Breeding and biotechnology in Argentina: a sugarcane genetics perspective

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Sugarcane is a major crop that provides sugar, bioenergy and biomaterials

Courtesy of Ledesma SAAIC

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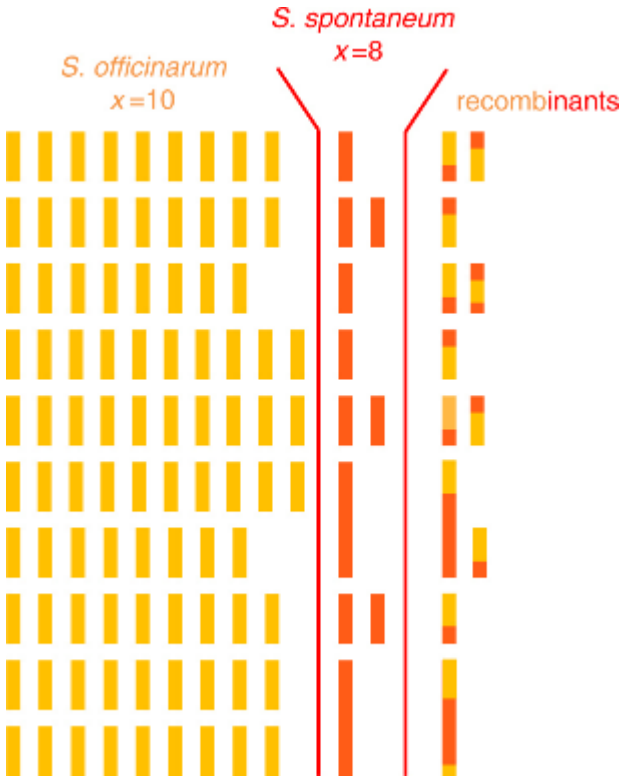
Chacra Experimental Agrícola Santa Rosa (est. 1951) develops sugarcane varieties for cultivation in Jujuy & Salta

Breeding sugarcane (*Saccharum* spp.) is a major challenge because its interspecific genome is highly polyploid, aneuploid, and heterozygous



Garsmeur et al Nat Commun. 2018; 9: 2638.
doi: 10.1038/s41467-018-05051-5

Breeding a sugarcane variety demands years (14 at Chacra) of hard work

Table 8 Clonal selection stages of Chacra's Sugarcane Breeding Program

Stage (number of clones)	Plot size/trial design	Years	Sites	Selection criteria
Stage I: seedlings (250,000)	Individual seedlings/mass selection	3	1	Visual assessment (agronomic type and resistance to diseases), brix and ratooning ability
Stage II: first clonal stage (3000)	1 row, 6 m long/ unreplicated	2	1	Visual assessment (agronomic type and resistance to diseases); stalk number, stalk weight and brix (3 records between early and mid-harvest season)
Stage III: second clonal stage (250)	3 rows, 5 m long/ unreplicated	3	3	Visual assessment (agronomic type and resistance to diseases); stalk number, stalk weight and sucrose content (early, mid- and late harvest season)
Stage IV: multi-environment variety trials (20–25)	3 rows, 10 m long/ RCBD* (3 replicates)	3	10	Visual assessment (agronomic type and resistance to diseases); cane yield, sucrose content (early, mid- and late harvest season); maturity curves and ratooning ability
Stage V: macroplot (3–5)	6 rows, 70–100 m long/ RCBD (3 replicates)	3	10**	Visual assessment (resistance to diseases); cane yield; estimated sugar yield; herbicide phytotoxicity and maturity and tillering curves

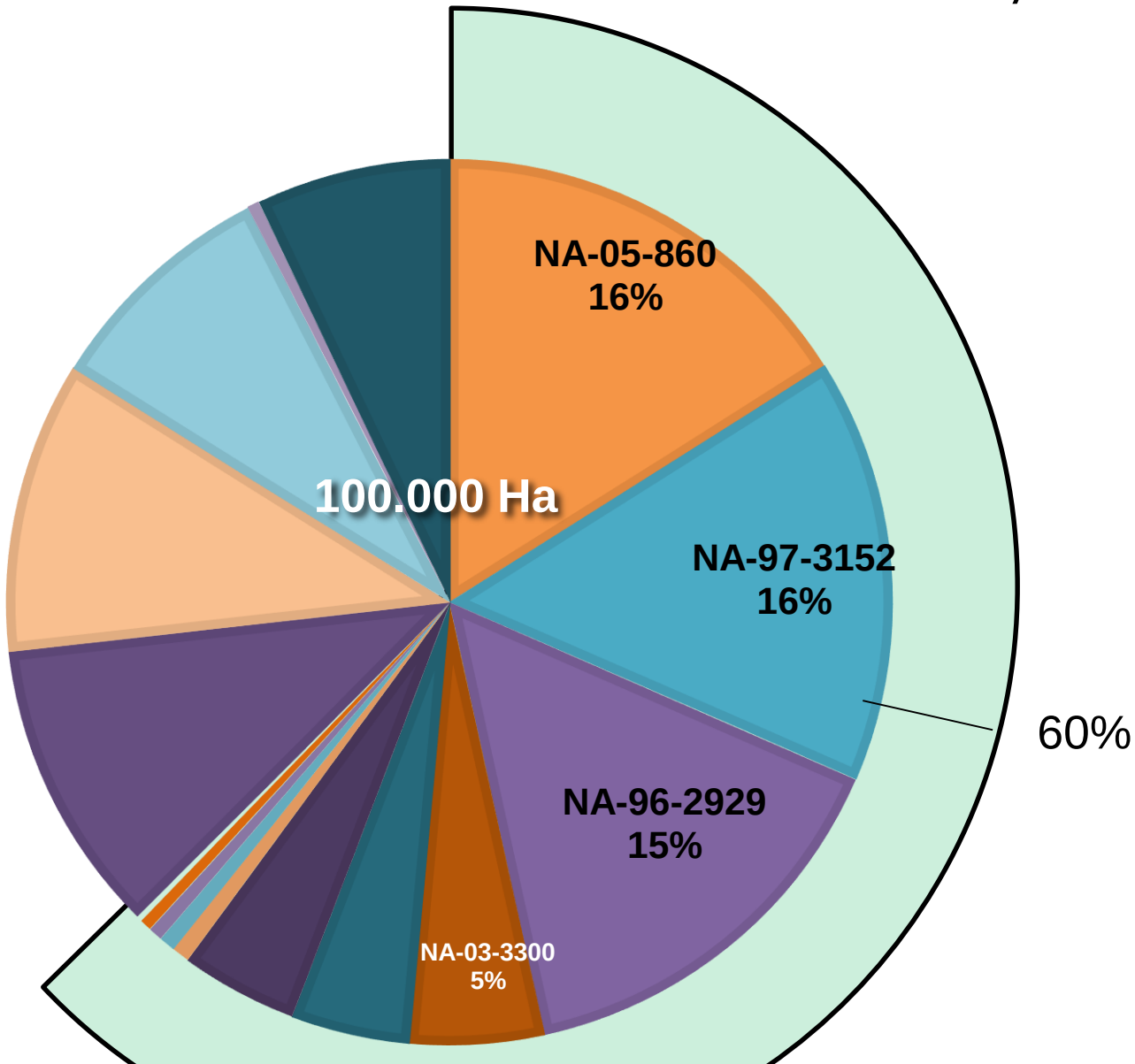
**RCBD* Randomized complete block design

