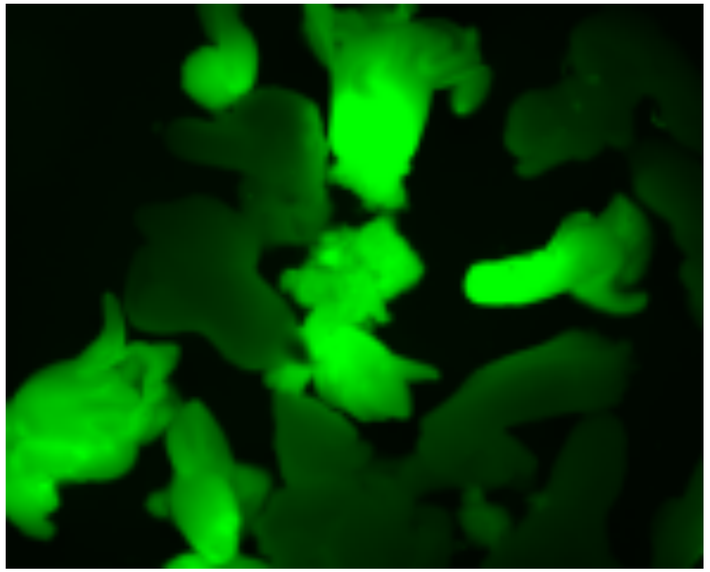
Oregon State University | COLLEGE OF AGRICULTURAL SCIENCES » Department of Horticulture

The Gene Editing Technology for grapevine

Satyanarayana Gouthu and Laurent Deluc



Edited microvines embryos expressing the GFP genes

Oregon State University
Oregon Wine Research Institute

Outline:

- Introduction and Background

- The two projects (MLO project and precise gene editing)

- Conclusions and perspectives

The Gene Editing technology

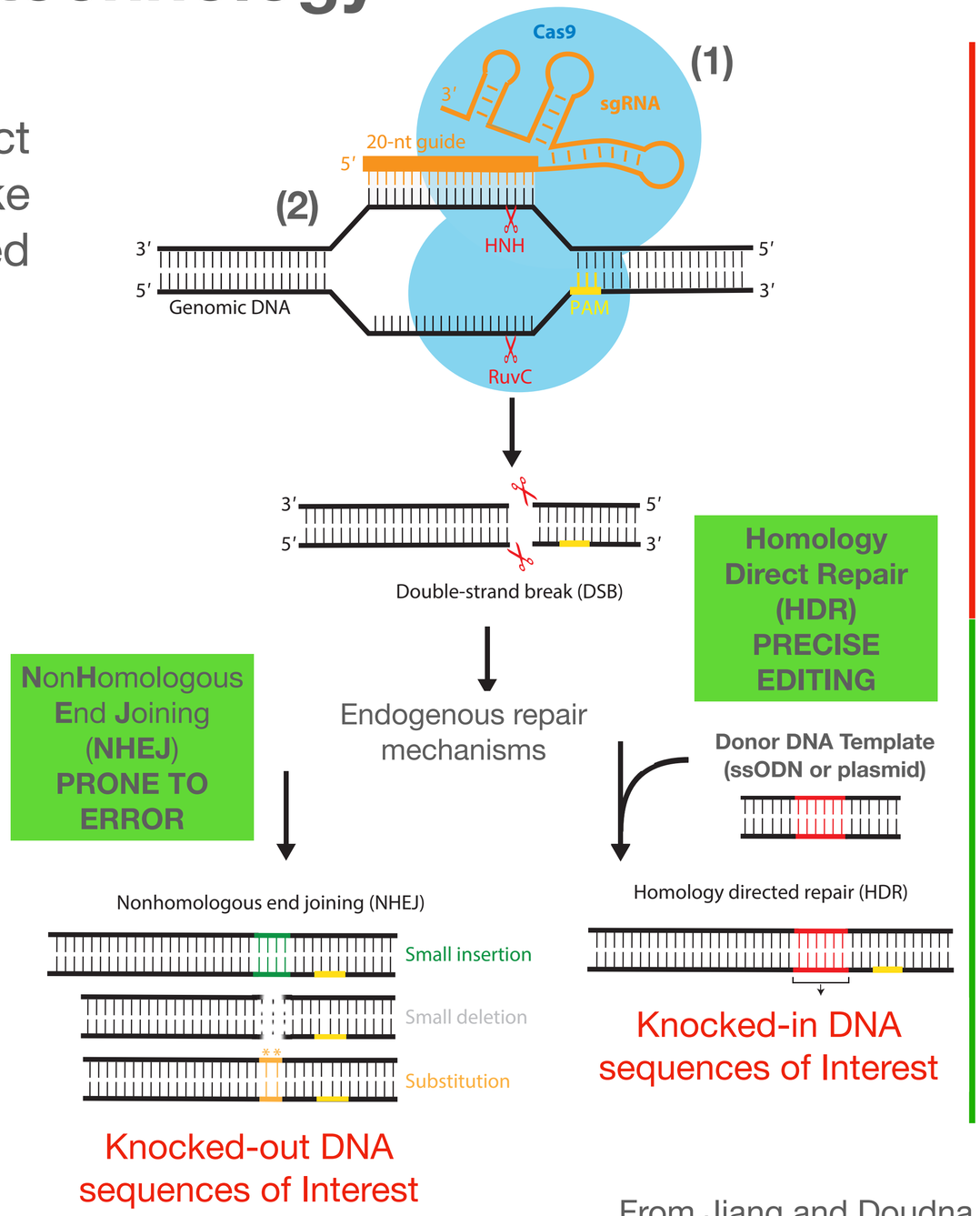
- Based upon the use of **R**ibo**N**ucleo**P**rotein (**RNP**) that act as “molecular scissors”. It consists of a Nuclease **(1)** like **Cas9, Cpf1,** and an RNA-structure **(2)** commonly named **single guide RNA or sgRNA**.

- The sgRNA **targets** the region to cut in the genome and then the Nuclease **cuts**.

- Two major repair mechanisms will “fix” the cut

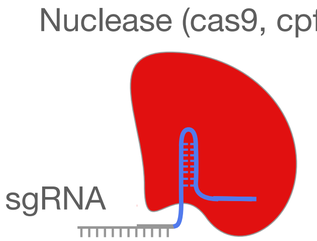
- **Non-Homologous End Joining (NHEJ) - Prevailing**: Prone to “error” (substitution, deletion, insertion) leading to the generation of “**knock-outs**”.

- **Homology-Direct Repair (HDR) - less frequent**: The process can also be engineered by providing the repair template (Donor Template), and creating **“knock-ins”**.



