Detection of Genome Edited Plant Products- Opportunities and Challenges

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Presentation Outline

- CRISPR system and Genome editing (GE)
- Comparison of molecular characterization of GM and GE plants
- Detection methods for GE crops
- Challenges in detecting GE plant products
- Summary

CRISPR system and Genome editing

Major CRISPR-Cas enzymes

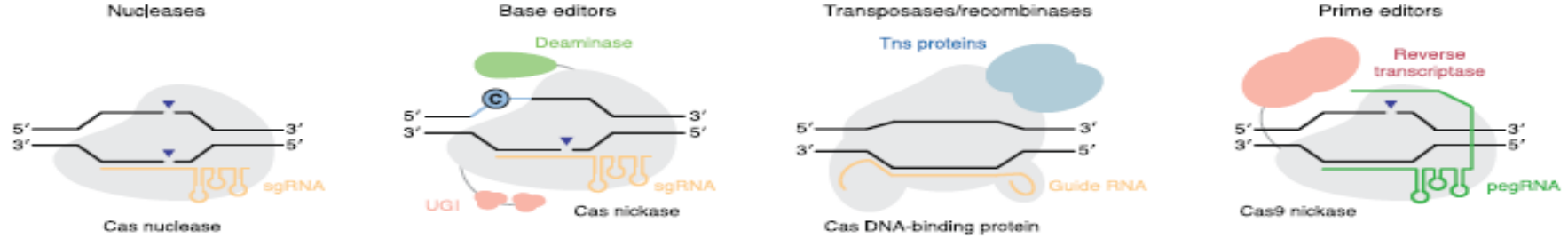
Table 1 Naturally occurring major CRISPR-Cas enzymes

	Size	PAM sequence	Size of sgRNA guiding sequence	Cutting site	Reference
spCas9	1368	NGG	20 bp	~ 3 bp 5′ of PAM	Jinek et al.[42] Gasiunas et al.[43]
FnCas9	1629	NGG	20 bp	~ 3 pb 5′ of PAM	Hirano et al.[60]
SaCas9	1053	NNGR RT	21 bp	~ 3 pb 5′ of PAM	Mojica et al.[57]
NmCas9	1082	NNNNG ATT	24 bp	~ 3 bp 5′ of PAM	Hou et al.[53]
St1Cas9	1121	NNAGA AW	20 bp	~ 3 bp 5′ of PAM	Gasiunas et al.[43] Cong et al.[45]
St3Cas9	1409	NGGNG	20 bp	~ 3 bp 5′ of PAM	Gasiunas et al.[43] Cong et al.[45]
CjCas9	984	NNNNACAC	22 bp	~ 3 bp 5′ of PAM	Kim et al.[56]
AsCPf1	1307	TTTV	24 bp	19/24 bp 3′ of PAM	Yamano et al.[50] Kim et al. 2016
LbCpf1	1228	TTTV	24 bp	19/24 bp 3′ of PAM	Yamano et al.[50] Kim et al. 2016
Cas13	Multiple orthologs	RNA targeting	28 bp		Abudayyeh et al. 2017

- Two classes of CRISPR systems: each contains multiple types.
- Class I contains type I and type III systems, commonly found in archaebacteria.
- Class II contains type II, IV, V and VI systems.
- Widely used Cas enzymes: spCas9- type II, Cas12 (Cfp1)- type V.