**Trials are not planted at every location every year

Chacra´s breeding program has developed varieties that have been widely adopted in Argentina, Brazil and Bolivia

NA56-79, widely adopted in Brazil, was arguably the most planted sugarcane variety in history

In 2022, NA cultivars occupied nearly 60% of Jujuy and Salta.
Adoption of NA varieties has increased in the last 7 years



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A herbicide tolerant GM variety is in the regulatory pipeline

Field efficacy trial demonstrating herbicide tolerance in sugarcane NA varieties (2005)

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Breeding a highly polyploid, aneuploid, and heterozygous interspecific hybrid currently poses challenges:

Conventional

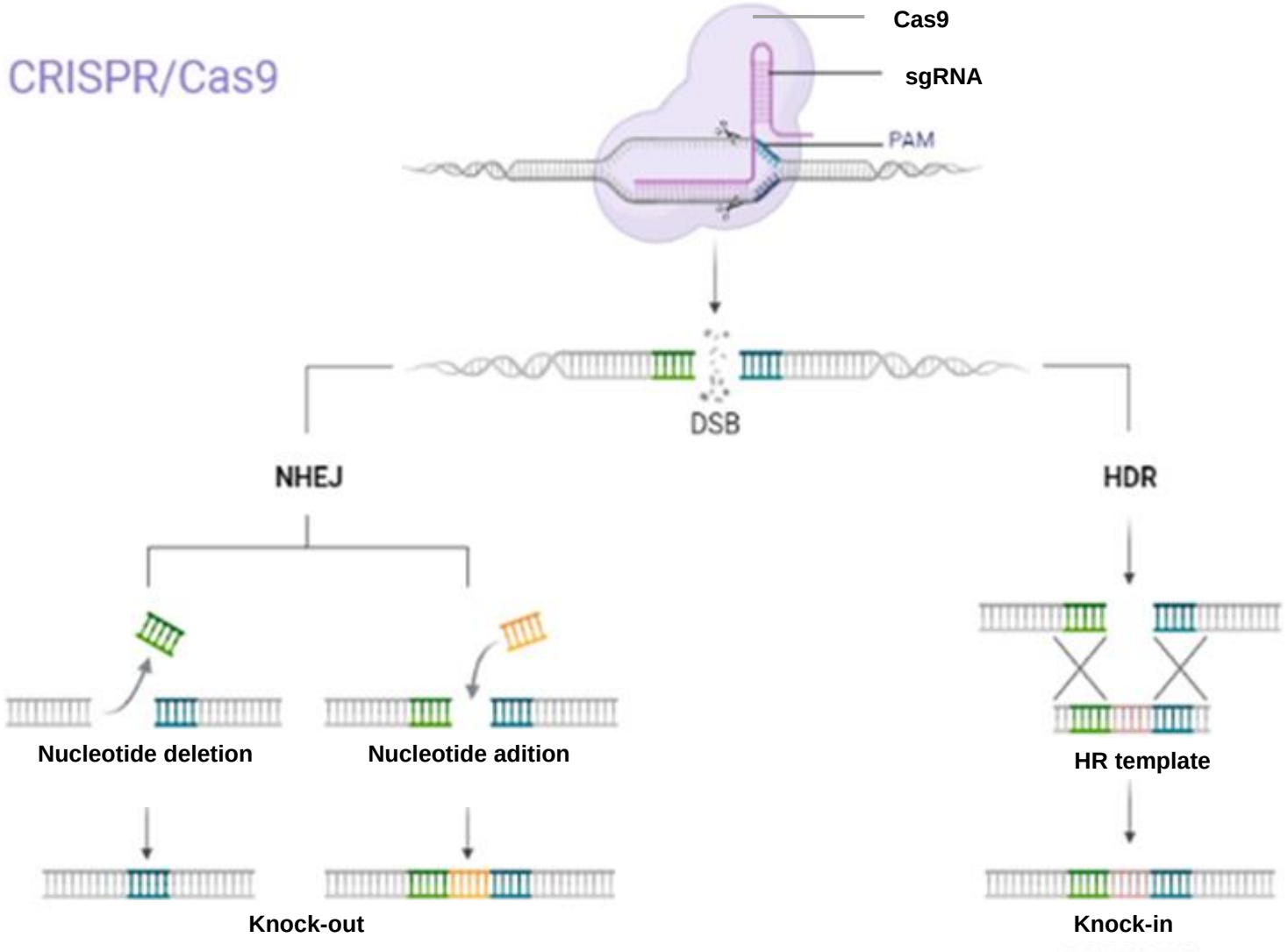
- No inbred lines
- No heterotic groups
- Hybrid vigor unexploited
- Slow introgression of traits from wild germplasm

Genetic modification

- New “Events” must be generated for each genotype (no introgression)
- Each trait implies deregulating several events (see CTC´s Bt sugarcane)
- Impossible to transform recalcitrant genotypes

New breeding technologies that enable simpler and safer breeding alternatives are critical to leverage sugarcane breeding

CRISPR/Cas genome editing allows precise and targeted genome modifications that may not be distinguished from naturally occurring genotypic variations



