Oregon State University
Oregon Wine Research Institute

Transgene-Free Gene Editing Methodology for grapevine

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Outline

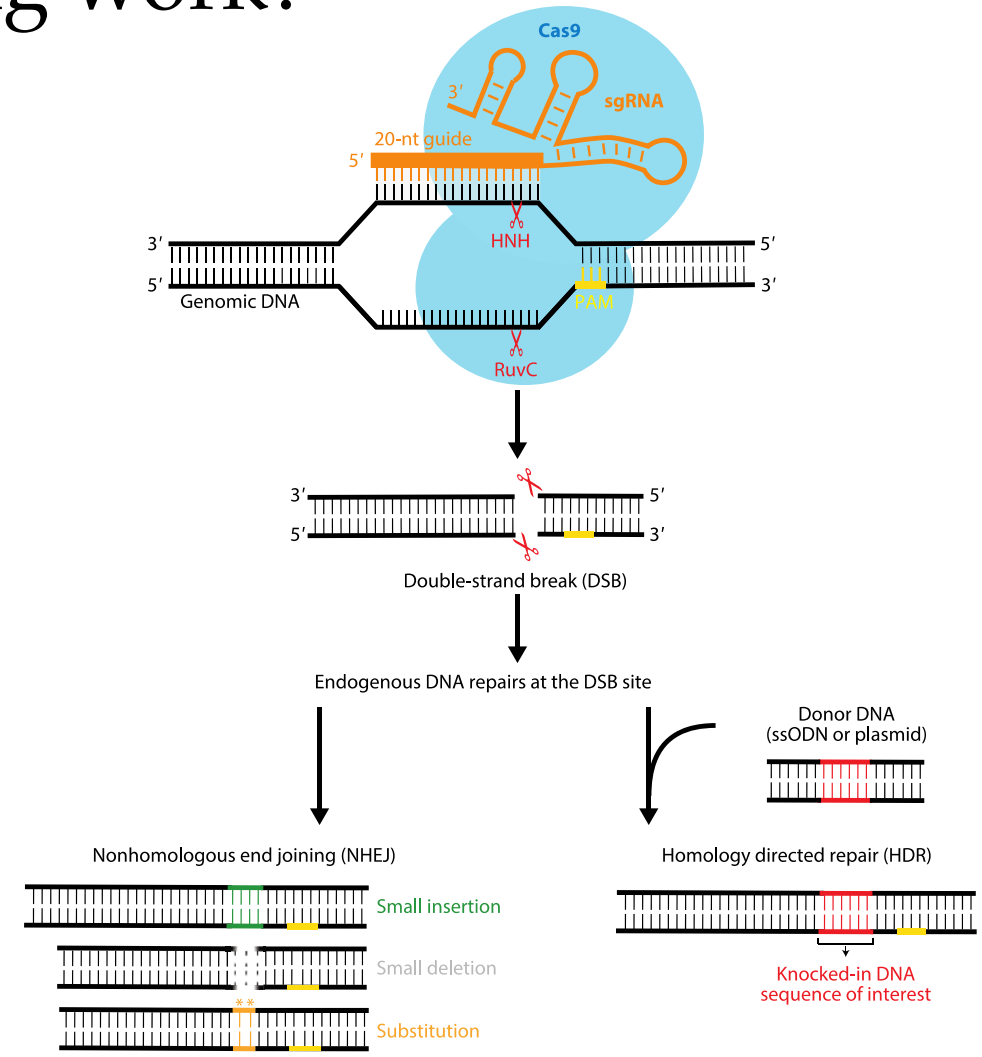
- Background
- Project Concept
- Current status of the research
- Conclusions

The Gene Editing

- Promising biotechnology for crop improvement.
- Simplicity, precision, and power offers opportunities to accelerate conventional breeding.
- Genetically edited crops are regarded as products of plant breeding under certain rules and conditions.

How does gene editing work?

- Use of a natural "molecular scissor" containing two elements (a guide RNA and a nuclease)
- Once delivered to plant cells, the nuclease guided by the RNA will cut the DNA (double-strand break) and the plant itself will repair with a relative success.
- Implementation of the system to a "precise" editing by providing the repair template.
- Different types of scissors (specificity, targeted molecules [RNA, DNA]) – the most popular system being the CRISPR-Cas9 complex.