Adli M, Nature Communications (2018): 1911

Overview of genome editing strategies and enzymes



Edit type	Starting sequence	Desired product	Reagent(s), method(s)	Byproducts
Stochastic indels	PAM	Indel / Indel PAM	Cas nuclease	Diverse indels (predictable, but not controllable)
PAM-distal transition point mutations	C / G PAM	T / A PAM	Base editors (CBEs, ABEs)	Possibility of bystander mutations
PAM-proximal point mutations	G / C PAM	T / A PAM	Cas nuclease HDR Prime editors	Indel byproducts, especially from nucleases
Small insertions (e.g., 1–40 bp)	PAM	1 bp PAM	Cas nuclease HDR Prime editors	Indel byproducts, especially from nucleases
Small deletions (e.g., 1–80 bp)	2 bp PAM	PAM	Cas nuclease HDR Prime editors	Indel byproducts, especially from nucleases
Large insertions (>30 bp)	PAM	1 kb PAM	Cas nuclease HDR Cas nuclease EJ Cas transposases/ recombinases	Indel byproducts, wrong insert orientation, multiple inserted fragments, vector insertion
Large deletions (>40 bp)	PAM 200 bp PAM	PAM PAM	Cas nuclease EJ Cas nuclease HDR	Indels at individual cut sites, inverted intervening sequence
Chromosomal translocations	Chr 3 Chr 7	Chr 3 Chr 7	Cas nuclease EJ Cas nuclease HDR	Indels at translocation junctions, indels at individual cut sites without translocation

Anzalone AV et al., Nature Biotech (2020) 824-844

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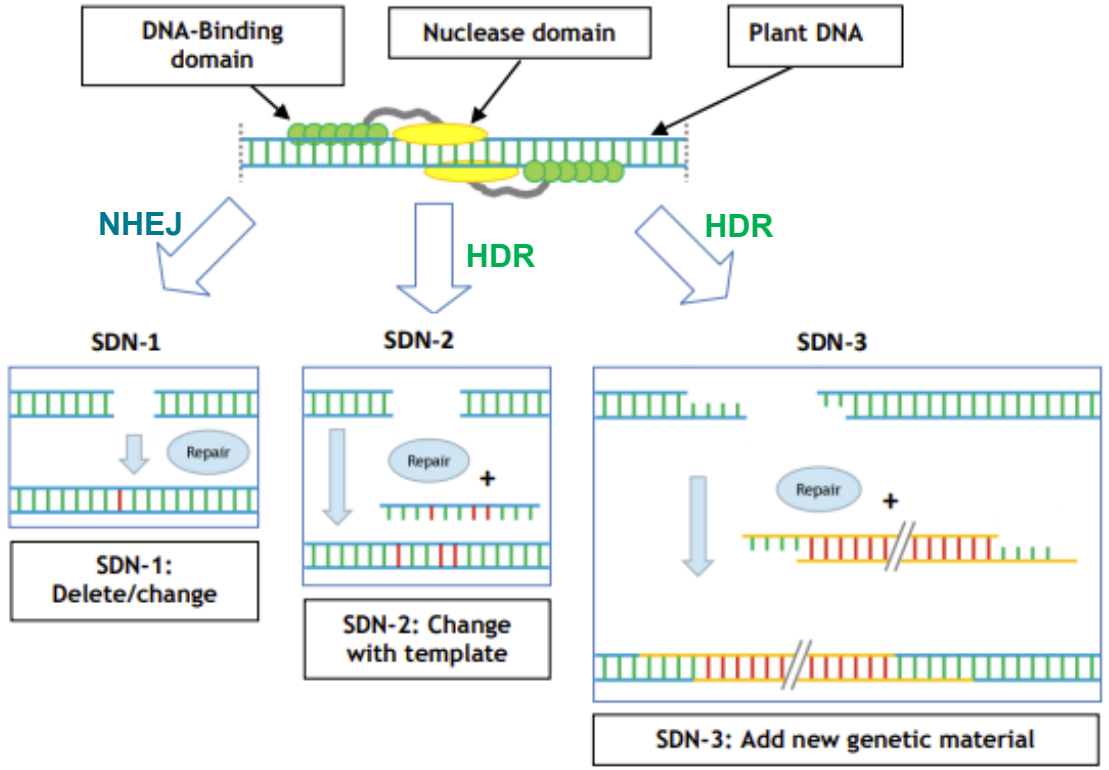
CRISPR-Cas system and genome editing