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ALS herbicide tolerance is a model for gene editing in sugarcane

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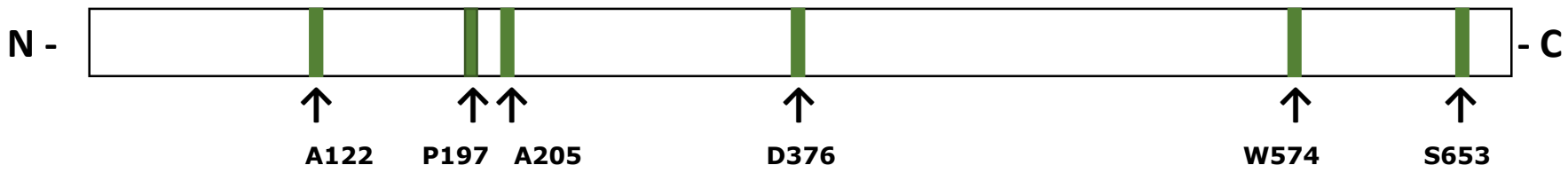


Linear representation of the ALS protein. AA positions according to standardized *Arabidopsis thaliana* ALS protein sequence. (Tan *et al*. 2006; Li *et al*. 2008; Merotto *et al*. 2009)

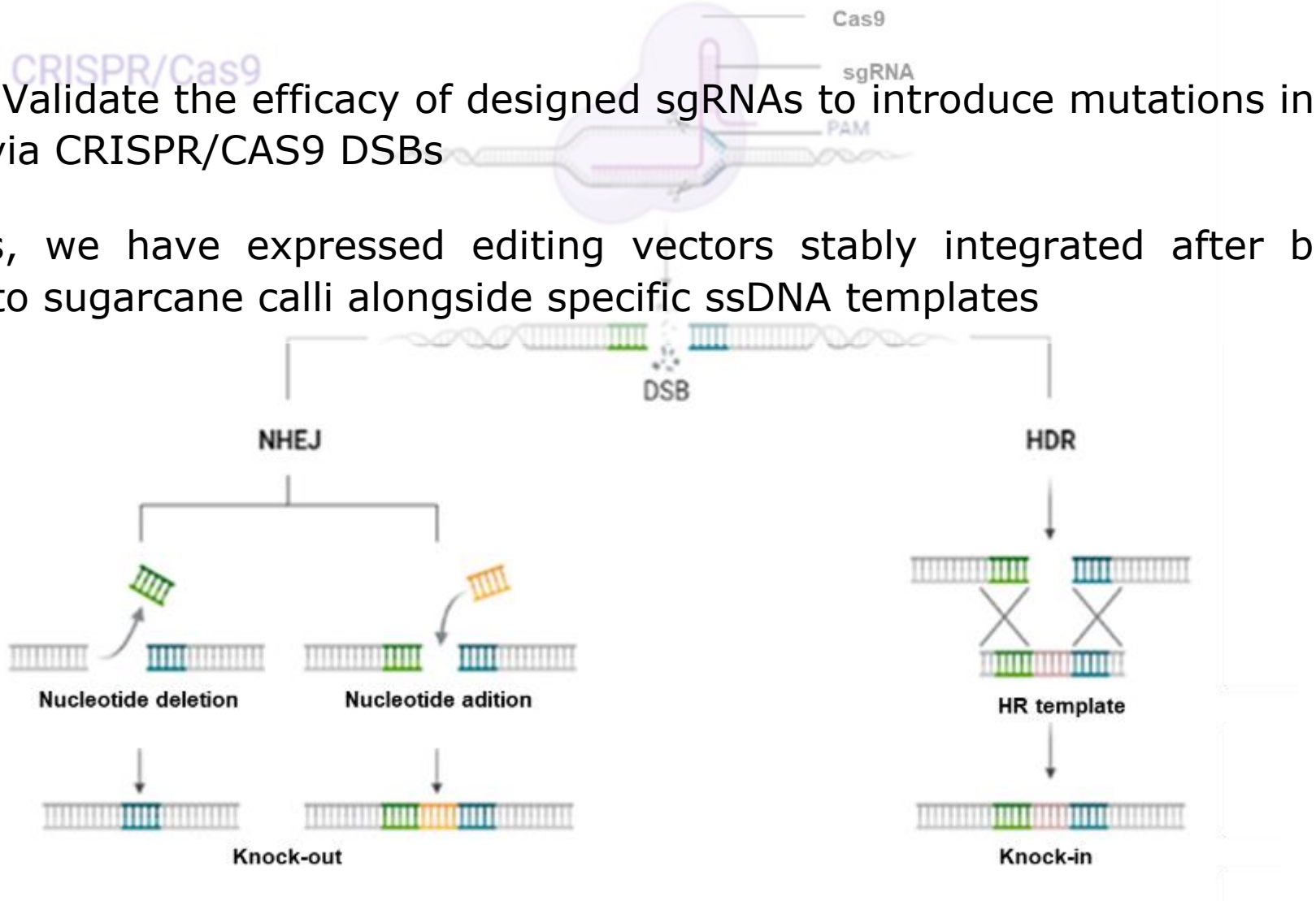
Mutation	Tolerance
A122T	IMIs
P197S	SUs
A205V	IMIs
W574L	all families of ALS inhibitors
S653N	IMIs

<u>Families of ALS Inhibitors</u>
SUs: sulfonylureas;
IMIs: imidazolinones;
POBs: pyrimidinylthiobenzoates;
TPs: triazolopyrimidines, and
SCTs: sulfonylamino-carbonyltriazolinones
(Tan *et al*. 2005; Tan *et al*. 2006)