How is gene editing currently used?

- **Stable expression via transgenic method (most popular)**: Insertion of the gene editing ingredient in the target genome to perform the editing.

  - **Poor acceptance of GMO and regulation of GMO-based products is a problem to fully embrace the technology with grapevine.**

- **Transient way to express the gene editing ingredient (emerging)**: Delivery of the ingredients either as a nucleic-acid based molecule or a RiboNucleoProtein (RNP).

  - **Crossing the cell wall and the plasma membrane is still a major hurddle that tends to decrease the editing efficiency. The use of naked plant cells (protoplasts) as material is an option but it is not a simple methodology to regenerate individual plants.**

Can we develop a sustainable method for transgene-free gene editing in grapevine?

A stepwise methodology to generate transgene-free gene edited grapevine

- **Phase 1**: Conventional genetic engineering to generate stable transformants ***via*** *Agrobacterium tumefaciens* transformation containing an ***"excisable"*** genetic cassette aiming at editing one or several target genes in the grapevine genome.

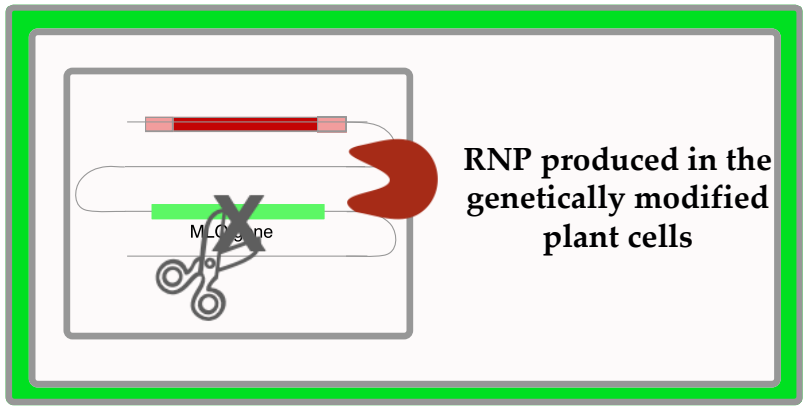
- **Phase 2**: Use of a peptide carrier (Cell Penetrating Peptide) to ease the entry of a RiboNucleoProtein (RNP) into intact a ***regenerable edited plant material*** to excise the inserted genetic cassette during Phase 1.

- Transgene-free gene editing for Mildew Locus O genes to confer Powdery Mildew resistance

