Current technical limitations and future developments in plant genome editing

Luisa Bortesi – Novi Sad – 06/11/2019

cost
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Genome editing limitations

Efficiency: nr. of positive events per 100 analyzed

Accuracy (specificity): nr. of events with the desired outcome compared to the nr. with off-target mutations

of the genome editing tool/approach

➔ From the mammalian field; for therapeutic applications in humans

Genome editing limitations: efficiency?

Gene KO via stable transgenic integration of CRISPR: most species approach 100% efficiency
(e.g. Arabidopsis, soya bean, tomato, potato, tobacco, petunia, poplar, maize, rice)

Supplementary Table 1. The efficiency, accuracy and structure of on/off-target mutations induced by CRISPR systems in different plant species.

Species and generation/tissue[1]		Input[2]	Repair[3]	Percentage efficiency and accuracy of editing[4]						Type and size of mutations[5] (bp)				Zygosity	Comments	Ref
				CRISPR/Cas9		Cas9 mod		sgRNA mod								
				ON	OFF	ON	OFF	ON	OFF	DEL	INS	SUB	COM			
DICOTS																
Arabidopsis (*Arabidopsis thaliana*)	P	G	NHEJ	1.1-5.6	NA					1	1	1			Induced 7.7% small deletions (48 bp) between two sgRNAs.	Li *et al.* (2013)
	L	A	NHEJ	2.7	NA					1	1	1-2				
	T1	A	NHEJ	71.2	NF					1->100	1-100		X	100% chimeric in T1, 22% homozygous in T2	Predominantly 1-bp insertions and short deletions; 53% of the mutations detected in T2 plants were not found in T1 plants.	Feng *et* (2014)
	T1	A	NHEJ			42.8	NA			6-111	1-82		X		Double nickase (52-nt overhangs) generated ~70% deletions and ~30% insertions, larger than those generated by intact Cas9. In most cases, the insertions originated directly from the sequence upstream or downstream of the insertion site.	Schiml (2014)
	T1	A	NHEJ	84.7	NF					1-28	1	1			Off-target mutations not found at three sites with 3-bp mismatches using the INCURVATA 2 promoter which is active in proliferating tissues.	Hyun *e* (2015)
	T1	A	NHEJ	35.6	NA									20.3% heterozygous, 8.5% chimeric, 6.8% biallelic, 1.7% biallelic	A sgRNA with 25% GC achieved a lower editing efficiency.	Ma *et* (2015)
	T1	A	NHEJ			12.5	NA			1-54	1-2			44% homozygous or biallelic in T2	*St*Cas9:sgRNAs with NNAGAA-PAM: 8.6% insertions (mostly single nucleotide) and 3.8% deletions; *St*Cas9:sgRNA with NNGGAA-PAM: 11.6% insertions (primarily single nucleotide) and 3.8% deletions.	Steiner *et al.* (2015)
	T1	A	NHEJ			58.3 - 76.3	NA			1-51	1-3			34% homozygous or biallelic in T2	*Sa*Cas9: mostly insertions for NNGAA-PAM (52.1%, predominantly single nucleotide), whereas NNGGGT-PAM generated 46.7% deletions and 21.6% insertions. Both insertions and deletions were larger than those associated with NNGAA-PAM. The *Sa*Cas9 generated DSBs with at least the efficiency of *Sp*Cas9.	
	T1	A	NHEJ	8.3	NF					1-52	1-16		X	Up to 8.3% homozygous mutation at three loci in T1 plants	Cas9 driven by an egg-specific promoter. Off-target mutations not found at three sites with fewer than three mismatches.	Wang *e* (2015b
	P	PNP	NHEJ	16	NF					1-4	1				A 223-bp deletion between two targets. Indels were not detected at any sites that differed	Woo *et*

Bortesi et al., 2016. Plant Biotech J

Lower efficiencies for base editing, gene insertion and replacement

➔ but we can select!

Genome editing limitations: Off-target effects?

