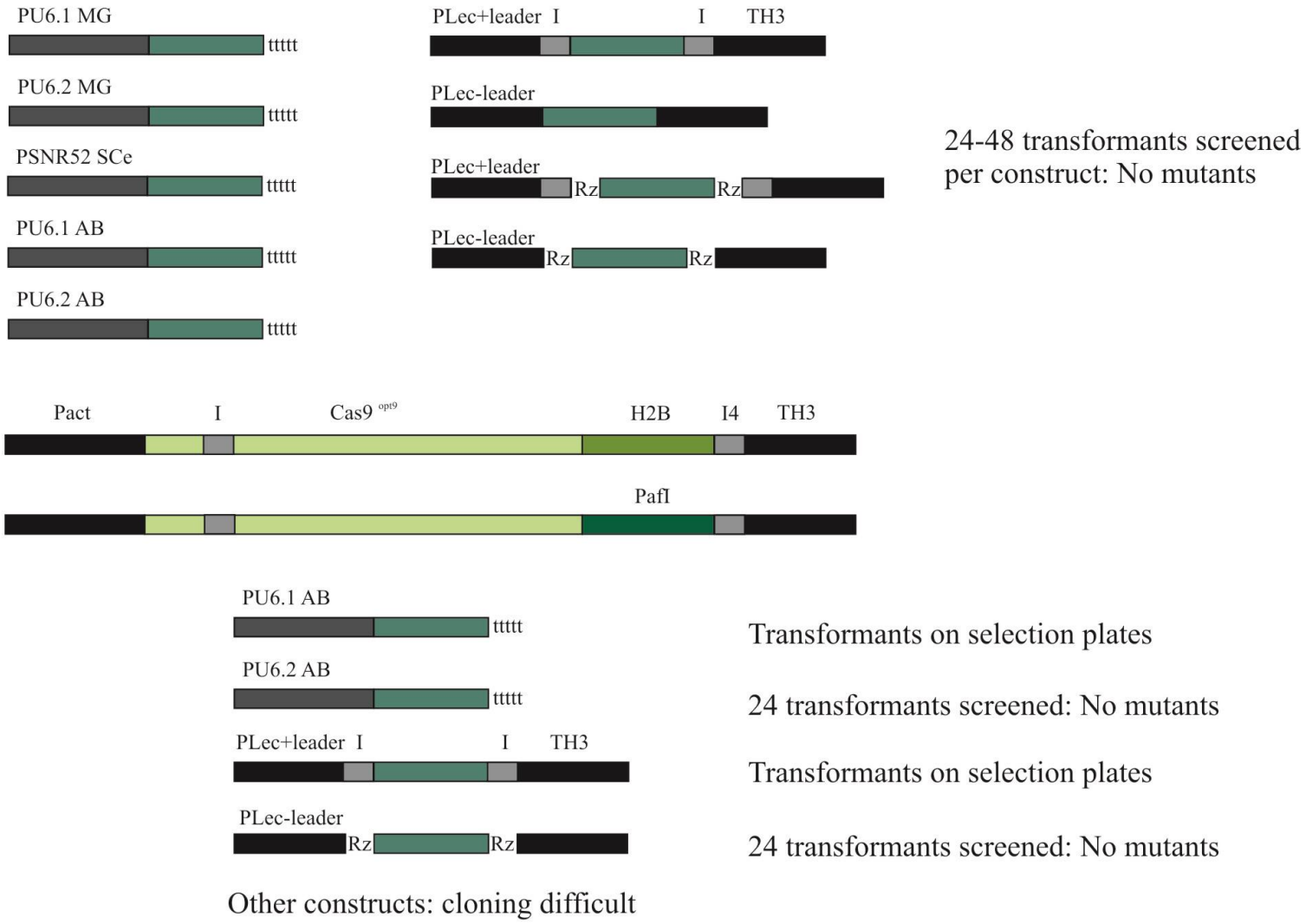

Despite many attempts no mutants obtained with CRISPR/Cas9 in button mushroom.....