Grapevine susceptible to Powdery Mildew

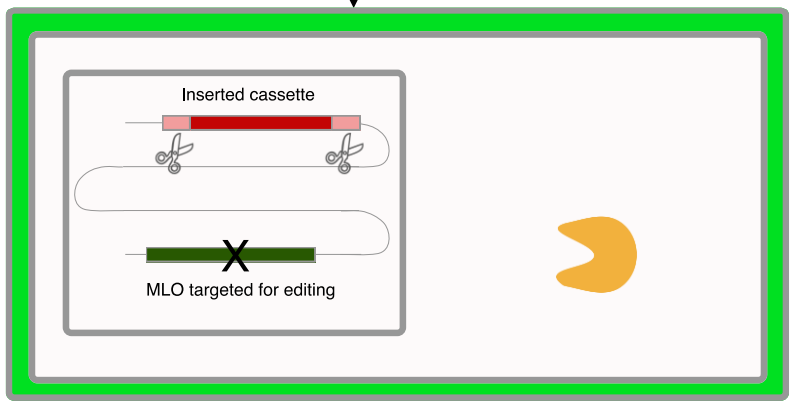
Genetic Engineering

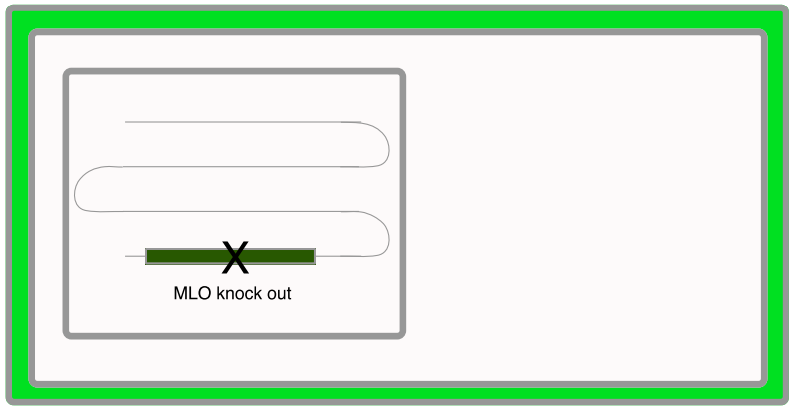
Phase 1
INTEGRATION



Knockout mutants supposedly resistant to PM

Phase 2
EXCISION





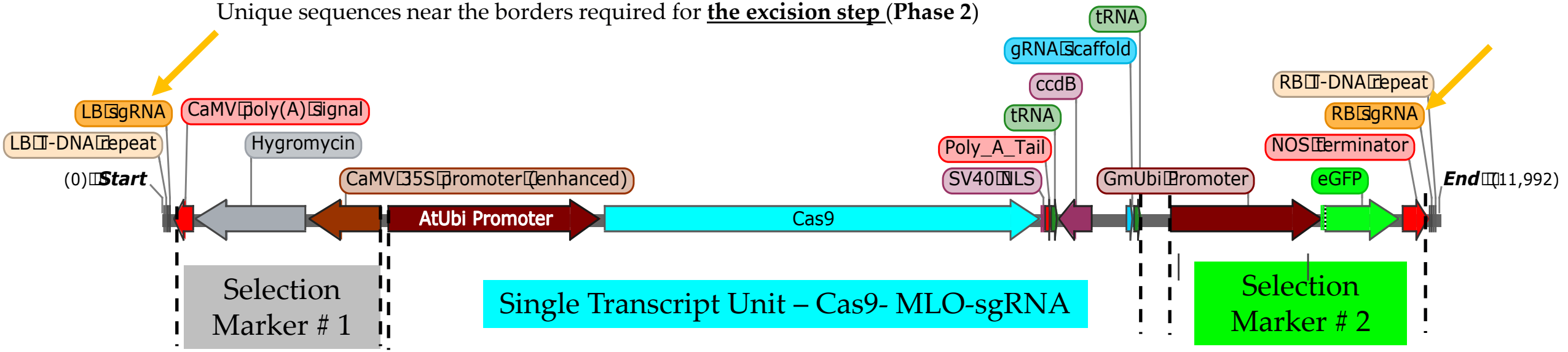
Transgene free edited grapevine material resistant to GPM

Objective 1: Characterize the **unique sequences** in the inserted genetic cassette for further excision.

Objective 2: Produce the specific **MLO-gene edited knockouts** grapevine plants and evaluating their resistance to *Erysiphe necator*.

Objective 3: Validate the **CPP-mediated RNP delivery to intact transgenic grapevine cells** to excise the inserted cassette in the MLO-edited plants.