From Jiang and Doudna, 2017

Non Homologous End Joining mechanism (Prevailing but prone to error):



Protospacer-Adjacent Motif

Complementary sequence to sgRNA

ACCGTCGAGCGTTACTGATGTAGCC

TGGCAGCTCGCAATGACTACATCGG

Selected site for editing (coding region)

After Repair

Gene Status

ACCGTCG-GCGTTACTGATGTAGCC

TGGCAGC-CGCAATGACTACATCGG

→ Silenced Mutation

Deletion of one base- Generation of a frameshift (-1)

ACCGTCGAGCGTTACTGATGTAGCC

TGGCAGCTCGCAATGACTACATCGG

→ No functional change

Original sequence - no frameshift

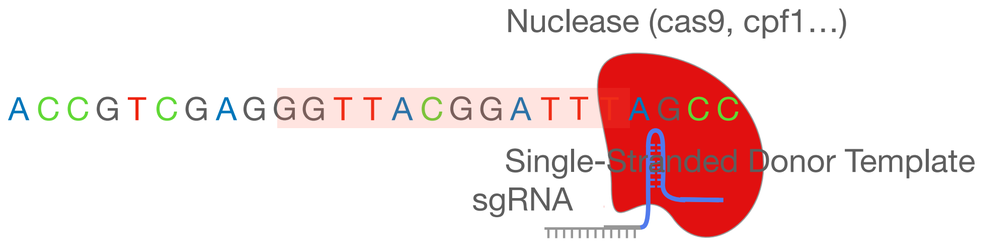
ACCGTCGTAGCGTTACTGATGTAGCC

TGGCAGCATTCGCAATGACTACATCGG

→ Silenced Mutation

Insertion of one base- Generation of a frameshift (+1)

Homology-Directed Repair:



Gene Status

Value of Gene Editing technology

- It is fast, versatile (multiplexing, targeting coding regions or non-coding [promoter]).
- It can accelerate breeding program.
- Creating new elite cultivars (***making a "white" Cabernet-Sauvignon or a "Red" Chardonnay***).
- Regarding in U.S. as breeding process.

Limitations of its application

- Pre-required knowledge about the relationship between the gene function and the performance trait of interest.
- Mostly focused right now on "Negative Regulators".
- Off-target is a risk with unintended effects on other parts of the genome.

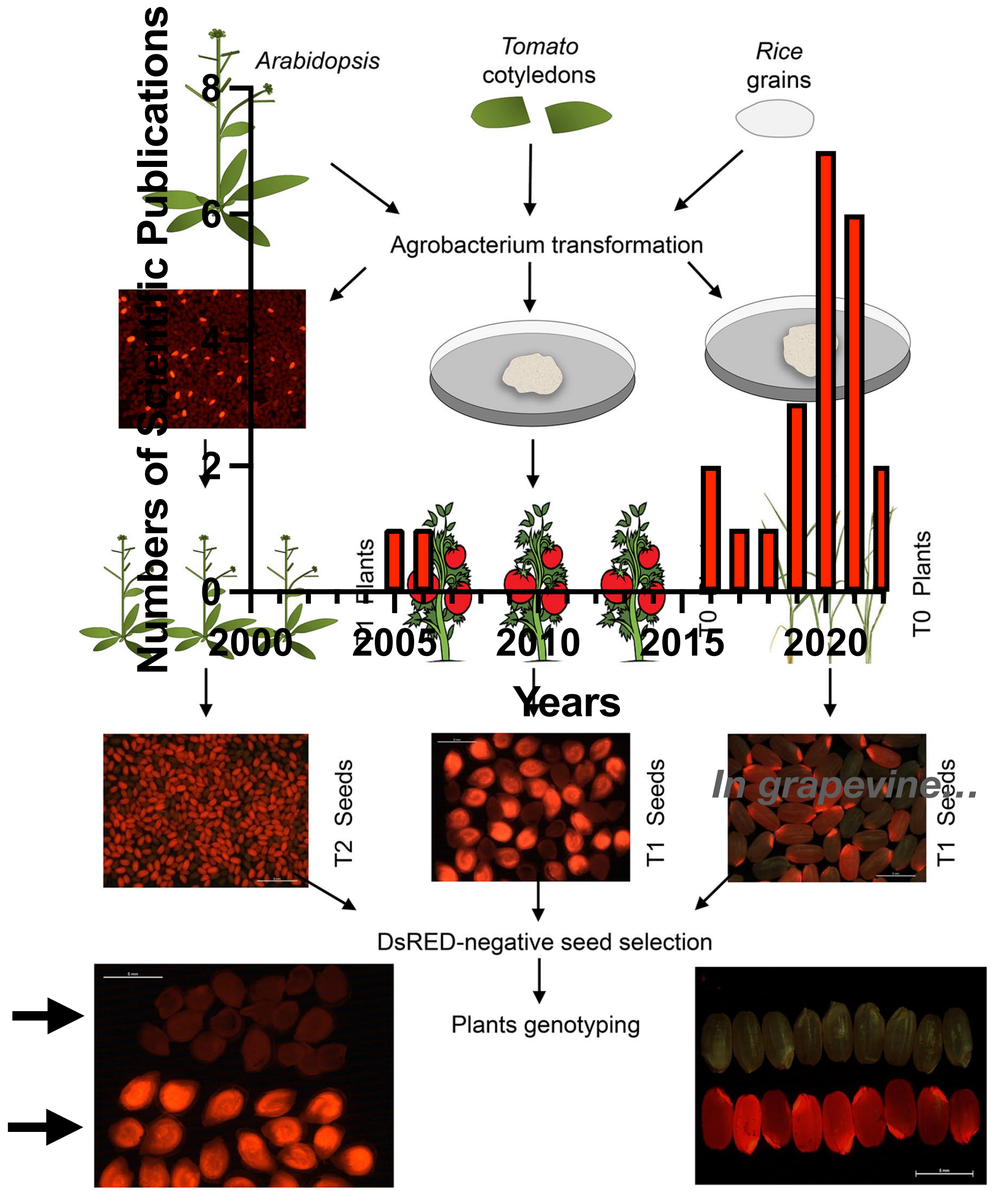
How is genome editing performed in plants ?

Transgenic method:

- **Physical insertion** in the plant genome of a DNA-based molecule containing all the gene-editing ingredients (endonuclease and sgRNA structure targeting a Gene of Interest), and the selection markers) - **Conventional Genetic Engineering**.

- BUT **you can also eliminate** the transgenic cassette…most well-documented strategy in plants is **selfing/backcrossing**.