➔ Are **rare** in plants

http://canacopegdl.com

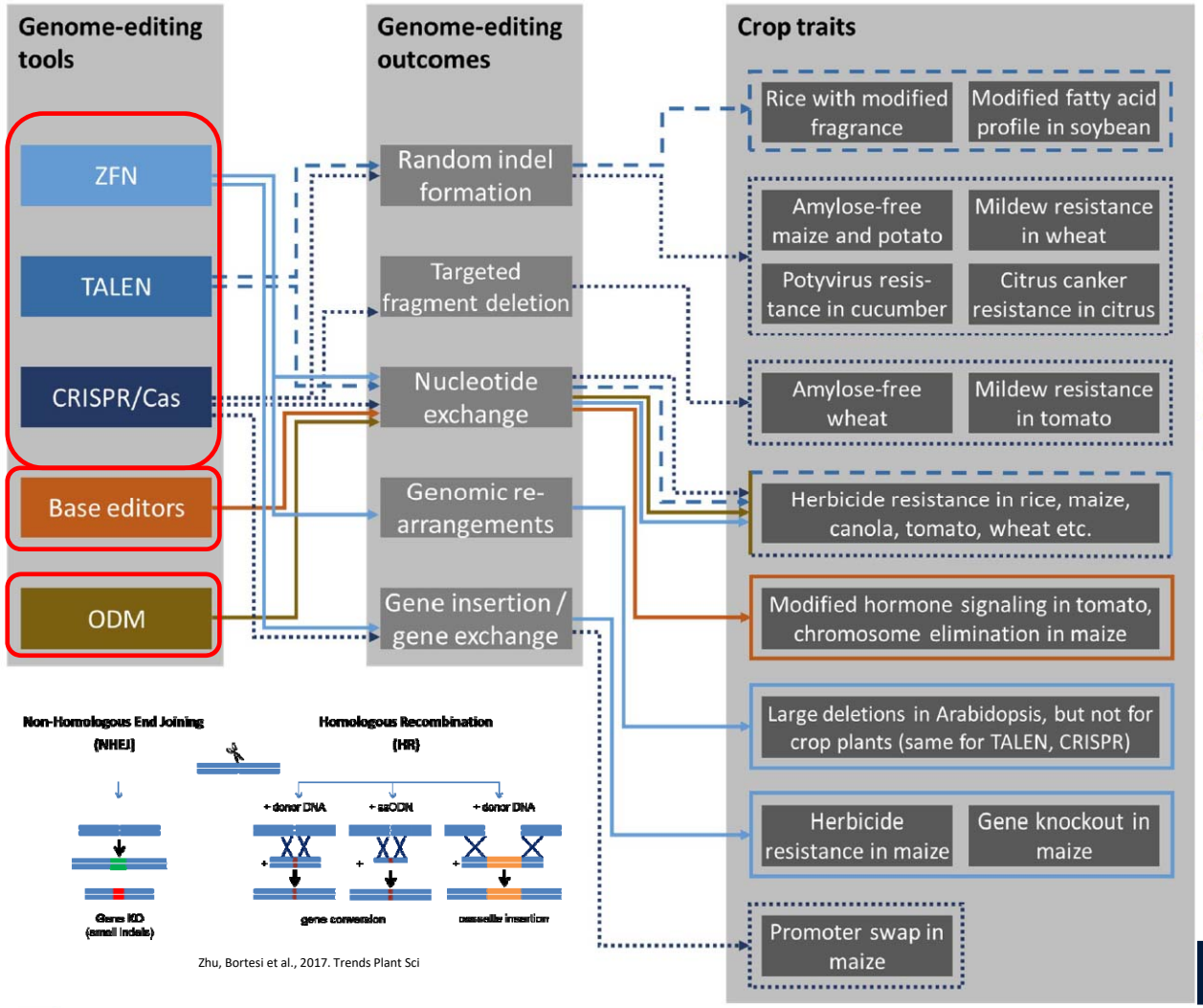
- Where reported, they tend to involve a minority of gRNAs
- Careful gRNA design can ensure specific targeting
- Likelihood of off-target mutations:
  - Integrated DNA >> transient DNA > RNA > RNP complex
- Off-target effects might be reduced or avoided by selecting appropriate progeny without Cas9/gRNA transgene integrated
- The frequency of off-target mutations is much lower than that of on-target mutations, allowing the recovery of solely on-target mutations in all experiments