Two new oil crops, crambe and camelina

- *Camelina sativa* (camelina, NL: huttentut, deder)
- *Crambe abyssinica* (crambe, NL: Afrikaanse bolletjes kool)
- *Brassicaceae*
- both allohexaploid

Changing oil profile, improving seed meal

- Reduction in poly-unsaturated fatty acids (PUFA)
  - PUFA < 10 % desired
  - higher level of C18:1, C20:1, C22:1 needed
  - Target: fatty acid desaturase (*FAD2) should be knocked out*
- Co-products should be higher value (animal feed)
  - elimination of anti-nutritional factors
  - *no glucosinolates, no sinapine*

Challenges

- Multiallelic deletions required
  - allohexaploids; A,B,C-genome
    (but self-pollinators; 2-3 generations/year)
- Recalcitrant in transformation
  - Camelina: floral dip of greenhouse plants
  - Crambe: '*in vitro*' transformation

Camelina: floral dip, DsRed as visual marker

- Optimized transformation protocol:
  - 2-3% transformation efficiency
  - 5,000-10,000 seeds/transformation; DsRed selection

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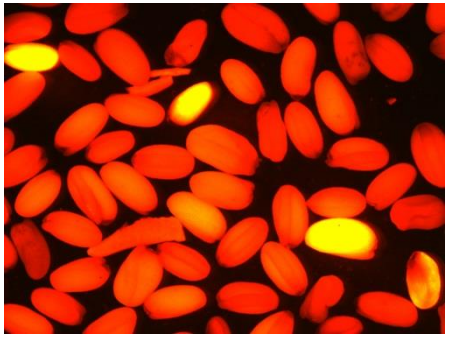
Transformation with 35S-Cas9 en DsRed

T0: flowerbud transformation



T1: select seeds

Mutations accumulated in part of somatic tissue

T2: high u**niformity** of transgene seeds

Accumulation of mutations in somatic tissue

pEC-Cas9 Mutation detection in (end) T1 generation!

Mutation detection (ongoing)

Changed oil composition?

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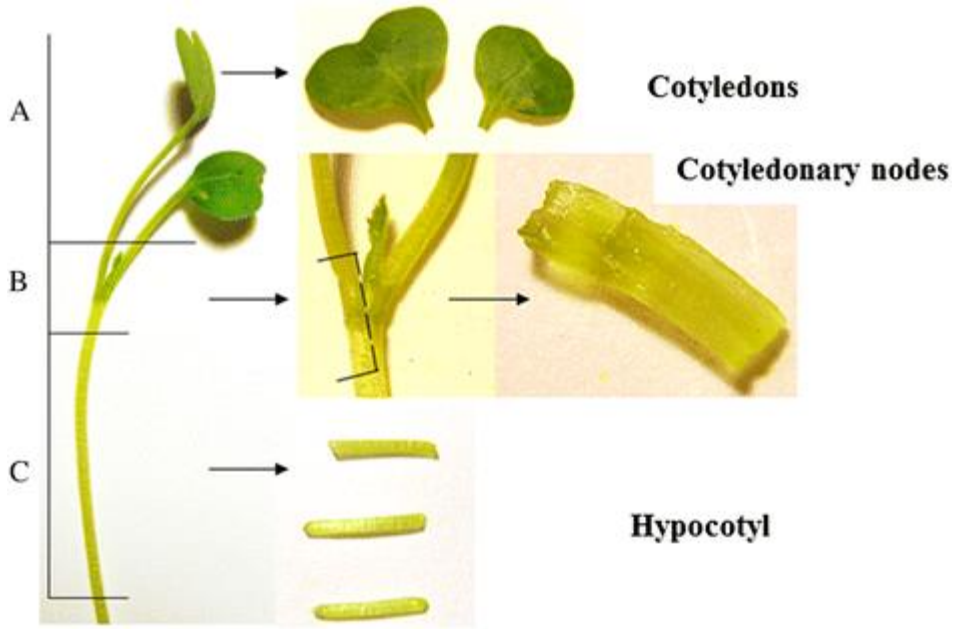
Outcome sofar in Camelina

- Many problems with creating constructs with measurable activity
- Promotors seem to be very crucial
- Different guides in one construct seems to be a must
- Many different transformants in the making
- Literature is available where mutants have been obtained in this crop.....but not straightforward