This approach is not a viable in vegetatively propagated crop like grapevine…



Steps describing one methodology to remove the selection markers after gene editing via *Agrobacterium* transformation
Aliaga-Franco et al., 2019

How is genome editing performed in plants ?

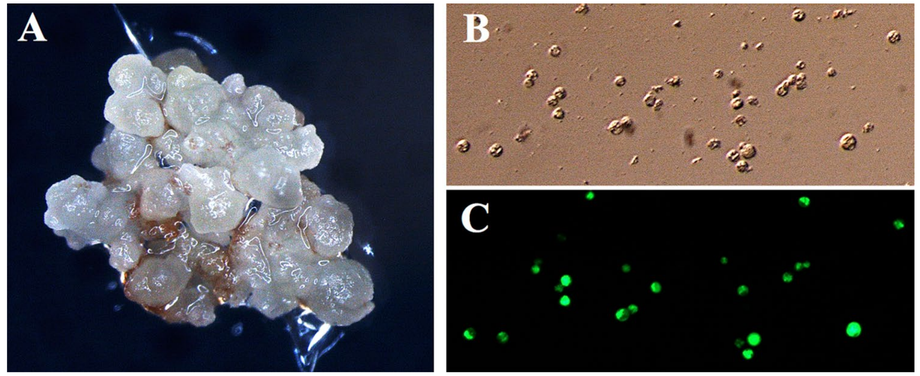
Non-transgenic method:

- Delivery of Gene Editing ingredients as either a Protein or a DNA-based molecule to an intact cell is almost **IMPOSSIBLE**….

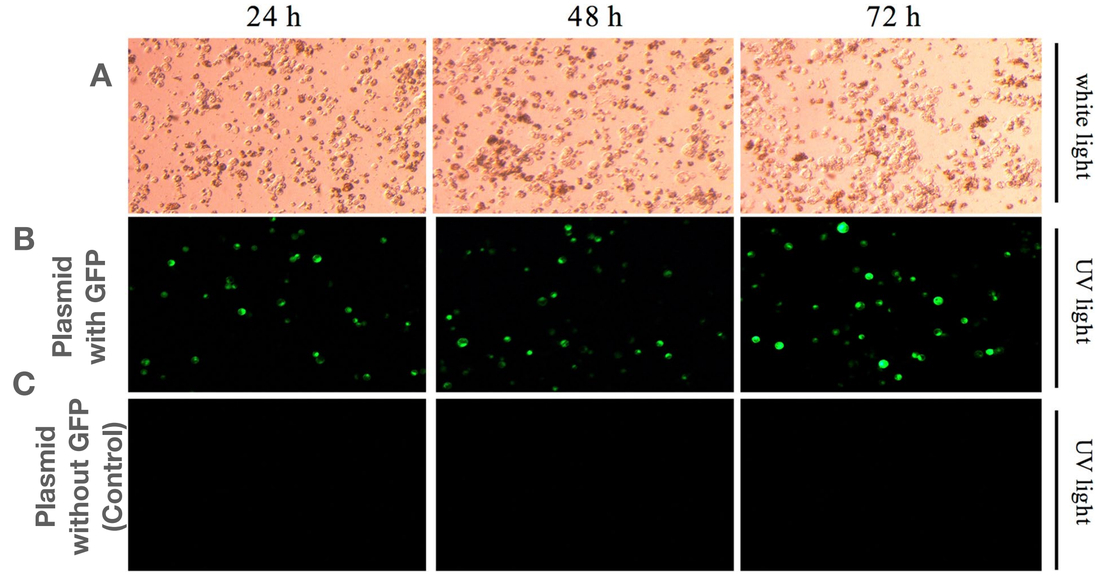
- One popular technique is to use **protoplasts** (naked cells without **Cell Wall**) to “transform” the cells. The expression of the genetic cassette is **transient**, not stable. So, **not regarded as GMO**…

- Plant regeneration from protoplasts is still difficult to streamline in many models and the progenies may be prone to *widespread genome instability* (Fossi et al., 2018).

- Difficult to trace edited protoplasts among an entire population.



Protoplast isolation from Embryogenic callus: **A**) Garganega embryogenic callus, **B** Protoplasts isolated from the callus, **C**) Protoplasts isolated from the callus and labelled with green dye FluoroDiacetate (FDA)—UV light.



Protoplast transfection: **A**) Protoplast transfected with plasmid DNA containing the Green Fluorescent Protein expression cassette (white light), **B** Protoplast transfected with plasmid DNA containing the Green Fluorescent Protein expression cassette (UV-Light) **C**) Protoplasts transfected with empty vector.

GMO and Gene Editing in grapevine

Drawbacks:

- Time-length and investment for regulation of GMO-based products are substantial.
- Poor public acceptance of GMOs.
- Continued expression of the editing system (constitutive expression) in the genome may result **in increased off-target effects and unintended impacts.**
- Precise gene editing (HDR) is still relatively inefficient.

Opportunities:

- If no foreign DNA is contained in Genetically Edited crops, then it is regarded by USDA as a product of plant breeding. Less regulatory hurdle.
- New **“transient” delivery systems and approaches** to deliver proteins to intact plant cells exist.

Two projects in grapevine

Project 1 (2019-2022): How to produce transgene-free gene-edited (knock out) grapevine material?

Proof of concept: Knockout of “negative regulators”, **susceptibility MLO genes** (Grape Powdery Mildew)

Project 2 (2021-2023): How to improve Precise Gene Editing (HDR) in grapevine?