➔ (much) more precise than mutation breeding

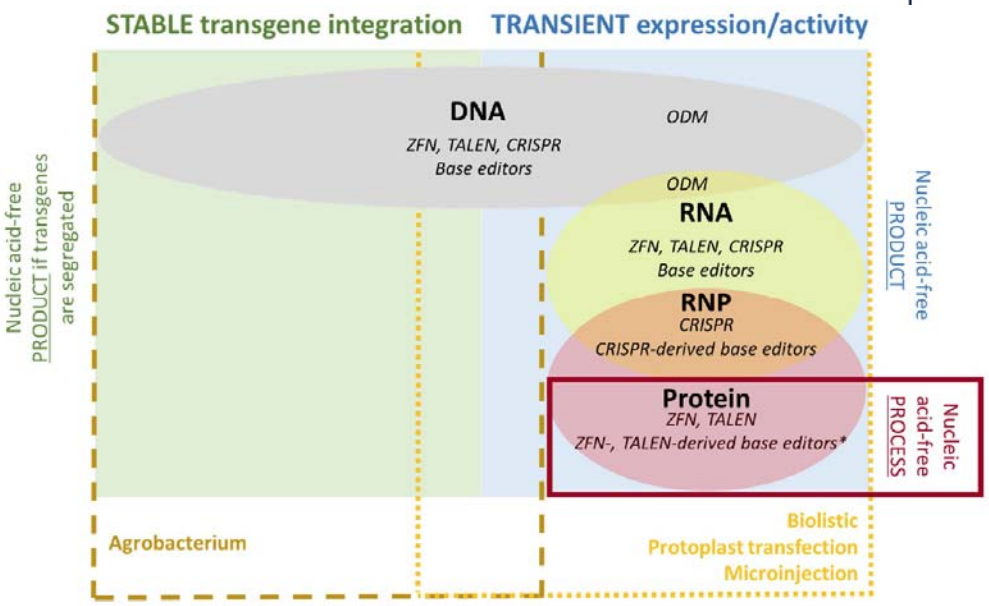
Genome editing limitations for agriculture

➔ **major** limitations are in the **application** of the technology, not the technology per se

Maastricht University | RWTH AACHEN UNIVERSITY | Fraunhofer | AMI Biobased Materials



Zhu, Bortesi et al., 2017. Trends Plant Sci



Jansing et al., 2019. Int J Mol Sci

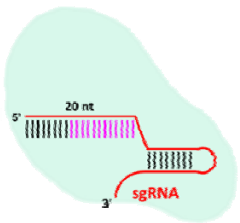
Genome editing in agriculture

1. Know the target
2. Introduce the genome editing reagents
3. Identify and select genome edited events
4. Regenerate a whole plant

3⇔4 2⇔4

Efficiency } of the genome editing tool/approach

} of delivery



ribonucleoprotein complex (RNP)

Regulatory advantage (?)

More efficient
Easier to dose
Less stressful for the plant cell
Well suited for high throughput applications

2. Introduce the genome editing reagents

DNA (stable or transient)

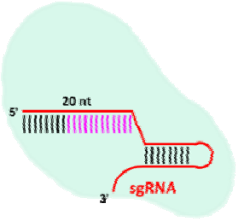
- Agrobacterium-meditated transfection ➔ works very well for many but not all species
- Particle bombardment
- Protoplast transfection/electroporation ➔ challenging to generate and manipulate

RNA

- Particle bombardment
- Protoplast transfection/electroporation ➔ challenging to generate and manipulate

Proteins and RNPs

- Particle bombardment ➔ special equipment, scattered delivery
- Protoplast transfection ➔ challenging to generate and manipulate



3. Identify and select genome edited events

➔ DNA modification itself

➔ Presence of genome editing tool

- High-throughput screening by DNA analysis
- Selectable marker genes (e.g antibiotic or herbicide resistance)
- Visual/screenable phenotype (e.g. fluorescent protein, phytoene desaturase)