Crambe: '*in vitro*' transformation

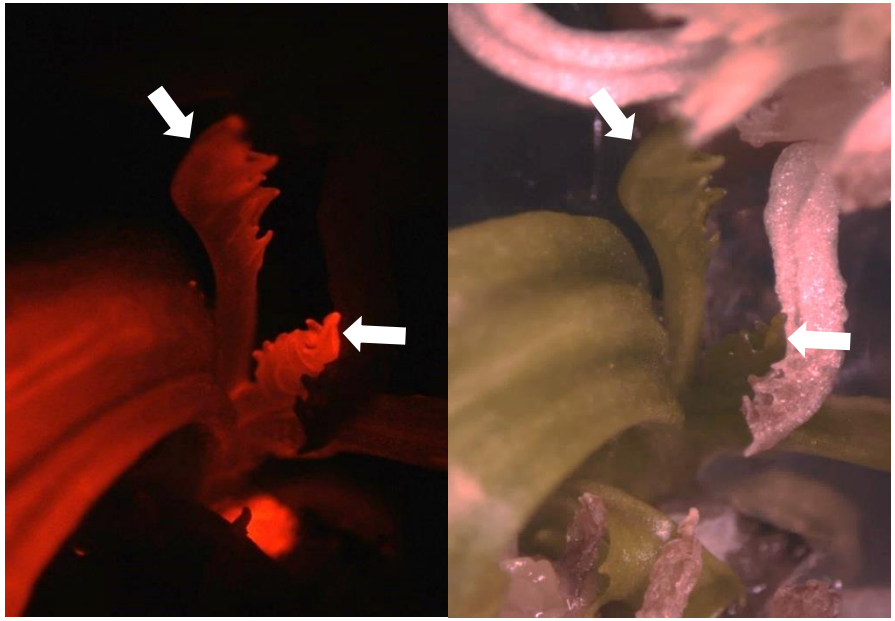
- Supervirulent *Agrobacterium* stain AGl0
- Binary vector with - *nptII* (kanamycin selection)
  - *Cas9* + *gRNA* (CRISPR-Cas)
  - *DsRed* (visual marker)

'*In vitro*' transformation starting with crambe seedlings



Crambe: '*in vitro*' transformation

- Crambe is very sensitive to kanamycin:
  - low kanamycin selection pressure; alternating cycles of selection/no selection: chimerism
  - repeated chopping of plants and selection to get rid of chimerism: subclones
  - DsRed for manual selection of transgenic tissue



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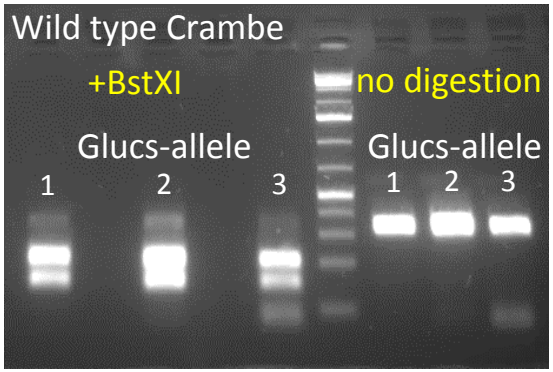
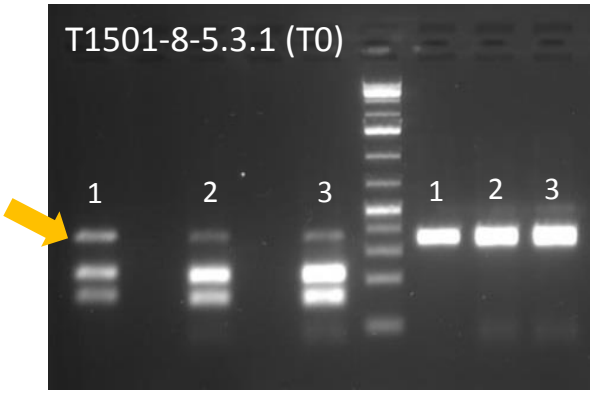
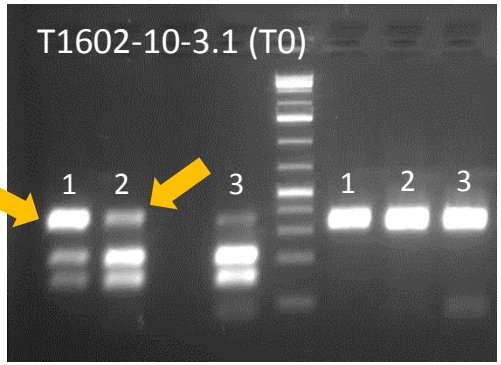
Kanamycin-resistant plants to greenhouse

Selected transgenic event (T0)	Nr of subclones tested	Nr of subclones with mutations	T1-offspring
T1501-2-3	41	0	
T1501-8-3	9	0	
8-4	9	0	
8-5	29	**2**	V
8-6	13	0	
T1602-10-2	2	0	
10-3	39	**19**	V
T1602-11-1	2	0	
T1603-10-1	26	0	
10-2	1	0	
T1604-11-1	1	0	
11-2	1	0	
11-3	1	0	
T1605-10-1	1	0	
10-2	1	0	
10-3	8	0	