- **Key components:**
  - Target sequence: 20-21 bases
  - SDN enzymes: Cas9, Cas12a endonucleases
  - sgRNA: directs SDN enzyme to the target
  - PAM: Protospacer Adjacent Motif
    - Cas9: 5'-NGG-3', cutting site at upstream of 3rd bp.
    - Cas12a: 5'-TTTN-3', cutting site at downstream of 19-24 bp

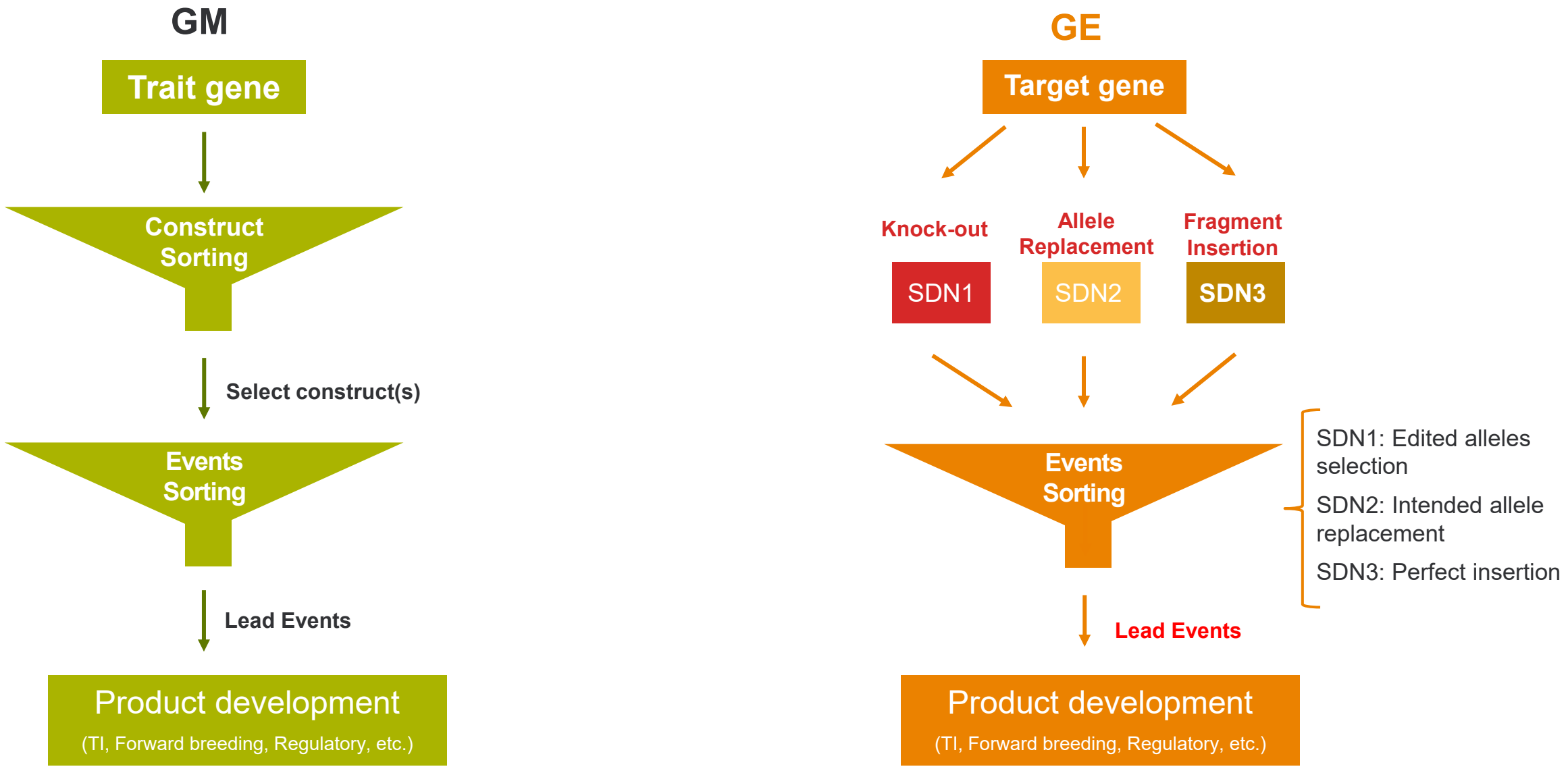
- **Mutations generated by CRISPR-Cas:**
  - SDN-1: induction of single point mutations or small In/Del, without donor;
  - SDN-2: short insertions or editing of a few base-pairs (allele replacement) with donor;
  - SDN-3: insertion of longer foreign DNA fragment (transgene) using donor;