We selected ALS herbicide tolerance as a model for genome editing

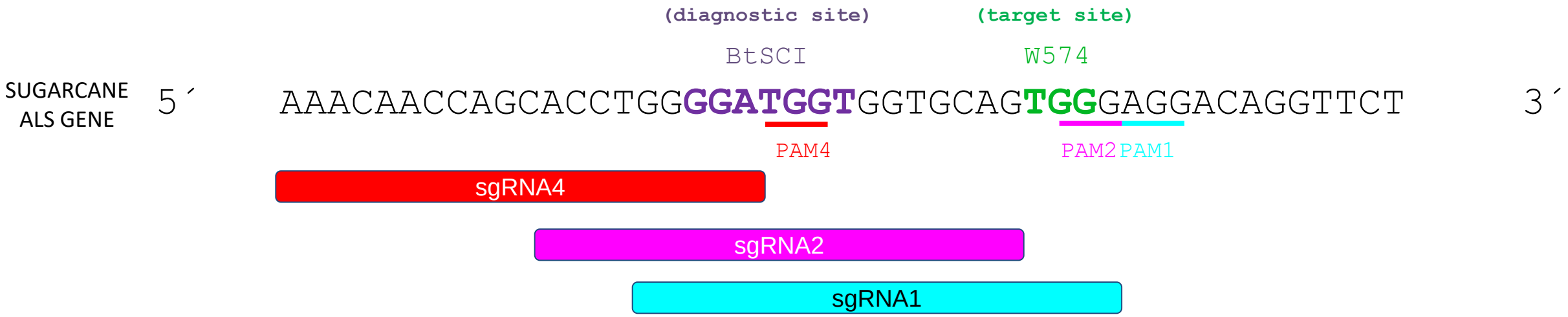
Objective: Validate the efficacy of designed sgRNAs to introduce mutations in the target sequence via CRISPR/CAS9 DSBs

To do this, we have expressed editing vectors stably integrated after biolistic co-delivery into sugarcane calli alongside specific ssDNA templates



sgRNA

Software-predicted sgRNA1, sgRNA2 and sgRNA4 with high target specificity and low off-target activity



Homologous repair templates (T)

Homologous repair ssDNA templates (T1, T2 and T3) were designed to functionally knock-in the *als* gene by modifying the target (W574), eliminate a molecular diagnostic *Bts*CI restriction site, and eliminate a specific **PAM** sequence

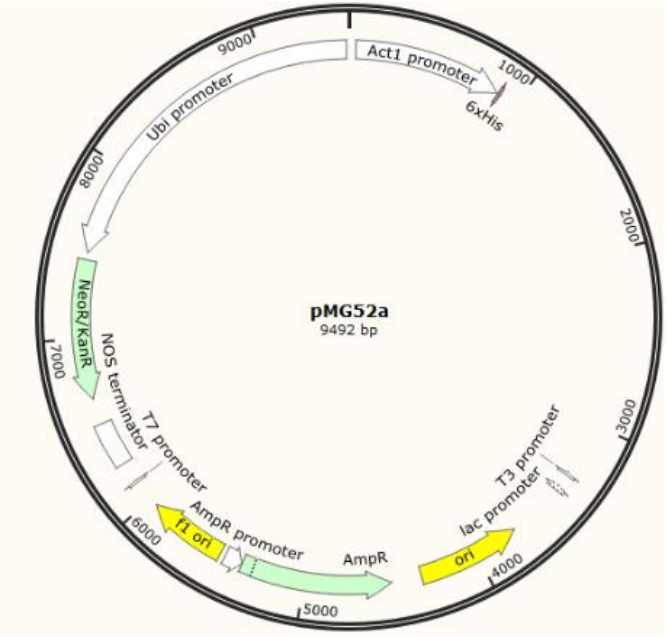
REVERSE COMPLEMENT SUGARCANE ALS GENE

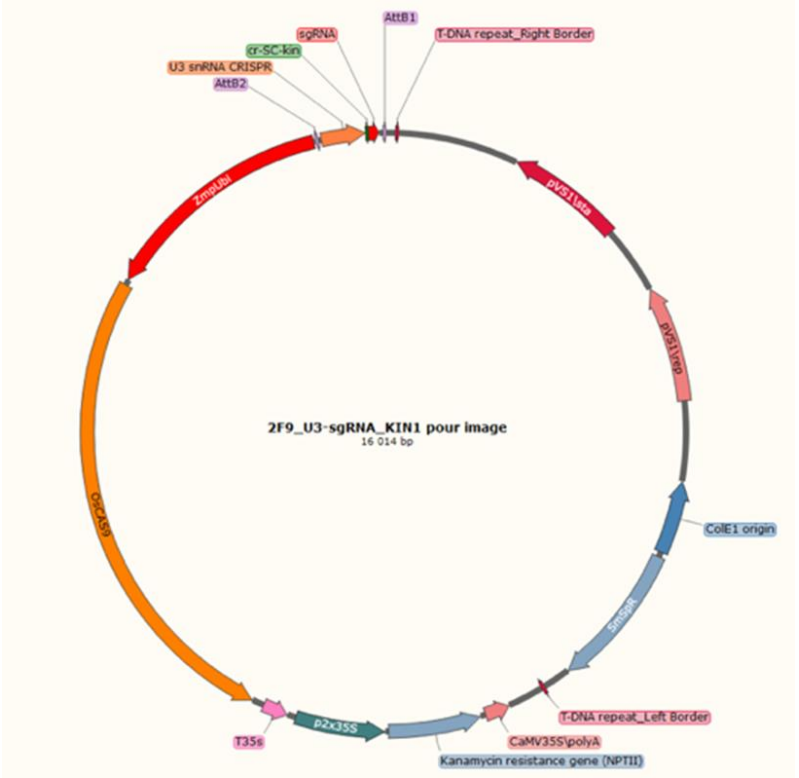
5´AGAACCTGTCCTC**CCA**CTGCACC**ACCATCC**CCAGGTGCTGGTTGTTT 3´

PAM1 PAM2 PAM4

T1	..TGTC**T**TC**GAG**CTGCACC**ACCATTC**CC..	127 nt
T2	..GGTC**T**TC**GAG**CTGCACC**ACCATGC**CCA..	92 nt
T3	..GGTCTTC**GAG**CTGCACC**AGCATGC**CCA..	92 nt

Embryogenic calli (*Saccharum* spp. Cv. NA 05-860) were transformed with editing vectors, HR templates and a selection vector through biolistic delivery