Testing selection: surrogate reporter

Molecular Plant

CellPress

Available online 18 October 2019

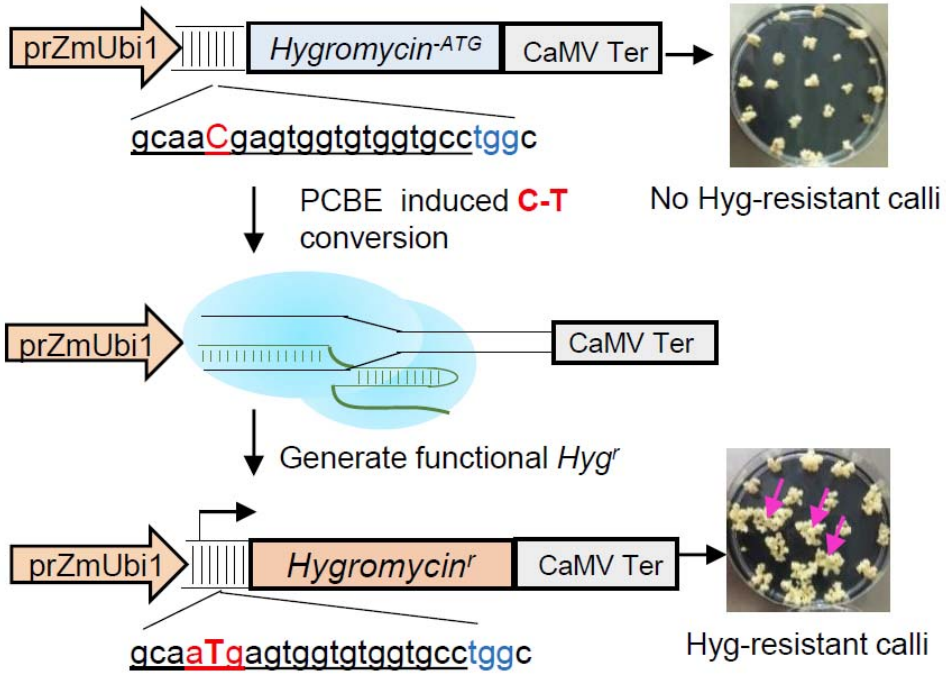
In Press, Journal Pre-proof

Research Article

Discriminated sgRNAs-based SurroGate System Greatly Enhances the Screening Efficiency of Plant Base-edited Cells

Wen Xu †, Yongxing Yang †, Ya Liu †, Guiting Kang, Feipeng Wang, Lu Li, Xinxin Lv, Si Zhao, Shuang Yuan, Jinling Song, Ying Wu, Feng Feng, Xiaoqing He, Chengwei Zhang, Wei Song, Jiuran Zhao, Jinxiao Yang

3-6 fold enhanced screening efficiency



➔ Develop DNA-free selection methods

4. Regenerate a whole plant

➔ The real bottleneck!

Delivery Method	Cargo	Plant Species	Tissue	Selection	Mutation Efficiency	Calculation	Reference
Protoplast transfection (PEG)	DNA TALEN	Potato *Solanum tuberosum*	Protoplasts	No	11–13%	Mutated callus/total protoplast-derived callus	[128]
Protoplast transfection (PEG)	DNA TALEN	Tobacco *Nicotiana benthamiana*	Protoplasts	n.a.	70.5%	Deep sequencing of protoplasts	[145]
	mRNA TALEN			n.a.	5.8–16.9%	Without/with UTR	
Protoplast transfection (PEG)	RNP CRISPR/Cas9	Lettuce *Lactuca sativa*	Protoplasts	No	46%, 6% mono-, 40% biallelic	Mutated callus/analyzed callus	[147]
Protoplast transfection (PEG)	RNP CRISPR/Cas9	Grapevine *Vitis vinifera* cv. Chardonnay	Protoplasts	n.a.	0.1%	Deep sequencing of protoplasts	[148]
		Apple *Malus domestica* cv. Golden delicious	Protoplasts	n.a.	0.5–6.7%	Deep sequencing of protoplasts	
Protoplast transfection (PEG)	RNP CRISPR/Cas9	Petunia *Petunia x hybrida*	Protoplasts	n.a.	5.3–17.8%	Deep sequencing of protoplasts	[149]

➔ In vitro regeneration of edited plant cells or tissues is genotype dependent AND time consuming

(Artificial) haploid induction-based genome editing

➔ To overcome the recalcitrance of most elite maize inbred lines to genetic transformation

one-step generation of genome edited monocot and dicot haploid plants

Molecular Plant
Research Report

IMGE

CellPress
PARTNER JOURNAL

Development of a Haploid-Inducer Mediated Genome Editing System for Accelerating Maize Breeding