(NBT platfom: https://www.nbtplatform.org/)

Comparison of molecular characterization of GM and GE plants

GM and GE Product Development



Consideration for GE event molecular characterization

	GM	GE
Transgene characterization	Transgene (i.e., trait gene) copy #;	Transgene (i.e., GE machinery) copy #
Absence of the transgene in the later generation	Confirmation of absence of vector backbone	Confirmation of absence of the transgene (GE machinery) and vector backbone
Trait or target gene expression	Temporal or spatial expression of trait gene	SDN2 and SDN3 editing
Editing at the target gene(s)- On-Target edit	Flanking sequence recovery (FSR)	SDN1: different editing allele with indels SDN2: Examine if the desired allele replacement is intact SDN3: Examine if the desired DNA template is intact
Inheritability	Transgene inheritability	Edited alleles or insertion DNA fragment inheritability

Detection methods for GE crops

Approaches for detecting On-target editing plants

- **PCR amplification-based methods:**
  - Cleaved amplified polymorphic sequences (CAPS)
  - Allele specific primer extension (ASPE)
  - Mismatch cleavage assay (T7E1 surveyor assay)
  - High resolution melting analysis (HRM)
  - TaqMan real-time PCR: LNA probe, rhPCR, castPCR, etc.
  - Digital PCR (dPCR)
- **Sequencing-based methods:**
  - Sanger sequencing
  - NGS: amplicon sequencing