Template dsDNA
92 – 10 bp

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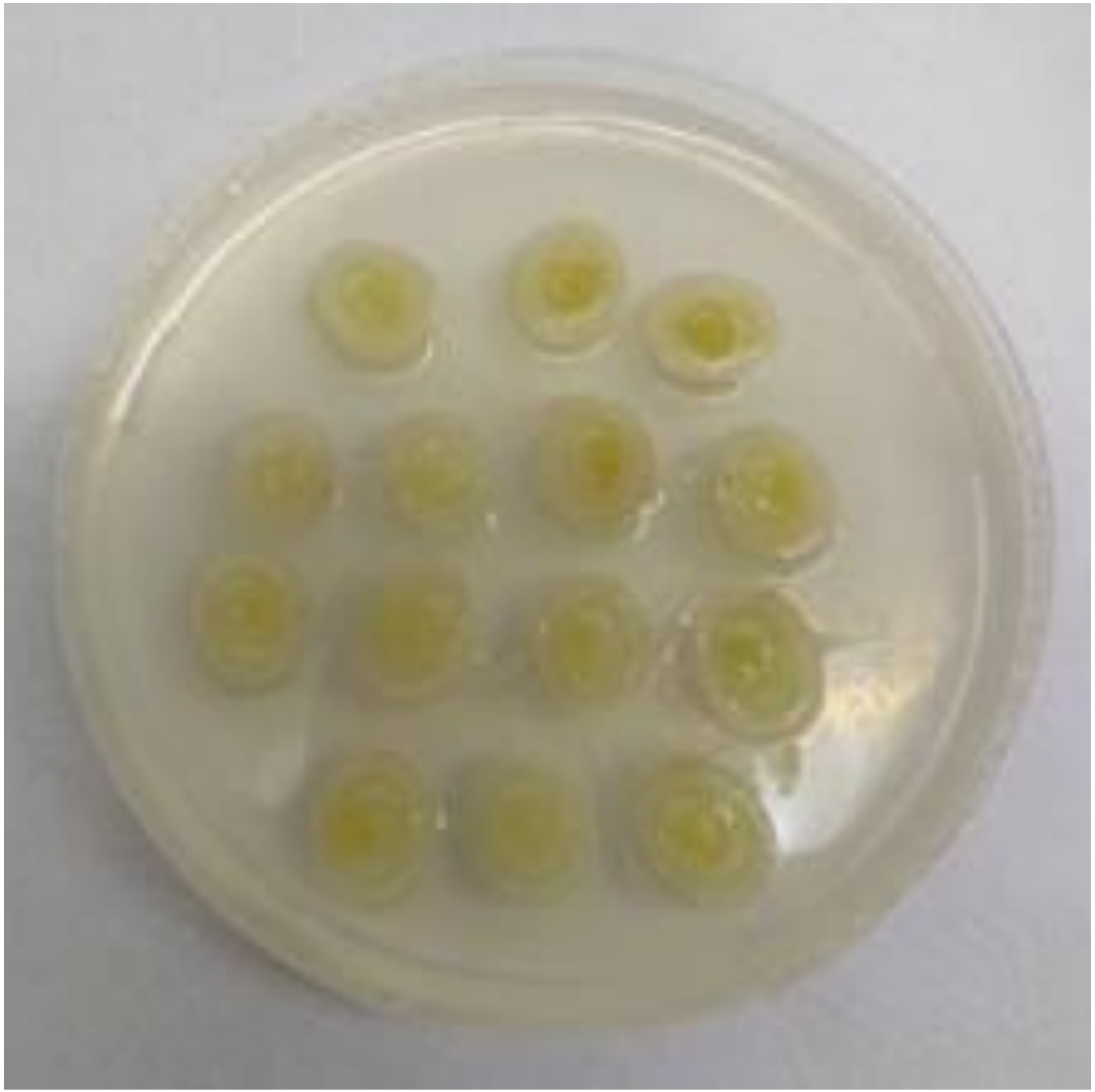

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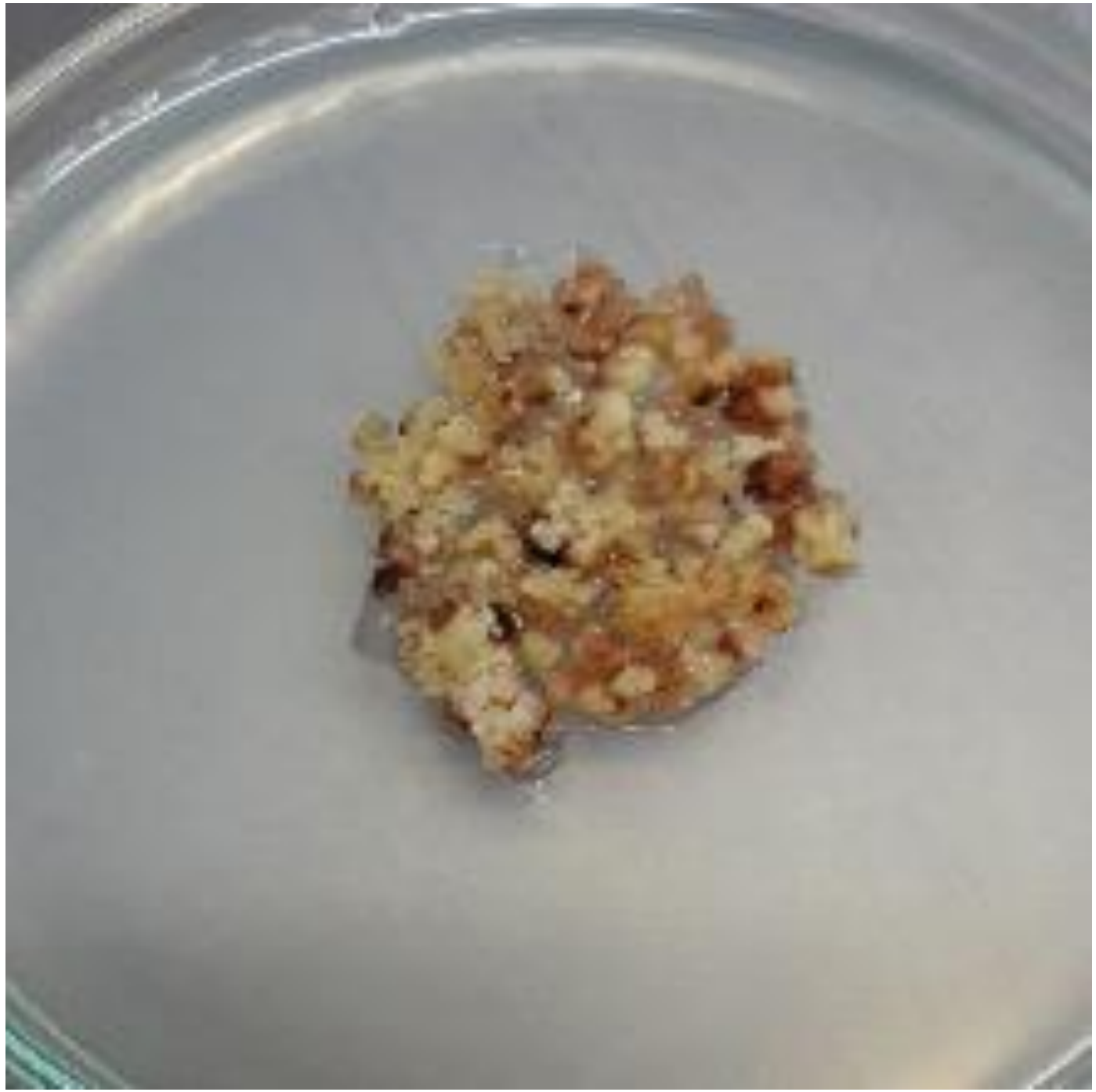