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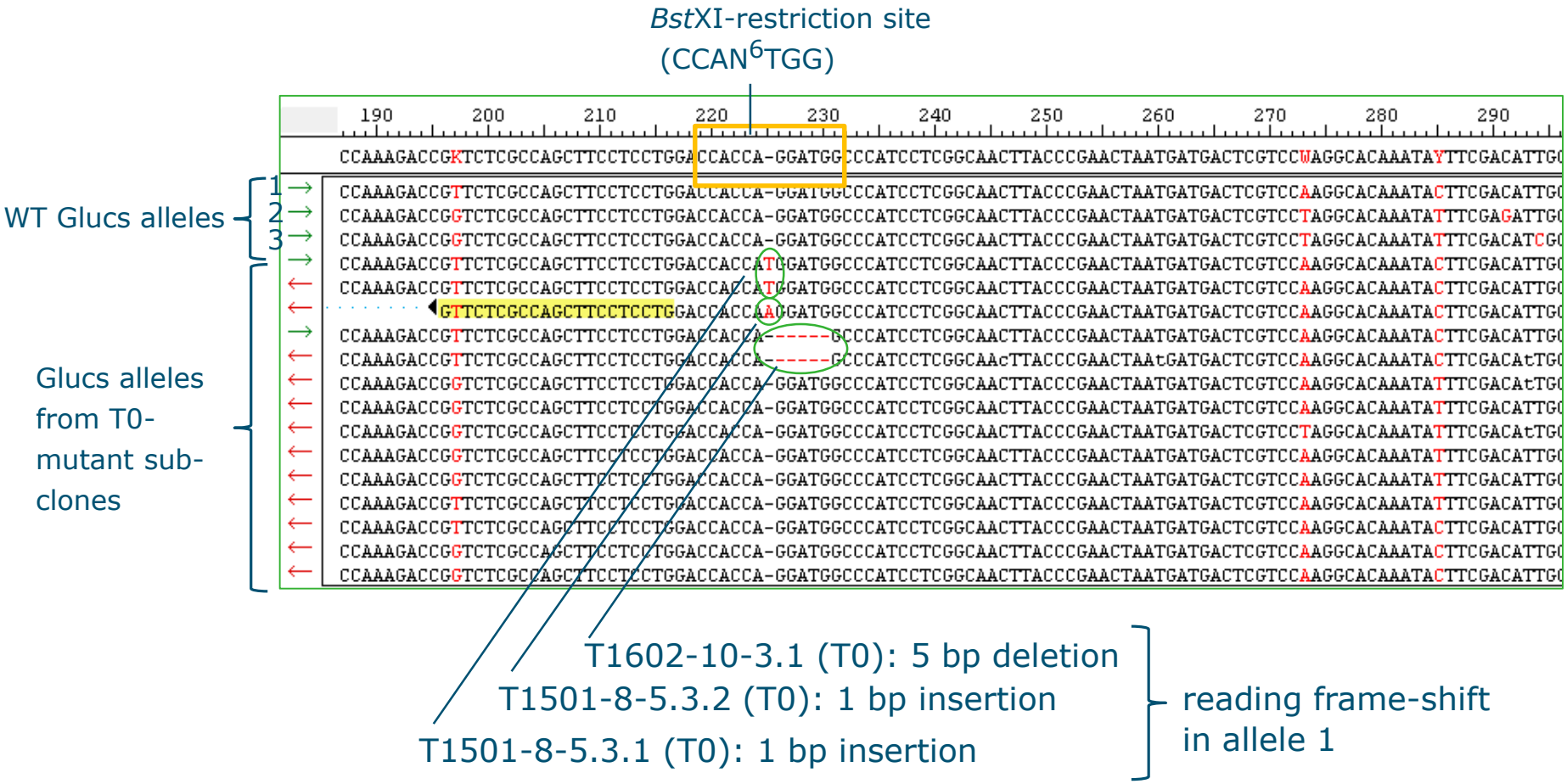
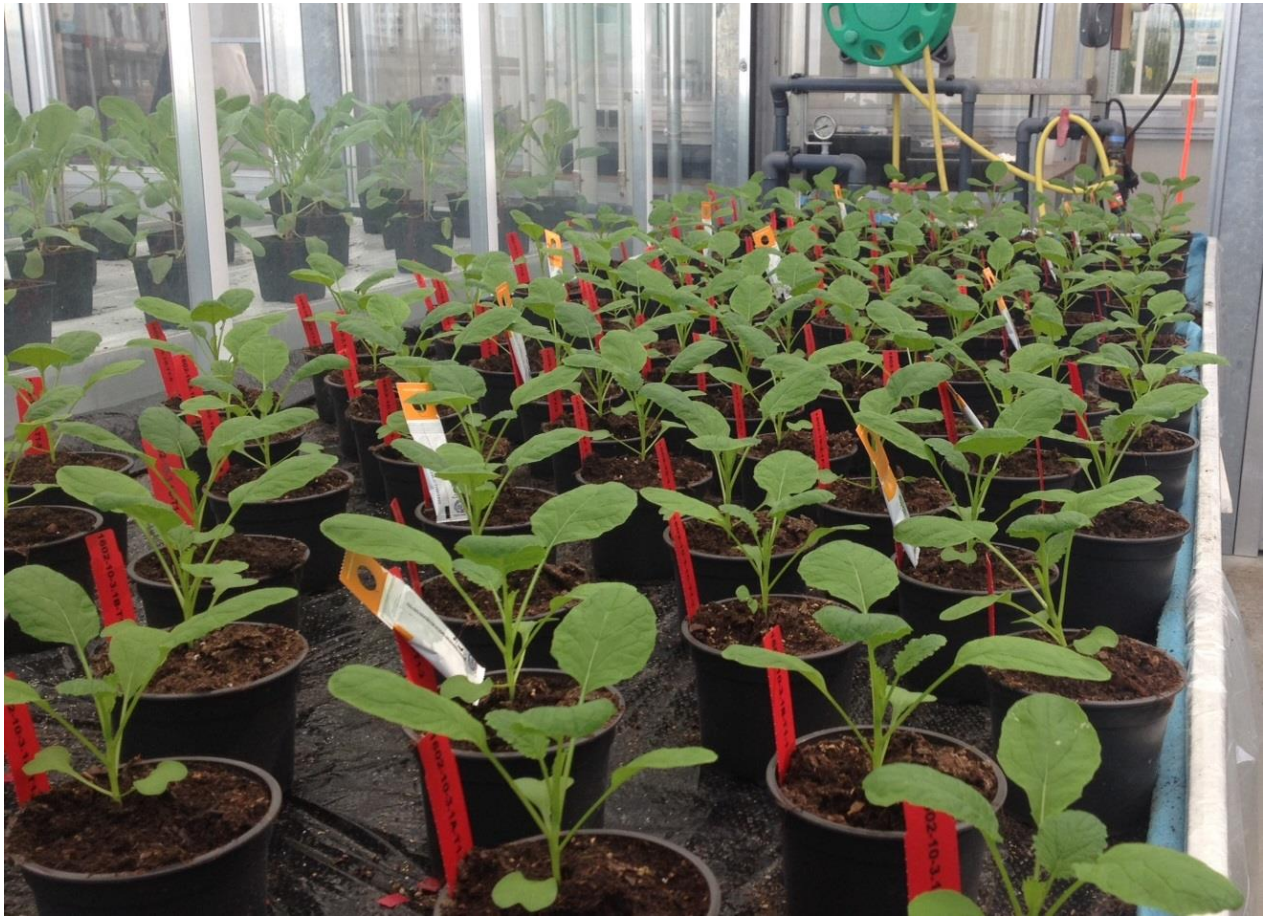
16 primary T0-events > 2x resulting in subclones with mutation