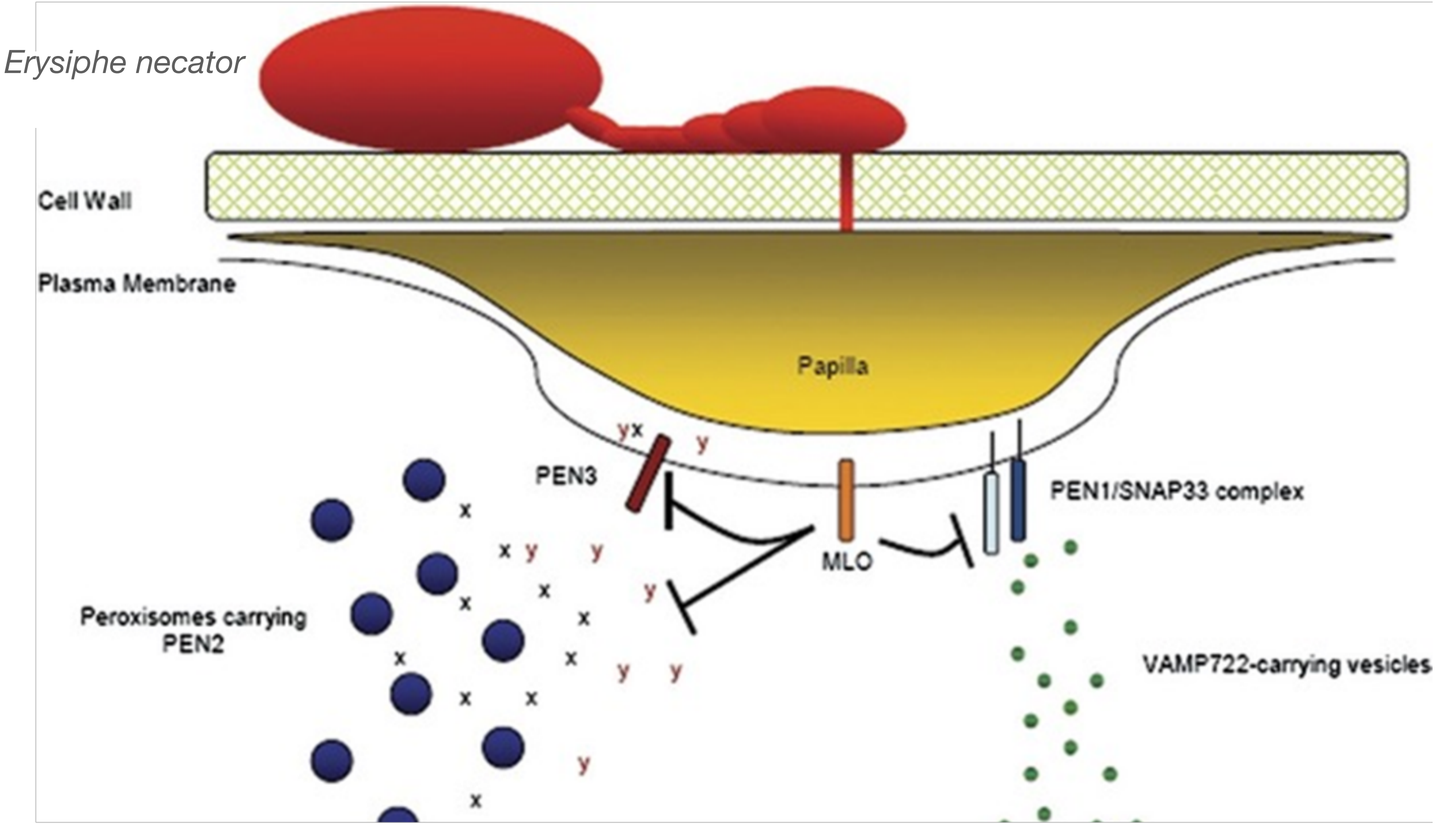
Proof of concept: *Edit* ***Green Fluorescent Protein****-expressing microvine lines to become* ***Blue Fluorescent Protein****-expressing lines.*

Project 1: Why MLO?

- MLO proteins ***negatively regulate*** the activity of plasma-membrane localized proteins involved in the formation of the papilla at the site of infection.

- ***Silencing MLO genes*** can positively affect the plant ability to combat powdery mildew.

- Identification of a natural MLO gene mutant in Barley still **confers resistance after 40 years** (durable resistance).



Underwood and Somerville, 2008

- **Knocking down** clade V-MLO genes (***VitviMLO3,4,13, 17***) confers relative resistance to GPM in grapevine

Transgene-free MLO edited microvines

Project 1

A two-step approach:

Phase 1) *Conventional genetic engineering to generate* ***stable transformants*** *with a visual marker and the editing ingredients in the genome aiming at editing MLO genes followed by*

Phase 2) *Use of Cell-Penetrating Peptides (CPP) to facilitate the entry of* ***RNPs*** *into intact regenerable gene-edited cells* ***to cut*** *the "foreign DNA" and make the edited materials transgene-free.*

Objective 1: Can we excise an inserted genetic cassette and leave few or no scares?

Objective 2: Which silenced MLO mutants (3,4,13, 17) would confer resistance to GPM?

Objective 3: Can we deliver the "molecular scissors (sgRNA-Cas9)" to intact grapevine cells that can be regenerated into an individual plant?

Poster 1: *Satyanarayana Gouthu*

Objective 2: Which KO MLO are resistant to GPM (**Phase 1**)?

Project 1

Where we are now…

Agrobacterium Transformation and Plant regeneration of transformants:

Using microvine embryogenic cells, antibiotic (Hygromycin) and visual (GFP) markers

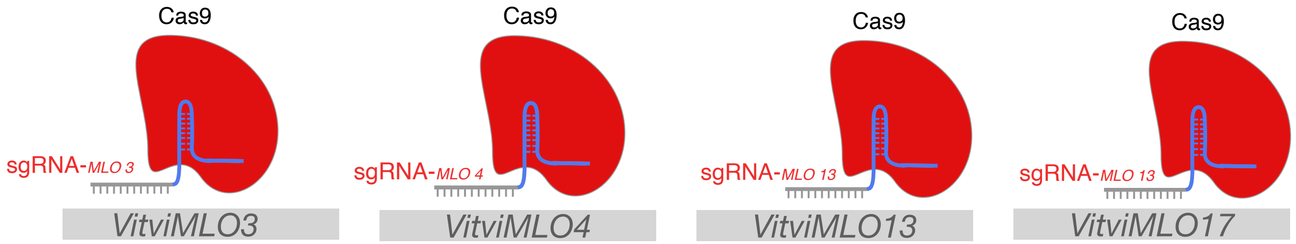
Genotyping:

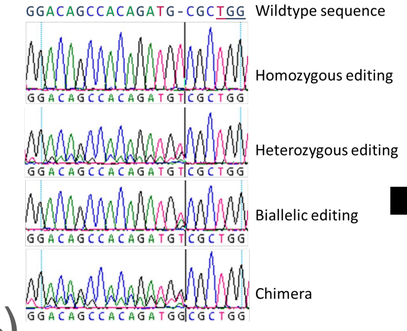
Checking the editing

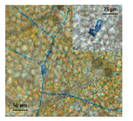
Phenotyping:

Checking the resistance to *Erysiphe necator*

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

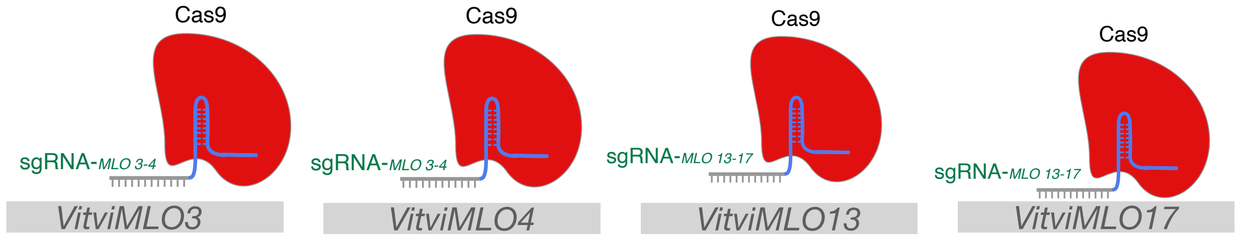


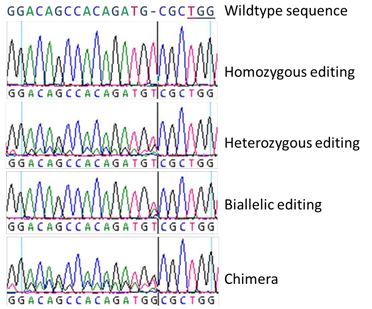




Single mutant evaluation in conferring PM resistance?