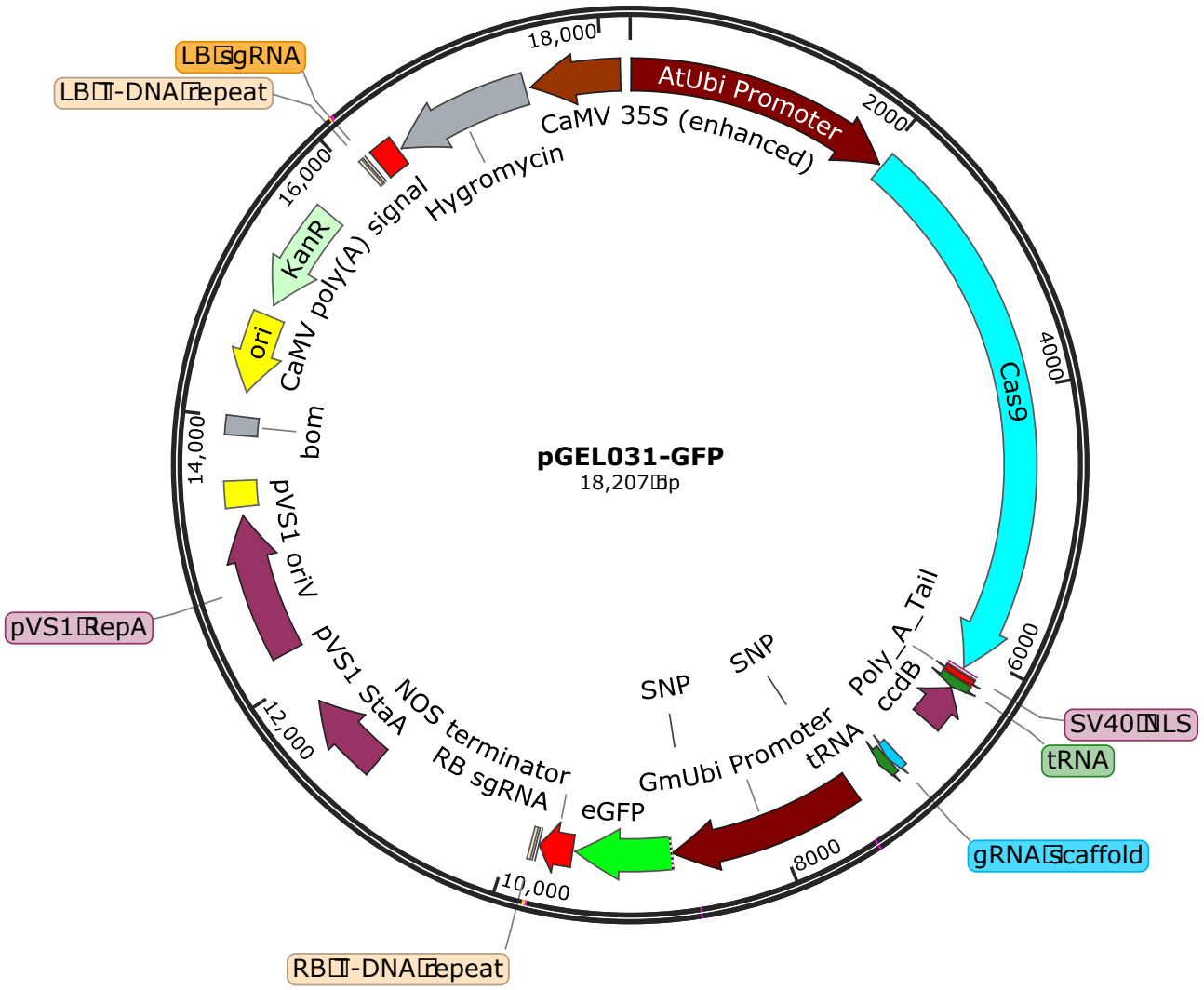
Map of the "excisable" genetic Cassette inserted in **Phase 1** (Transfer-DNA) – 11.2 kbases