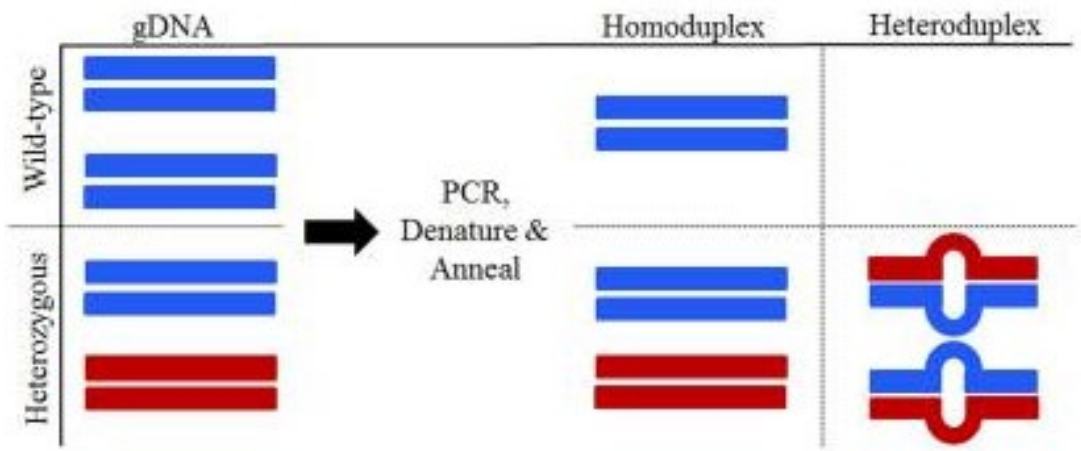
Mismatch cleavage assay

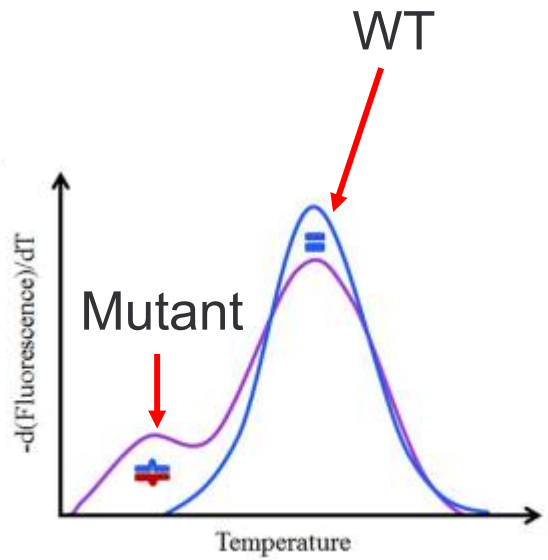
- Amplify target site and flanking sequence (~ 500 - 1000bp) by PCR
- Denature and anneal to form hetero-duplex DNA
- Digestion by T7E1 and run agarose gel
- Simple, cost-effective
- Can’t detect homozygous mutations

Zhu X et al., Scientific Reports 4, 6420 (2014).

High resolution melting analysis (HRM)

- Amplification of about 80-200bp with DNA binding dye
- Run melting curve analysis
- Can distinguish homozygous mutation

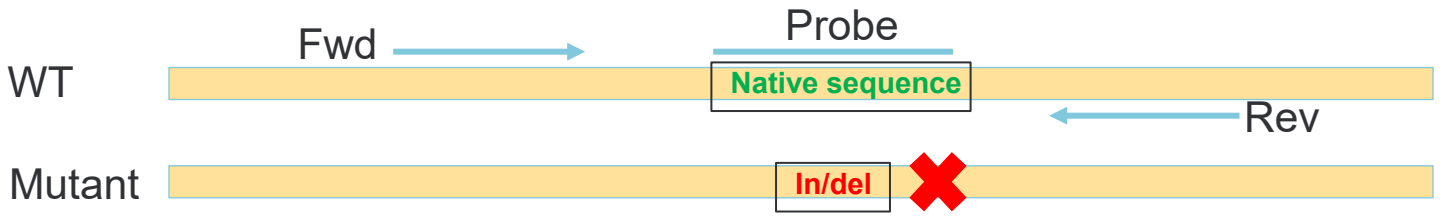




Thomas HR et al., PLOS ONE 9 (12): e114632 (2014).

TaqMan PCR approach for detecting SDN1 and SDN2 mutations

- Negative selection, also called drop-off assays
- Short probe is designed at SDN cutting sites
- High sensitivity, low cost



Examples of mutant

ATCGAGCTTACGACTACCGAATTCTGACGACTACCG (native sequence)
ATCGAGCTTACGACTACCGAATTCT- ACGACTACCG (1 bp deletion)
ATCGAGCTTACGACTACCGAATTCT- - CGACTACCG (2 bp deletion)
ATCGAGCTTACGACTACCGAATTCT- - - GACTACCG (3 bp deletion)

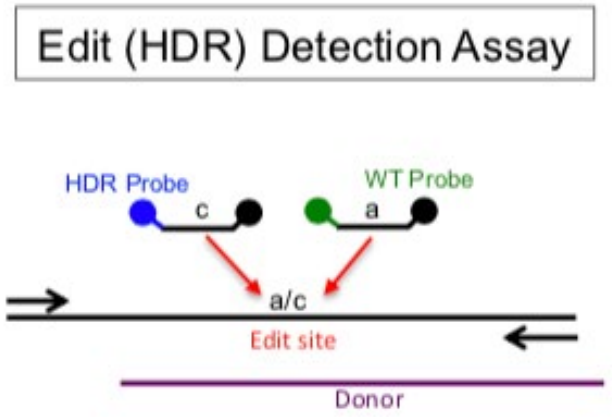
Interpretation of TaqMan results (e.g., diploid crop)