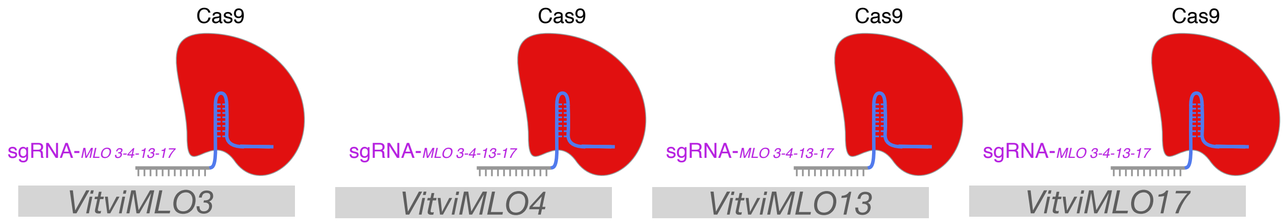
- VitviMLO 3,4, and 13-17 Double knockout mutants (**2 transformations**)

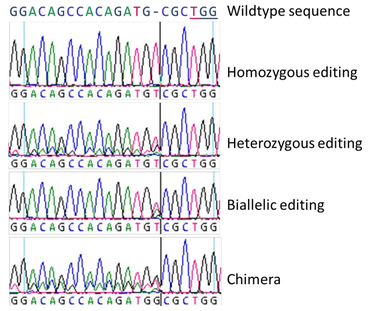




Double mutant evaluation in conferring PM resistance?

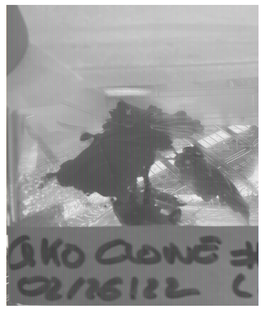
- VitviMLO 3-4-13-17 Quadruple knockout mutant (**1 transformation**)





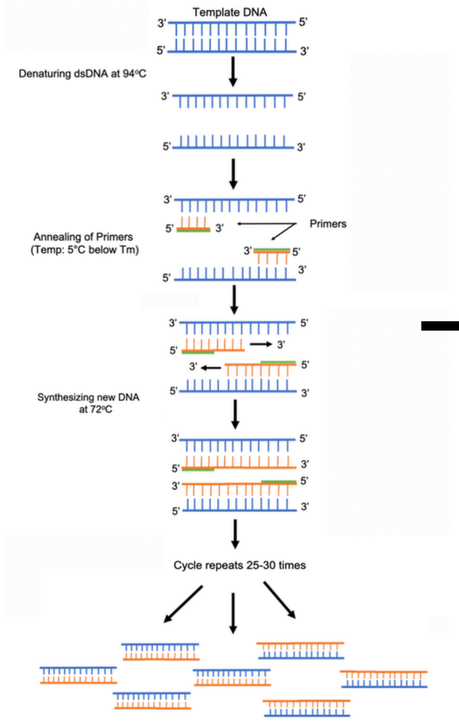
Quadruple mutant evaluation in conferring PM resistance?

Objective 2: Checking the editing by Sequencing?

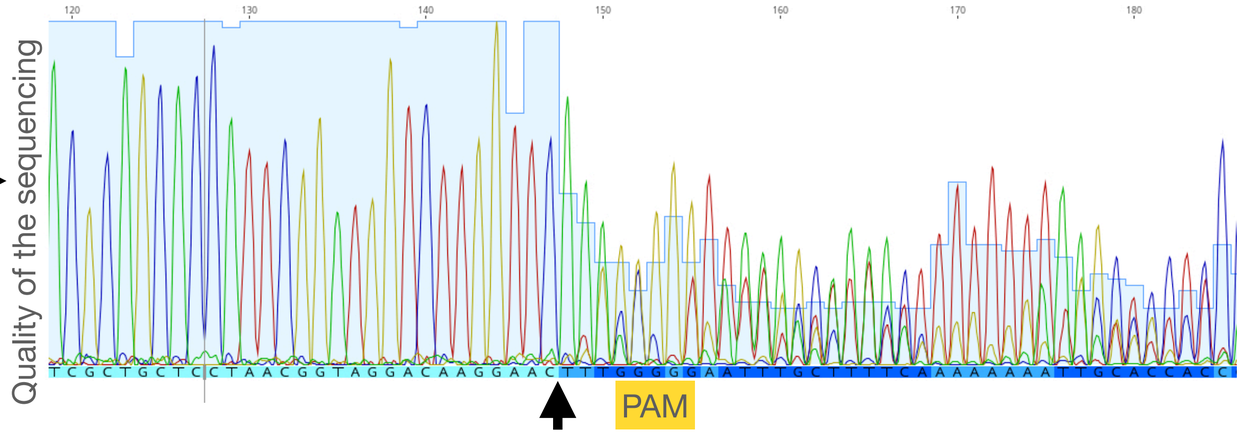


Transgenic microvine

Extraction of Genomic DNA from a single leaf



PCR amplification at the editing site



Sanger Sequencing for a Quadrupole Knockout targeting ***VitviMLO3***

That clone was edited but what is the extent of the editing? Does it affect the two alleles.

Poster 1: *Satyanarayana Gouthu*

Objective 2: Genotyping of the KO mutants.