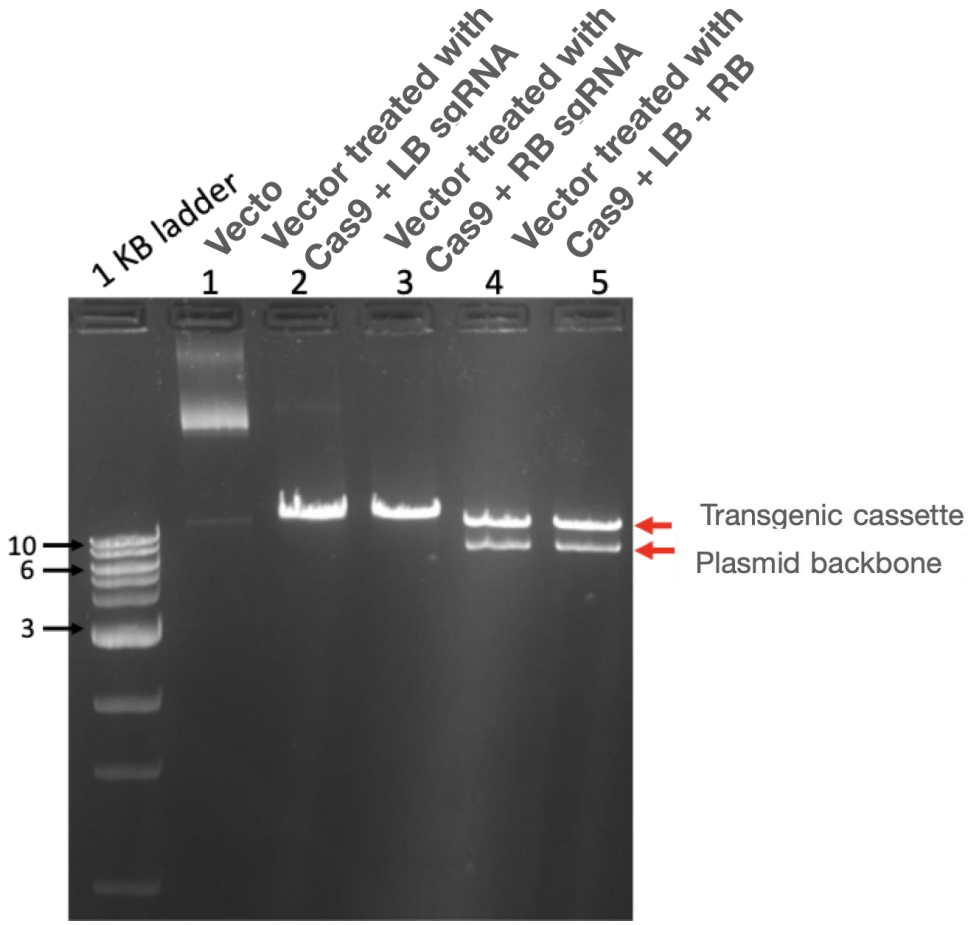


Two selection markers: Green Fluorescent Protein and Hygromycin B phosphotransferase

Expression system: Single Transcript Unit of CRISPR-Cas9 with sgRNA designed to target MLO genes

In vitro cleavage assay to test the release of the inserted genetic cassette



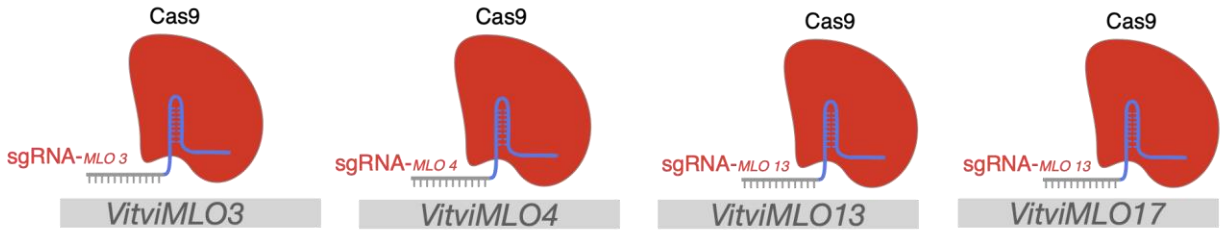


Agarose gel showing the release of the transgenic cassette following the incubation of Cas9 with two sgRNA candidates (LB and RB sgRNA) **complementary to the DNA sequence** near the **Right and Left Borders**.

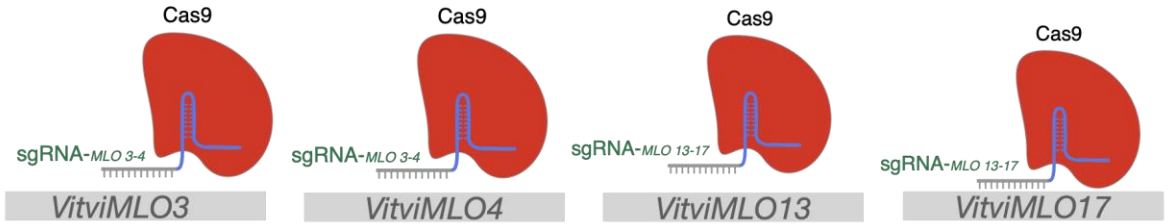
Map of the binary vector used for *Agrobacterium Tumefaciens* transformation (**Phase 1**)

Knockout generation of 4 MLO genes of the clade V

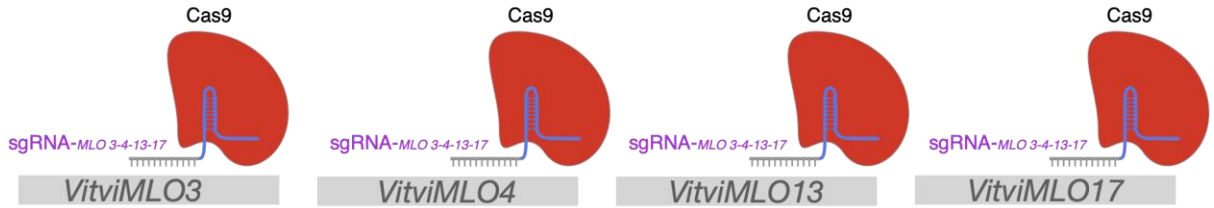
- VitviMLO 3,4,13, and 17 single knockout mutants (**4 transformations**)