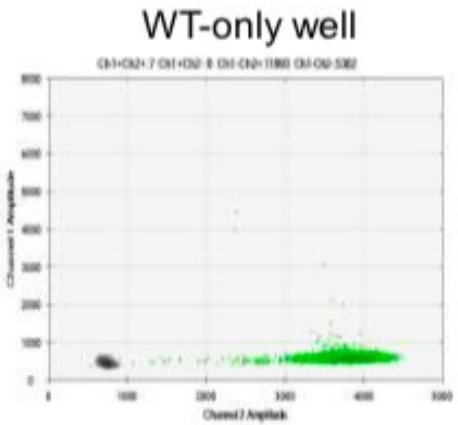
Copy number	Genotype
2 copies	wild type
1 copy	one allele is mutated
0 copy	two alleles are mutated

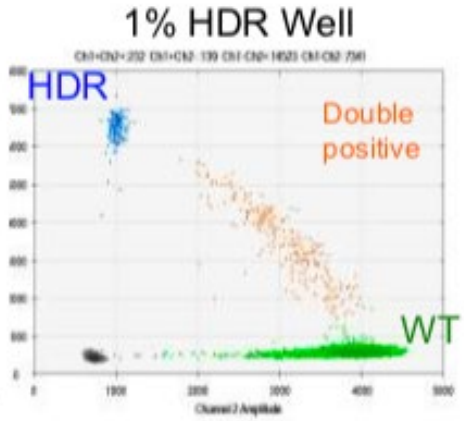
Digital PCR (dPCR) technology

Two different technologies:

- Droplet Digital PCR (ddPCR): PCR is performed in a water-oil emulsion in nano-scale reaction.
- Chip based: digital array and Open array.



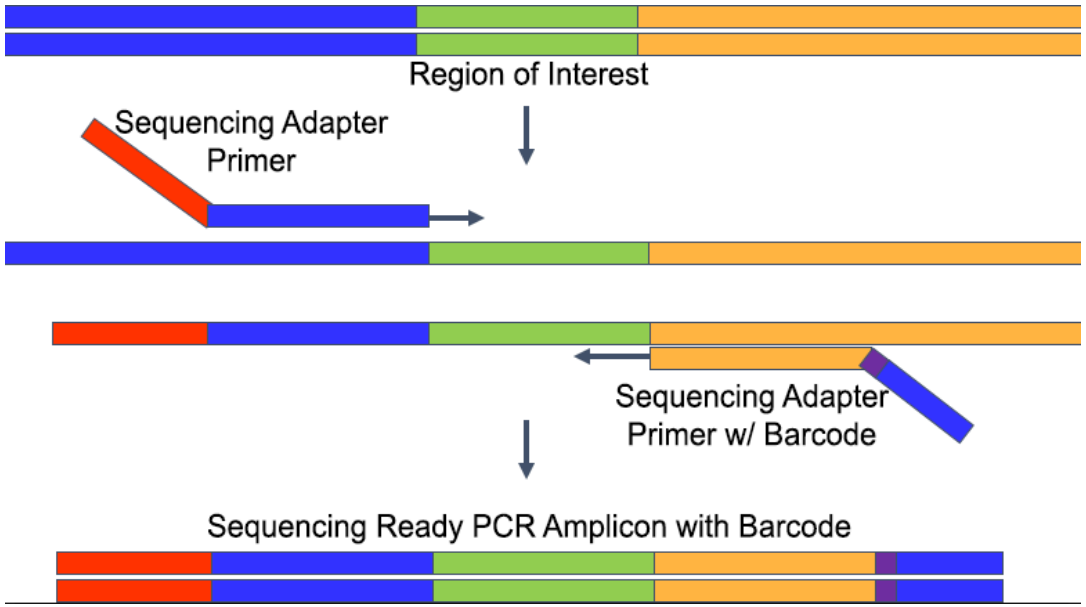




(BioRad technical report)