Baobao Wang[1,3,*], Lei Zhu[1,3], Binbin Zhao[1], Yongping Zhao[1], Yurong Xie[1], Zhigang Zheng[2], Yaoyao Li[2], Juan Sun[2] and Haiyang Wang[2,*]
[1]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[2]School of Life Sciences, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou 510642, China

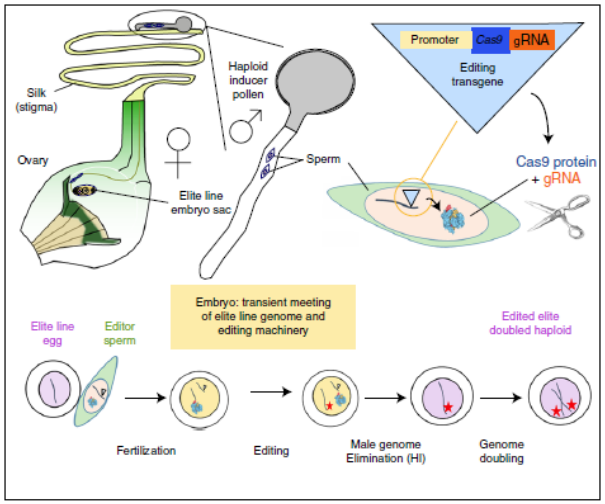
nature biotechnology

HI-Edit

LETTERS
https://doi.org/10.1038/s41587-019-0038-x

One-step genome editing of elite crop germplasm during haploid induction

Timothy Kelliher[1*], Dakota Starr[1,2], Xiujuan Su[1], Guozhu Tang[1], Zhongying Chen[1], Jared Carter[1], Peter E. Wittich[3], Shujie Dong[1], Julie Green[1], Erin Burch[1], Jamie McCuiston[1], Weining Gu[1], Yuejin Sun[1], Tim Strebe[1], James Roberts[1], Nic J. Bate[1,4] and Qiudeng Que[1*]



- Relevant but limited applicability to elite inbred lines
- Relies on the generation of a transgenic plant with the CRISPR genes ➔ DNA, in vitro regeneration
- Donor lines must be generated *de novo* for each new target

(DNA-free) induction of regeneration (in combination with selection?)

DNA-free editing of the germline

Future Developments

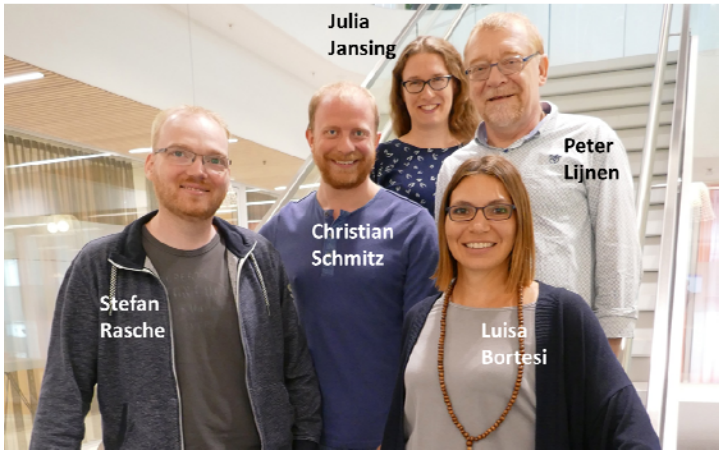
- **Regeneration of whole plants**
- **DNA-free germline editing**
- Efficiency of RNP delivery (for regeneration)
- DNA-free Selection

- Multiplexing with RNPs
- Targeted gene insertion and replacement by HR or NHEJ for transgenesis or cisgenesis, trait stacking

- Specificity and efficiency of base editors

- New enzymes with new specificities and new features
- New technologies (prime editing)

Acknowledgments



Institut für Molekulare Biotechnologie
RWTH AACHEN UNIVERSITY

Prof. Rainer Fischer
Dr. Sruthy Augustine
Markus Sack
Dr. Flora Schuster

Fraunhofer IME

Prof. Stefan Schillberg
Dr. Andreas Schiermeyer

Universitat de Lleida

Prof. Paul Christou
Dr. Changfu Zhu
Can Baysal