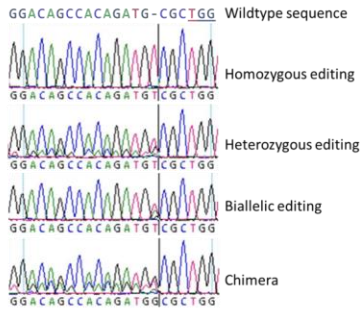
- VitviMLO 3,4, and 13-17 Double knockout mutants (**2 transformations**)



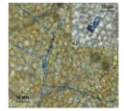
- VitviMLO 3-4-13-17 Quadruple knockout mutant (**1 transformation**)



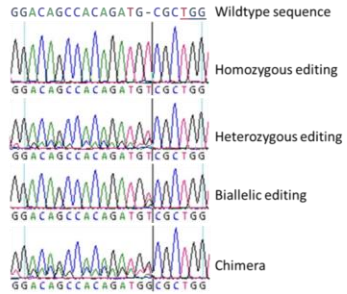
Identifying the edited microvine lines among the transgenic



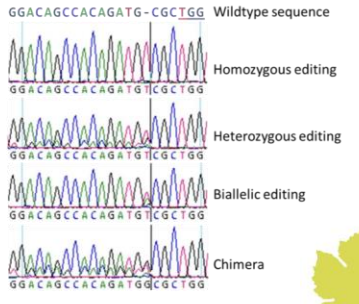
Erisyphe necator inoculation assay, disease severity assessment, histological analysis



Single mutant evaluation in conferring PM resistance?



Double mutant evaluation in conferring PM resistance?



Quadruple mutant evaluation in conferring PM resistance?

Number of transformants for each transformation event

Transformants confirmed by selection markers	Embryos at different differentiation stages	At plantlet stage for phenotypic test
VitviMLO3 - Single Knockout	40	2
VitviMLO4 - Single Knockout	15	-
VitviMLO13 - Single Knockout	2	-
VitviMLO17 - Single Knockout	31	-
VitviMLO3,4 - Double Knockout	32	2
VitviMLO13,17 - Double Knockout	26	-
VitviMLO3,4,13,17 Quadruple Knockout	80	1

Total: 226 transformants

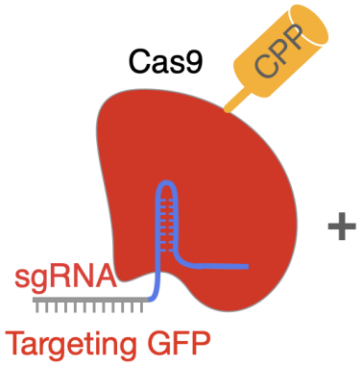
Preliminary results for the genotyping (Sanger Sequencing of the edited DNA region)

	Target Gene Locus Edited or Not			
Gene-edited MLO mutants Mutants	**VitviMLO3**	**VitviMLO4**	**VitviMLO13**	**VitviMLO17**
VitviMLO3 - Single Knockout	Yes			
VitviMLO17 - Single Knockout				Unclear
VitviMLO3,4 - Double Knockout	Yes	Unclear		
VitviMLO13,17 - Double Knockout			Yes	Yes
VitviMLO3,4,13,17 Quadruple Knockout	Yes	Unclear	Yes	Yes

In gray: the editing was not validated

- Only one transformant per mutant line has been analyzed for gene editing, still a lot to analyze…

Evaluation of cleavage of internalized CPP-RNP to edit GFP gene in transgenic Green Fluorescent Protein-expressing grapevine (positive control)