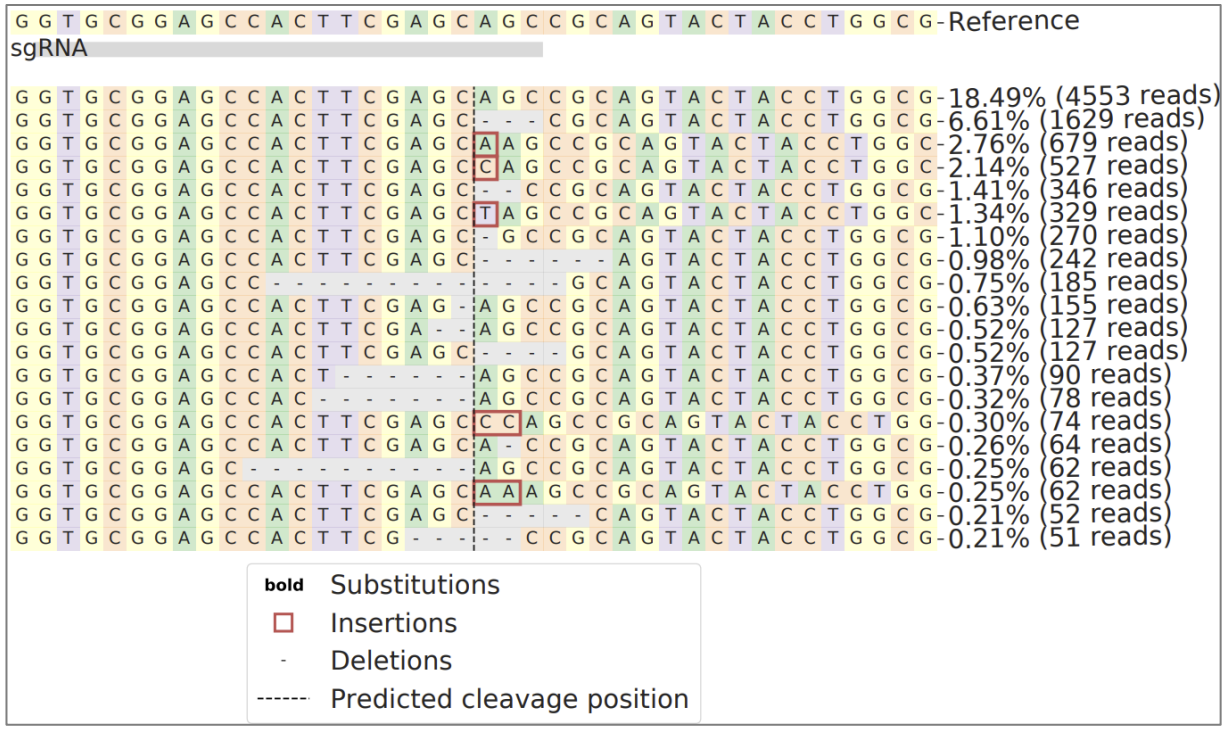
NGS amplicon sequencing

Targeted deep sequencing



Multiplexing using distinct barcode system

Example of edited sequences

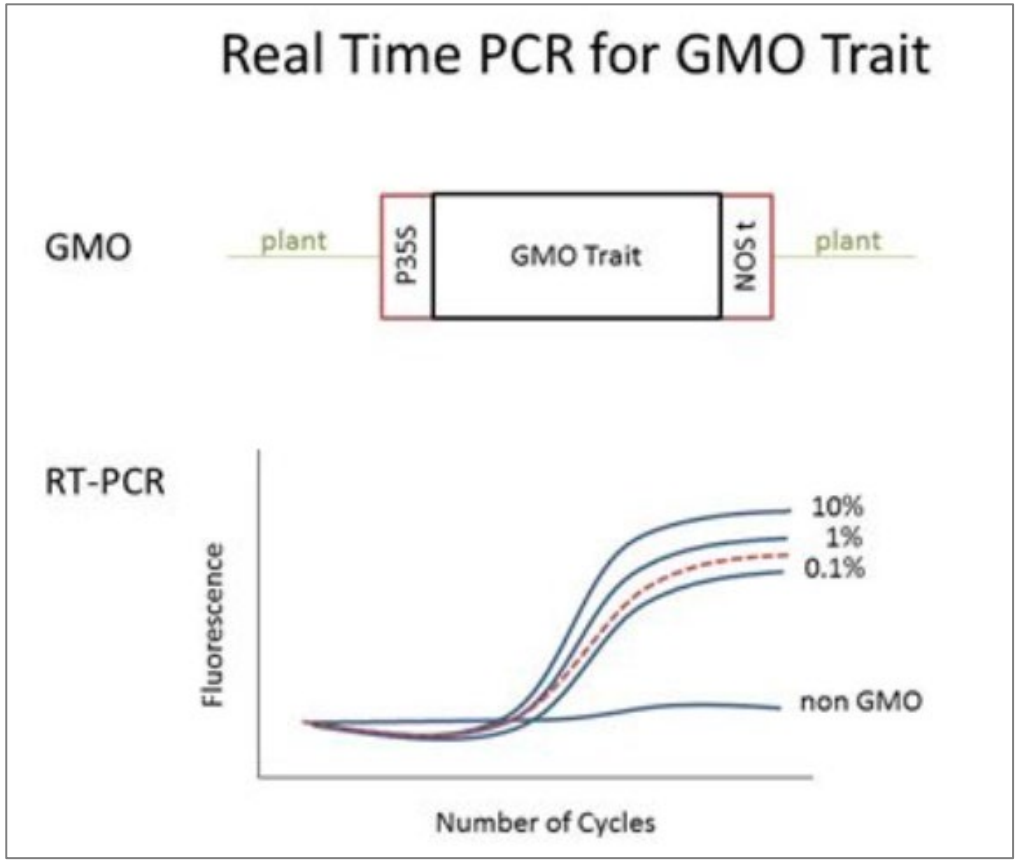


(Example from CRISPResso, https://crispresso.pinellolab.partners.org/)

Challenges in detecting GE plant products

Regular practice in detecting GM (genetically modified) plant products

- TaqMan real-time PCR is the gold standard for GM detection (individual or bulk samples).
  - Event specific assay (ESA): designed at the junction of T-DNA and flanking sequence from genome to detect a particular event.
  - Component assay: trait genes (e.g., PAT, EPSPS) or commonly used promoters and terminators (p35S, tNOS etc.).



https://www.indianacrop.org/Lab-Services/Low-Level-Presence/GMO-Non-GMO-Scr

Challenges in detecting GE (genome edited) plant products

- Develop a detection method with comparable sensitivity and specificity as for GM detection.
  - For unique editing sequences (small insertion, deletion or even one single nucleotide change) in the GE events, PCR-based assay may not be robust enough to differentiate from the WT sequence.
- The ability to routinely and reliably detect the GE-derived small indels in bulk grain sample (mixed or heterogeneous) as opposed to pure and homogeneous samples from the laboratory.
- Need a high-quality database: include all genotypes of genome-edited plants as reference.

Summary

- Genome editing technology can greatly accelerate trait development and crop breeding.
- As compared with GM detection method, GE product requires different analytical approaches during the trait development process to characterize editing alleles. There are more challenges in detecting GE plants.
- In the early phase of GE product development, PCR based and NGS based technology complement each other to distinguish the editing alleles from the WT allele derived from conventional breeding.

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Thank you!