Gene-Edited MLO mutants	Plant Regeneration stages	Individual Plants in Magenta Box	Expected Sanger sequencing from the mutants
VitviMLO3 SKO	19	4	23
VitviMLO4 SKO	55	2	57
VitviML13 SKO	-	-	-
VitviML17 SKO	16	1	17
DKO3,4	26	10	72
DKO13,17	13	5	36
QKO (3,4,13,17)	60	8	272

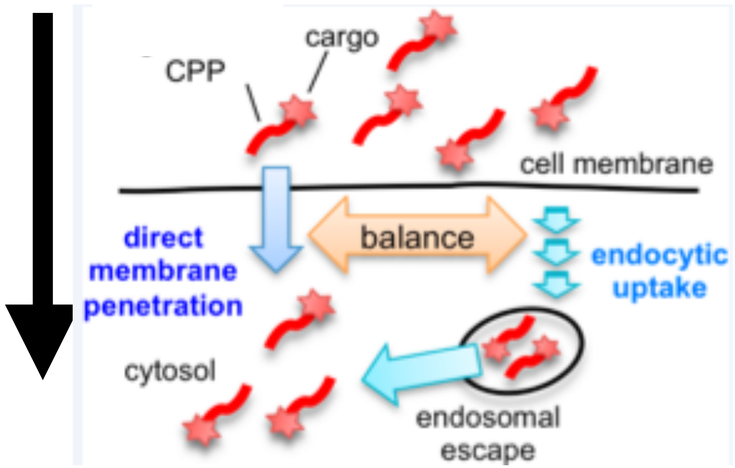
Total = 477

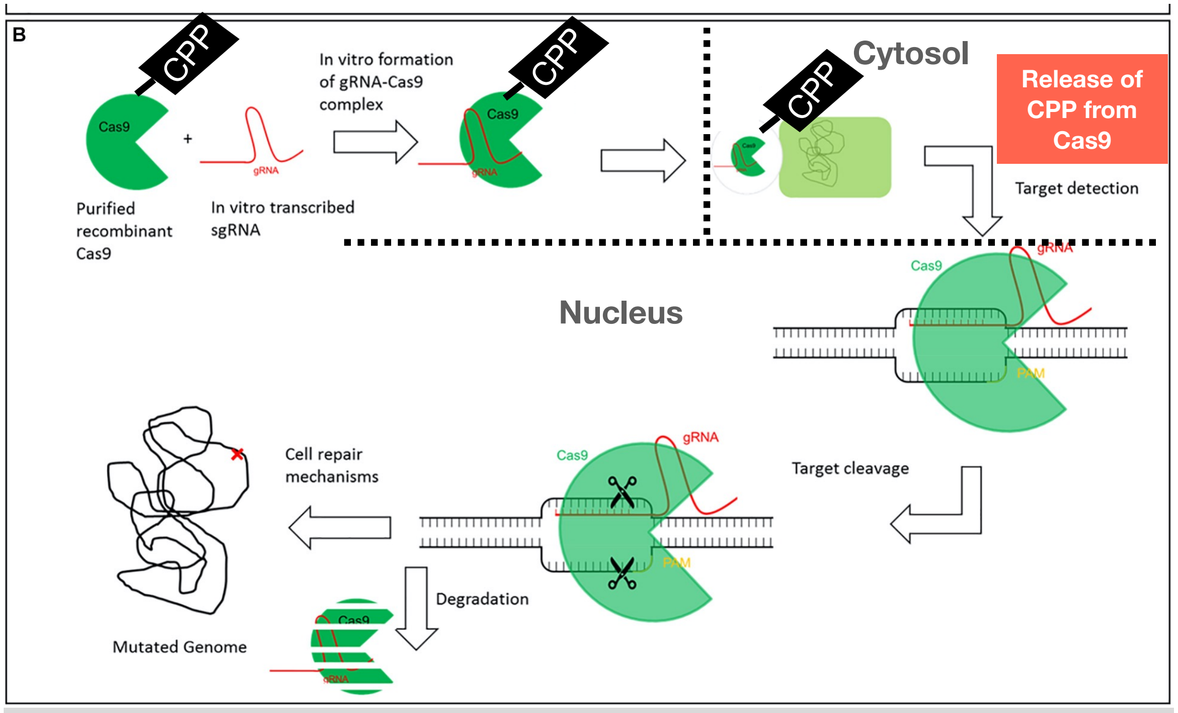
- Not all editing events will lead to a silenced mutation of the two alleles…
- Currently, we had sequenced ~ 10% of the mutants, more than 82% of transformants show editing events with a substantial number of mutants having the two alleles of the target genes affected.

Poster 1: *Satyanarayana Gouthu*

Objective 3: How can we deliver the “molecular scissors” to intact grapevine cells?

A Cell Penetrating Peptide (8 to 15 AA) is a small Peptide that facilitates the entry of cargo molecules like DNA, RNA, and protein into intact cells.





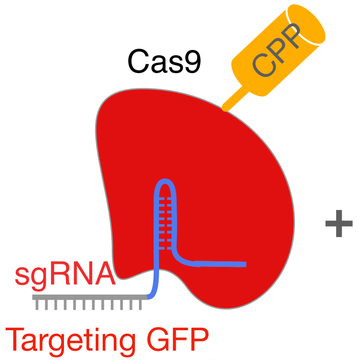
Modified illustration from Janina Metje-Sprink et al., 2019

Protein and Target cells	Reference	Validation
CPP-Cas9 through plasma membrane in animal cells	Ramakrishna et al., 2014	X
Internalization of CPP-protein in intact plant cells	Del-Guidice et al., 2018	X
CPP-Cas9 delivery in intact plant cells	-	O

Conjugated to Cas9 we expect the CPP will go **through the cell wall and plasma membrane** to deliver CRISPR/ Cas9 into the microvine cells

Poster 1: *Satyanarayana Gouthu*

Evaluation of cleavage activity of internalized CPP/RNP to edit GFP expression in transgenic microvine



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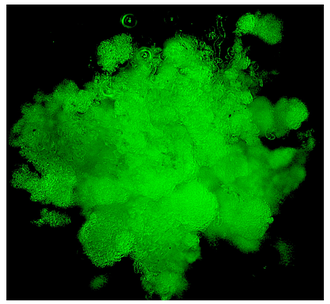
Transgenic GFP microvine cells