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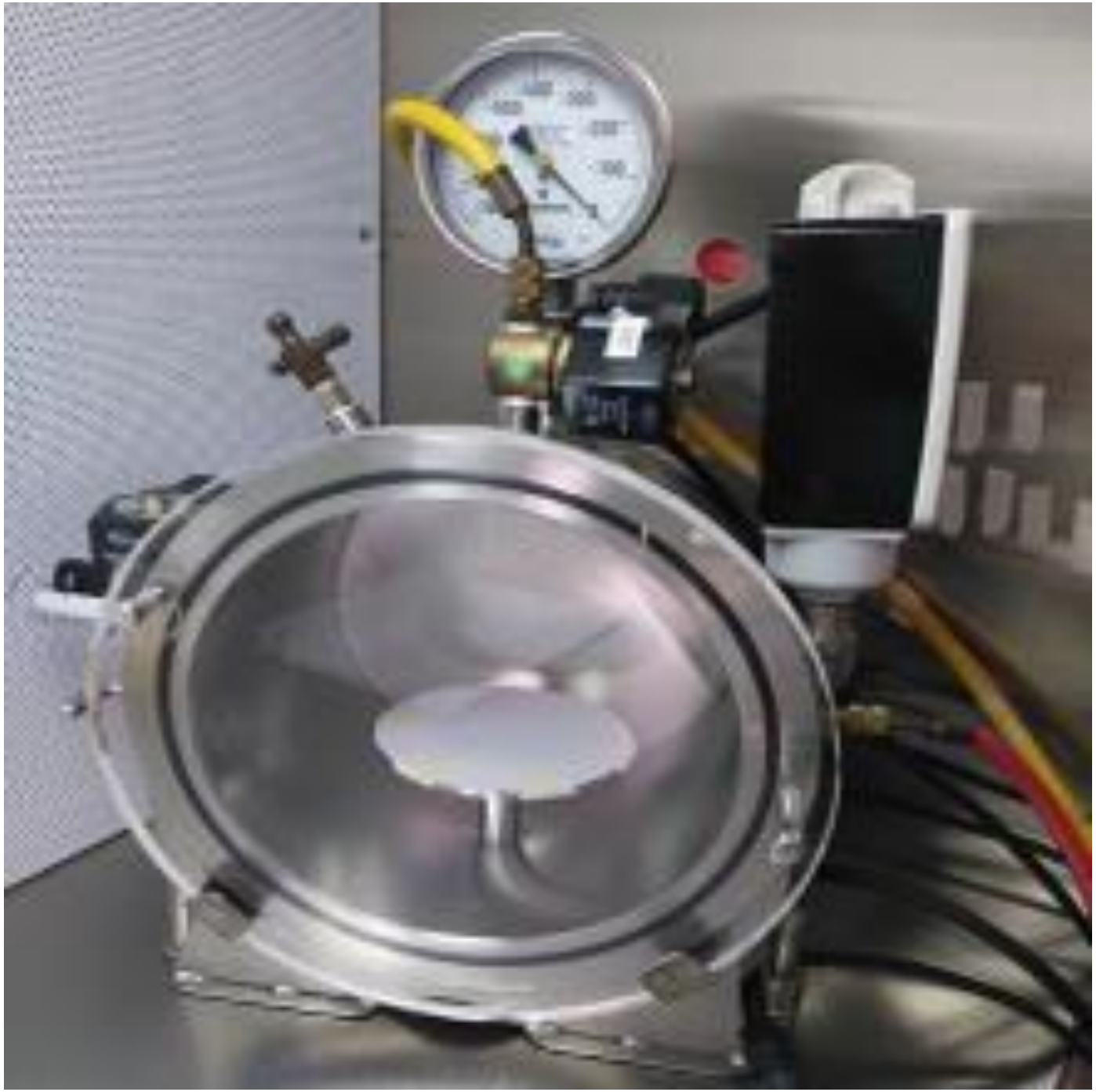