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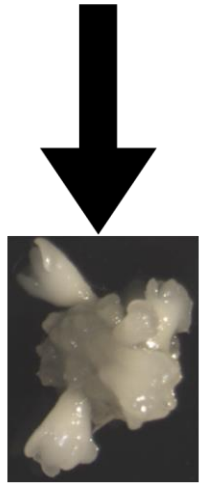
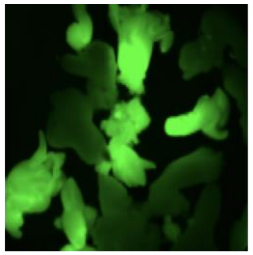
gDNA extraction of the cells 48 hours after their treatment with CPP-RNP + PCR on the target region

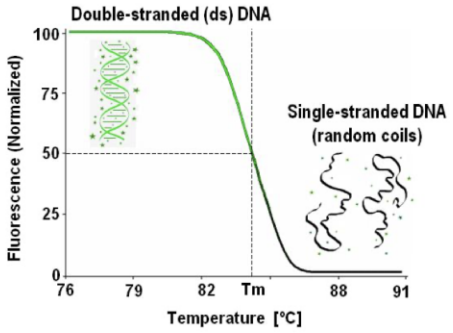


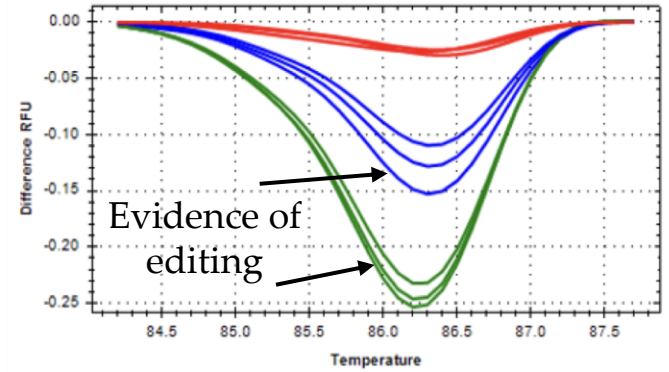
Embryogenesis



Negative Selection

High-Resolution Melting Analysis





Cas9/sgRNA treated cells

CPP-Cas9/sgRNA treated cells

CPP-Cas9/sgRNA embryos that lost GFP fluorescence

Ten edited embryos with clear loss of GFP activity, but out of hundred non edited GFP embryos… and the "transgene free" embryos stopped growing after a while

Objective 1:

- Modification of the inserted genetic cassette was **successfully engineered** for further excision and validated.

Objective 2:

- We generated transformants for single, double and quadruple knockouts,
- **Editing was confirmed** for a few of them, but so far, no presence of an editing across the two alleles of the loci, however, still a lot of transformants to screen,
- The phenotypic characterization of the plants still needs to be performed.

Objective 3:

- The **RNP can be delivered** to intact plant material for editing **using Signal Peptide (CPP)**, but low editing rate and cells don't seem to survive to the treatment,
- Need for **revisiting the delivery conditions** and potentially **the carrier system** to improve the editing rate and **long-term viability** of the transgene-free edited material

Acknowledgment to the funding agencies:

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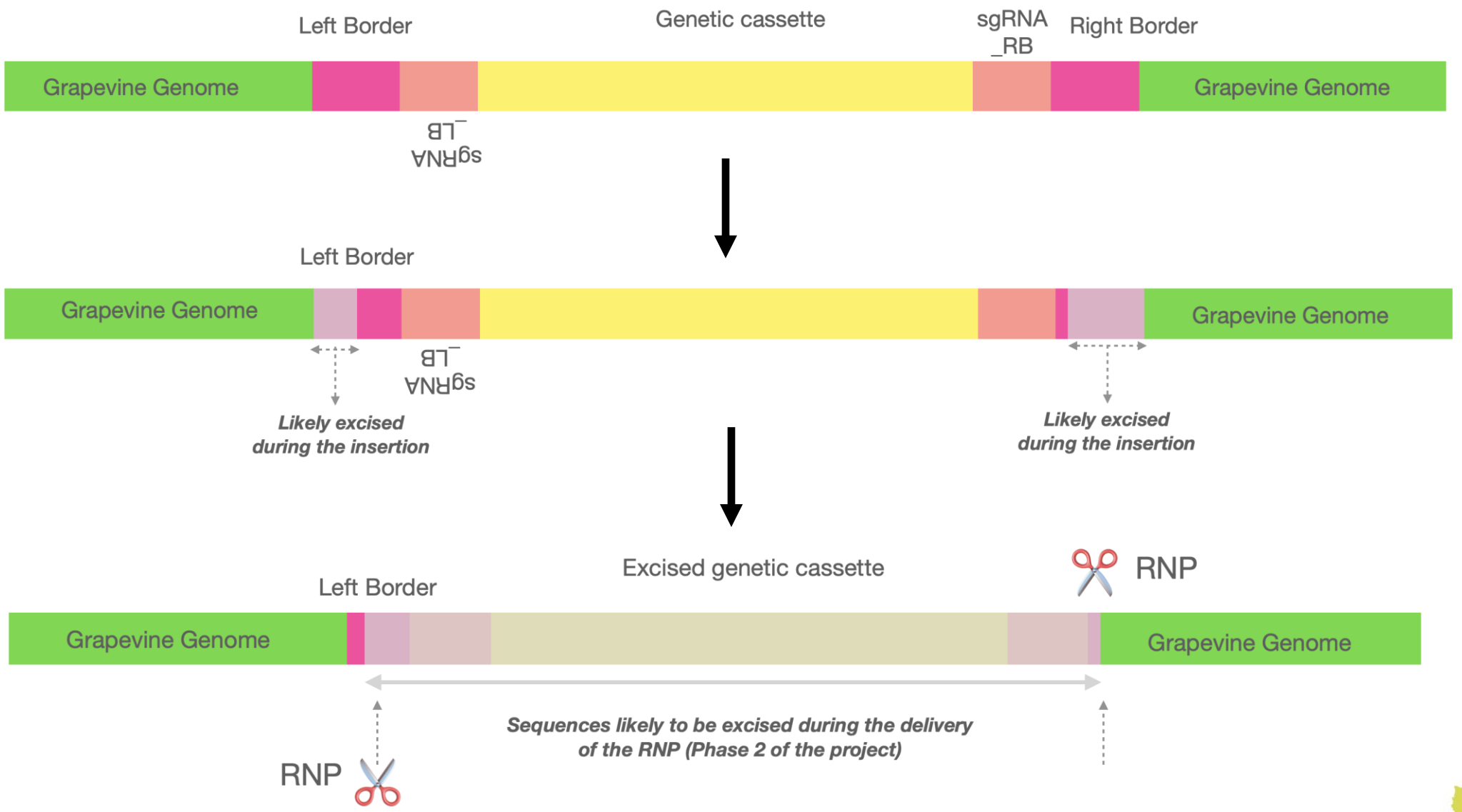
Application of Gene Editing

- Understanding gene-to-trait relationships
- Improving current cultivars that show “impaired genetic background”.
- Complementary tool to conventional breeding to accelerate the time for genetic improvement

Status of the Research: Objective 1



Before Insertion in the Genome

After Insertion in the Genome and before the Excision (Phase 2)

Expected product

Preliminary results for the genotyping of the MLO transformants

MLO13 in DKO_13-17 mutant

Allele1: AGATATGCATTTCTAAGAGT -GTTGGATCCACTTGG (WT)

Allele2: AGATATGCATTTCTAAGAGT**T**GTTGGATCCACTTG **(insertion)**

Reference: AGATATGCATTTCTAAGAGT -GTTGGATCCACTTGGTACCCTTGTGATGTTGATGAGAAAGAATTTAAAAA

MLO17 in DKO 13-17 mutant

Allele1: AAGATATGCATTTCTAAGAGT GTT GGGTCCACTTG (WT)

Allele2: AAGATATGCATTTCTAAGAG**G**G**GG**GGG**C**C----------CCCTTG **(substitution and deletion)**

Reference: AAGATATGCATTTCTAAG AGT GTT GGGTCCACTTGGCACCCTTGTAAAAAGGAAACCAAAGATTTTAAGA

MLO17 in QKO mutant

Allele1: GATATGCATTTCTAAGAGTGT TGGGT CCACTTGGC (WT)

Allele2: GATATGCATTTCTAAGAGTG**G**TGGG**G**CC**C**CTTGGC (**substitution**)

Reference: GATATGCATTTCTAAGAGT GTTGGG TCCACTTGGCACCCTTGTAAAAAGGAAACCAAAGATTTTAAGAAC

Sanger sequence chromatograms of MLO gene-target regions from gDNA extracted in transformants analyzed using a web-based tool for editing call (DSDecodeM by Liu et al., 2015)