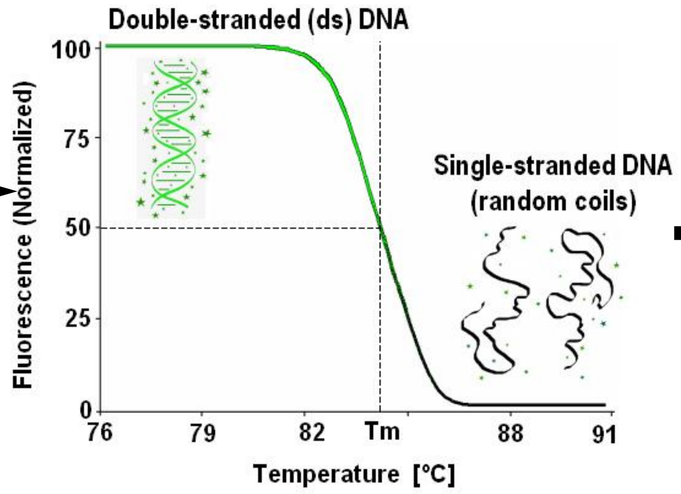


Few hours of incubation in a test tube

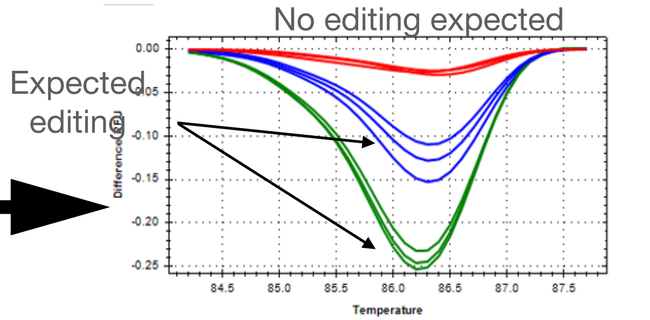
48 hours later

Extraction of Genomic DNA transgenic cells

PCR of edited region on thousand, millions cells



High-Resolution Melting Analysis on the "edited" DNA region of GFP

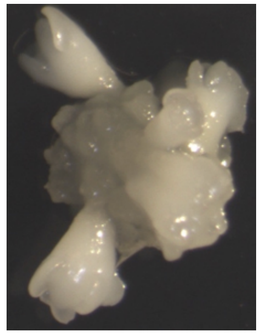


Cas9/sgRNA treated cells without CPP

CPP-Cas9/ sgRNA treated cells

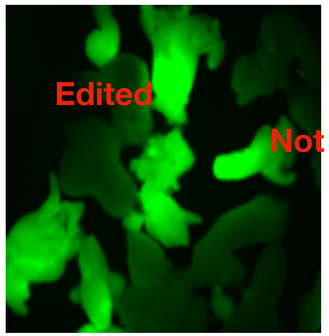
CPP-Cas9/sgRNA embryos that lost GFP fluorescence

Few weeks later…



Embryogenesis

Ten edited embryos (loss of GFP) but out of a hundred embryos that were not edited



Negative Selection

Edited embryos stopped growing….

Plant Regeneration

Project 1 on transgene-free MLO edited grapevine

Milestones

Objective 1: Can we insert a genetic cassette that can be excised later?

YES

Objective 2: Which silencing MLO mutants (3,4,13, 17) confer resistance to GPM?

We don’t know yet but we have plenty edited mutants

Objective 3: Can we deliver the “molecular scissors (sgRNA-Cas9)” to intact grapevine cells that can be regenerated into an individual?

YES and the CPP is a good helper but it might be toxic to cells

Project 2: How to improve DNA-free precise gene editing in grapevine (HDR)?

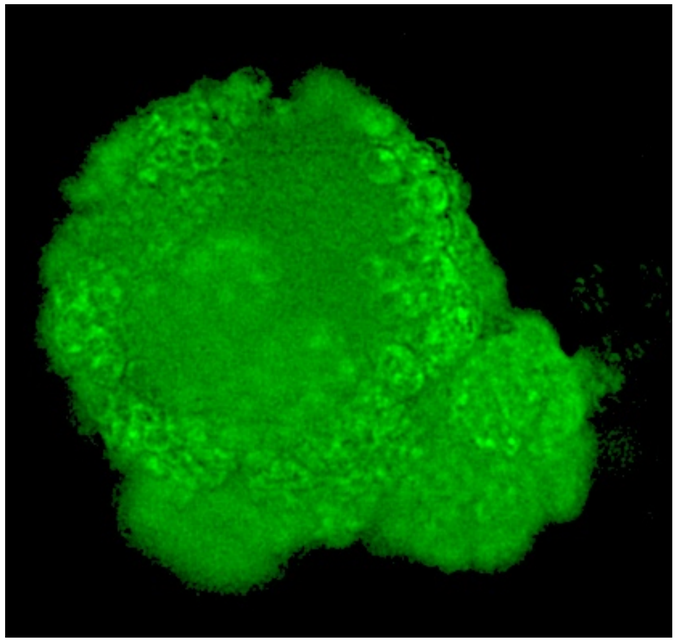
- 1) Favor the HDR by having the Donor Template in close proximity with the RiboNucleoProtein (RNP).

- 2) Optimizing the CPP-delivery of the Ribonucleoprotein with a donor template to grapevine regenerable cells.

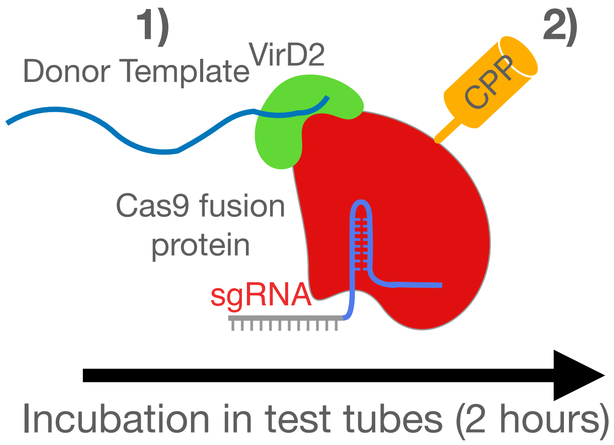
Proof of concept: *Perform precise editing to convert* ***Green Fluorescent Protein****-expressing microvine lines to* ***Blue Fluorescent Protein****-expressing lines.*

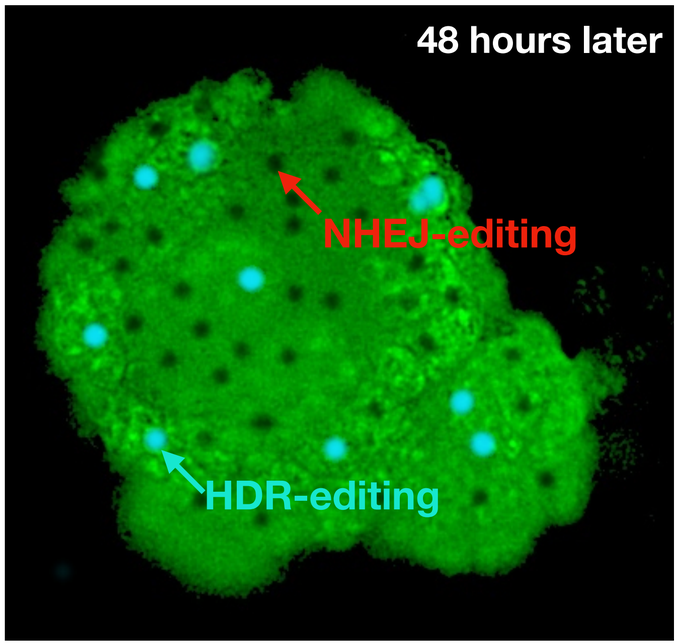
Poster 2: Satyanarayana Gouthu

Project 2: What do we expect?