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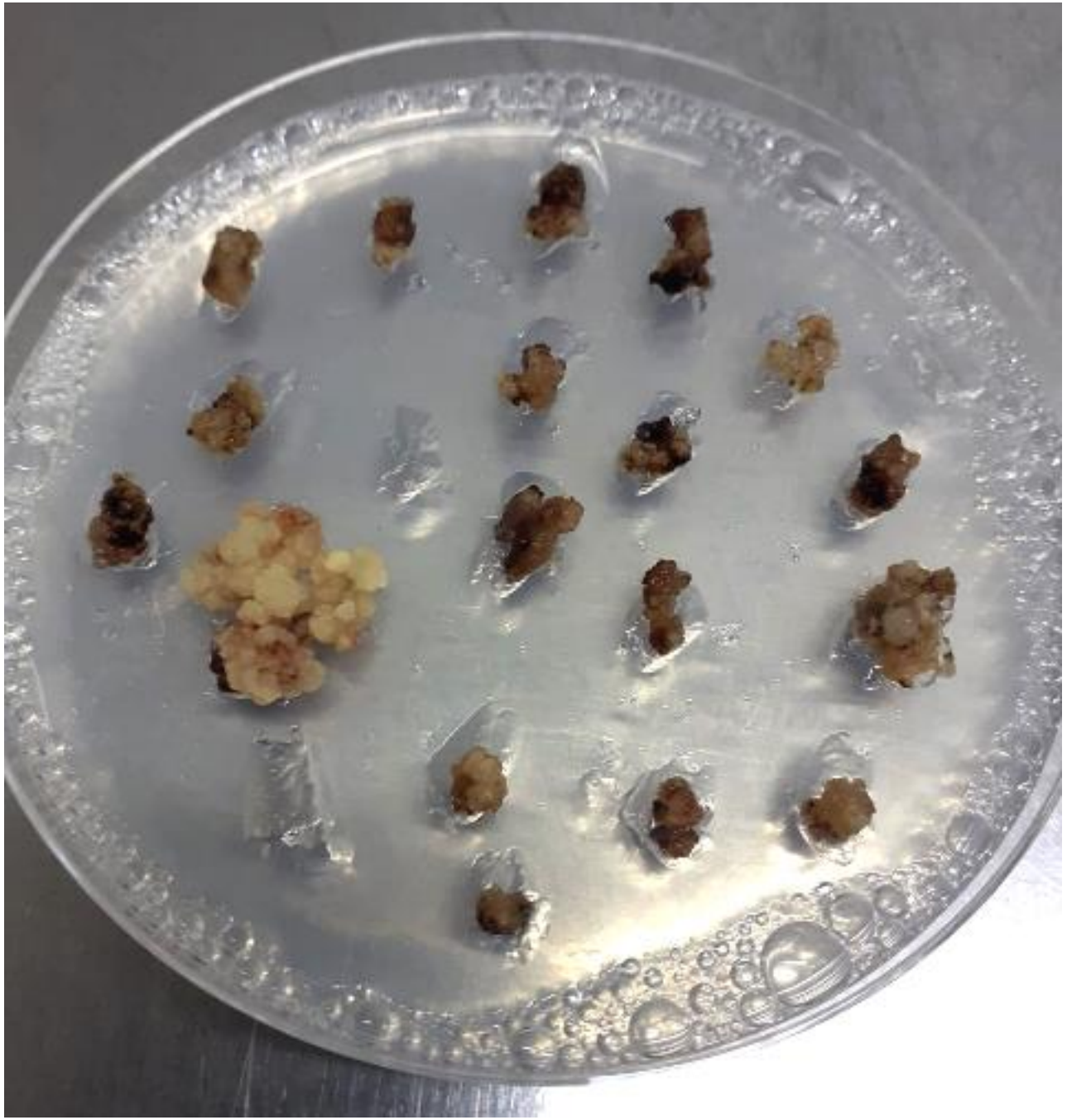

Transgenic calli were selected in culture media supplemented with G418

Transgenic calli regenerated in media containing with no 2,4-D

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Transgenic regenerants in rooting media

Transgenic regenerants in the greenhouse

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TOTAL TIME ABOUT 1 YEAR

Plants were diagnosed for the presence of the transgene

We have regenerated 65 stably independently transformed plants

Transgene integration of *nptII* and *Cas9* genes were confirmed using PCR

	Vector combinations	Number of Putative, in vitro selected events	*nptII* PCR	*Cas9* PCR
Stable expression	pNPTII + pEG_G1 + M1	16	16	15
	pNPTII + pEG_G1 + M2	15	15	15
	pNPTII + pEG_G2 + M1	11	11	10
	pNPTII + pEG_G2 + M2	5	5	5
	pNPTII + pEG_G4 + M3	18	17	15
		65	64	60
			98%	**92%**

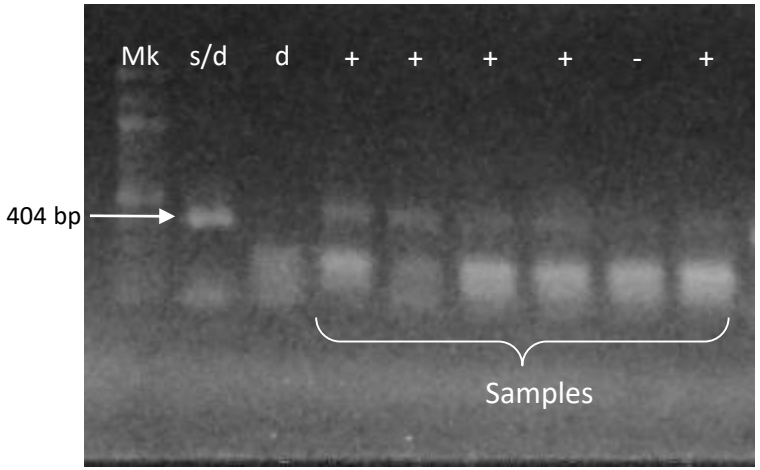
Putatively edited regenerants were diagnosed by PCR/RE

Specific primers were designed to amplify a 404 bp *als* gene fragment

PCR restriction enzyme (PCR/RE) detects the elimination of the *Bts*CI recognition site near the target codon

BtsCI W574

GG**GGATGGT**GGTGCAG**TGG**GAGGACA



Putatively edited regenerants were diagnosed by PCR/RE (cont.)

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13 independent events resulted positive for the PCR/RE assay