Preliminary screening: sequencing of cloned PCR products enriched for mutations



T1-offspring: screening for homozygous mutations, glucosinolates, selfing



Summary

- Targeted mutagenesis is possible in various crops
- Multi-allelic mutagenesis can be achieved in polyploids
- Limited species-specific genomic info complicates application of targeted information
- Fine tuning required in many systems; promoters, signal sequences, guides etc
- Effective transformation protocols for delivery are required
- Different legal status in many continents (US clear, Argentina new technique but clear regulation, Europe uncertain)

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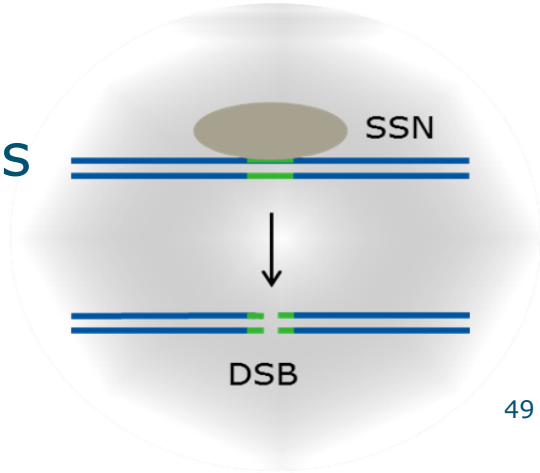

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