Embryogenic microvine cells expressing eGreen Fluorescent Protein.





Embryogenic microvine cells expressing eGreen Fluorescent Protein with edited cells expressing the Blue Fluorescent Protein

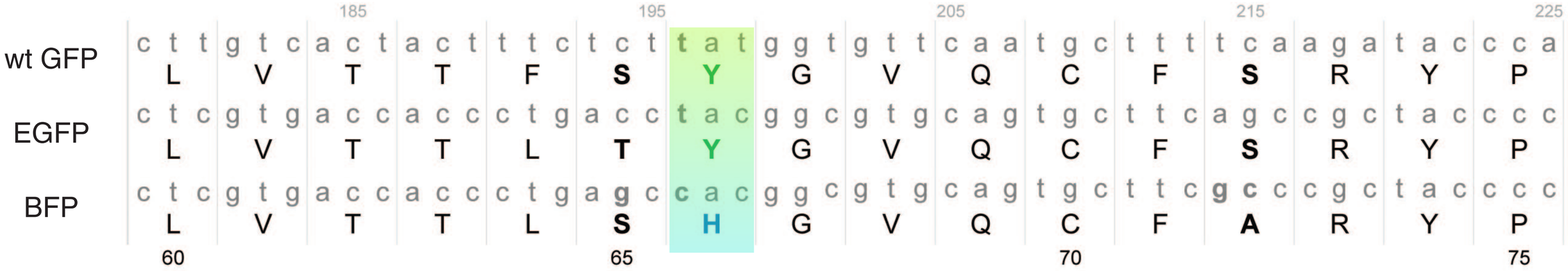
1) We hypothesize that **the close proximity** of the donor template will increase the Homology Directed Repair (precise editing) at the expense of NHEJ.

2) The **complexation and/or conjugation** of the CPPs would facilitate the delivery into the cells (overall editing rate)

Poster 2: Satyanarayana Gouthu

Project 2: How to improve DNA-free precise gene editing in grapevine ?

One single nucleotide change is enough to convert eGFP into BFP.



Region of the Enhanced GFP that can be precisely edited to convert the GFP protein into a BFP (Glaser et al., 2016)

Poster 2: Satyanarayana Gouthu

Conclusion and perspectives

Project 1: How to produce transgene-free gene-edited (knock out) grapevine material?

Milestone:

- The transgenic cassette can be excised leaving few scares, the CPP help the editing into plant regenerable material.

Remaining work:

-Characterizing the MLO edited plants that show degrees of resistance to GPM (inoculation assays).

-Render these transgenic resistant plants transgene-free by excising the transgenic

Project 2: How to improve Precise Gene Editing via delivery of the RNP.

Milestone:

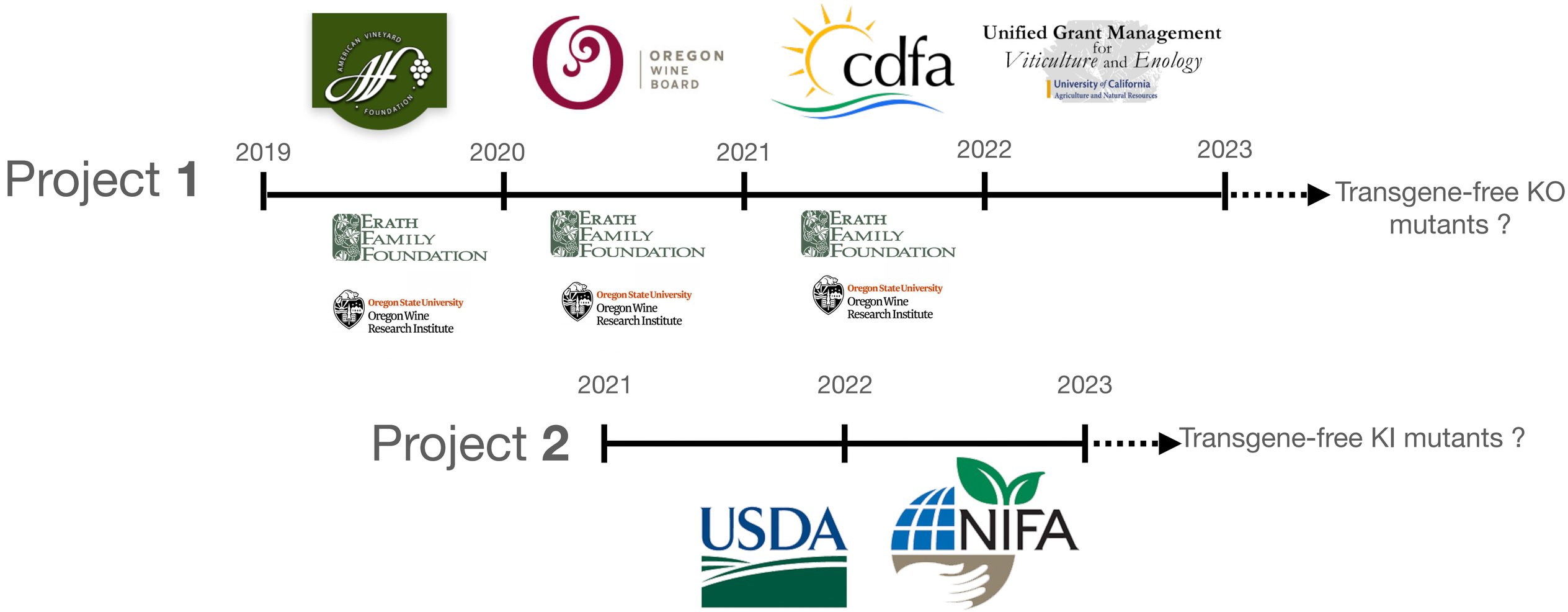
- The activity of fusion protein with the SSODN is currently being tested

Remaining work:

-Evaluating the CPP delivery of the SSODN::Cas9/VirD2 to the GFP cells.

Acknowledgement

Thanks to the various funding sources



Main Collaborator:

Walt Mahaffee -USDA ARS

Claudia Maier - Director of the OSU Mass Spectrometry Facilities

Thank you