RE-resistant band from six plants were purified and sequenced

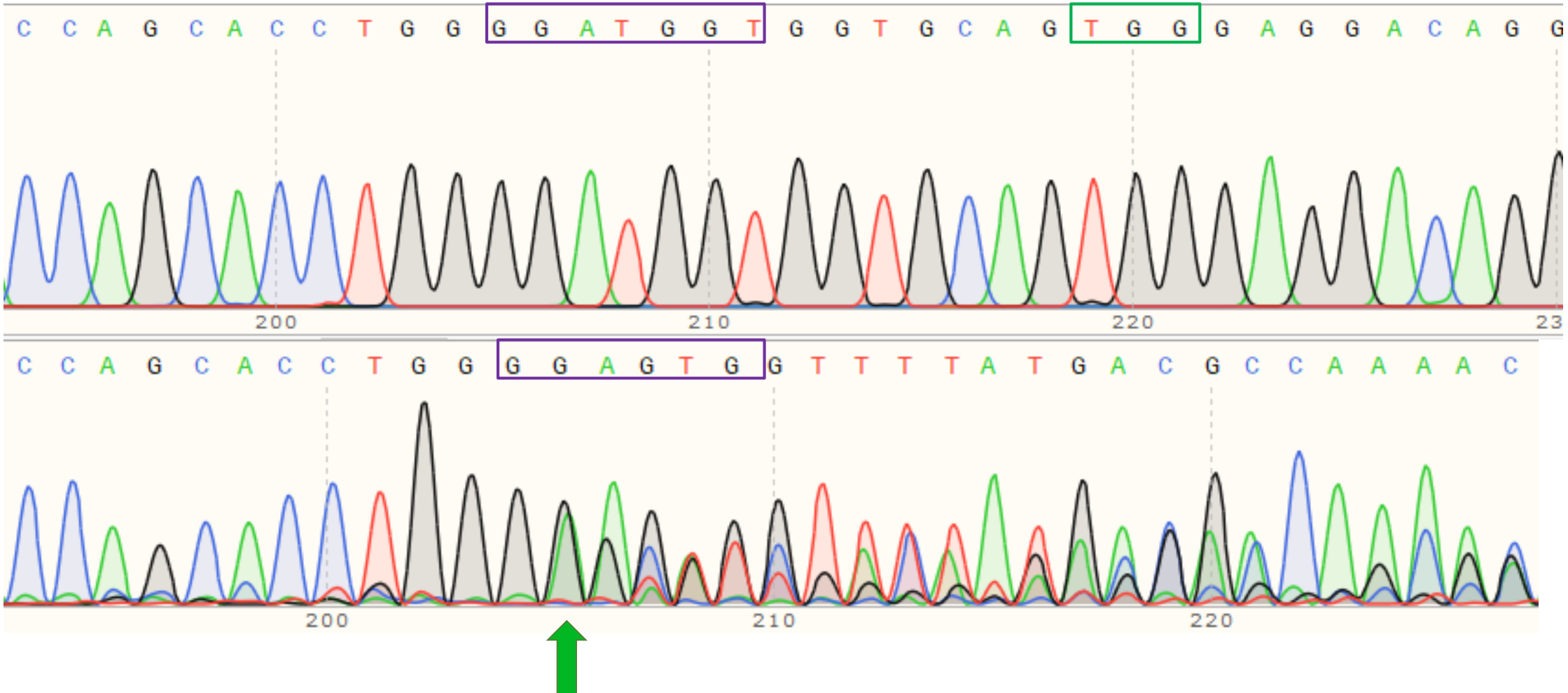
	Vector combinations	Number of independent events	*nptII* PCR	*Cas9* PCR	PCR/RE
Stable expression	pNPTII + pEG_G1 + M1	16	16	15	5
	pNPTII + pEG_G1 + M2	15	15	15	4
	pNPTII + pEG_G2 + M1	11	11	10	3
	pNPTII + pEG_G2 + M2	5	5	5	0
	pNPTII + pEG_G4 + M3	18	17	15	1
		65	64	60	13

G1 → 30%
G2 → 20%
G4 → 6,6%

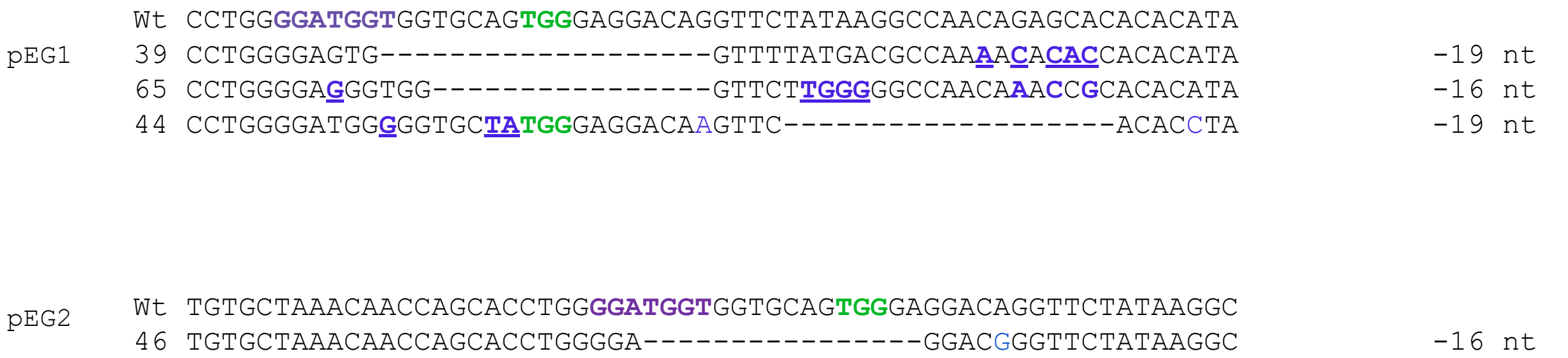
Editing was confirmed by DNA sequencing PCR products

BtsCI W574

GG**GGATGGT**GGTGCAG**TGG**GAGGACA



Sequencing


Wt CCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAACAGAGCACACACATA
pEG1
39 CCTGGGGAGTG----------GTTTTATGACGCCAAAACACACCACACATA -19 nt
65 CCTGGGGAGGGTGG----------GTTCTTGGGGGCCAACAAACCGCACACATA -16 nt
44 CCTGGGGATGGGGGTGCTATGGGAGGACAAGTTC----------ACACCTA -19 nt
Wt TGTGCTAAACAACCAGCACCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGC
pEG2
46 TGTGCTAAACAACCAGCACCTGGGGA----------GGACGGGTTCTATAAGGC -16 nt

Summary

- We have achieved sgRNA-targeted deletion of *als* gene fragments using CRISPR/Cas9 vectors through NHEJ
- Codelivering editing vectors into sugarcane results in foreign DNA insertion

Perspectives

- We may now be able to develop functional knockout phenotypes of suitable targets
- DNA-free techniques such as biolistic delivery of ribonucleoproteins must be implemented to generate transgene-free editing
- Template DNA activity must be optimized to allow for most functional knock-ins

More generally

- Sugarcane´s complex genome poses a major challenge for breeders and biotechnologists
- Sugarcane breeding is a demanding, knowledge intensive activity
- This activity will benefit from complementary tools that enhance cultivars by engineering specific traits into well developed germplasm


Thank you!