Protoplast-Mediated Gene Editing for Disease Resistance

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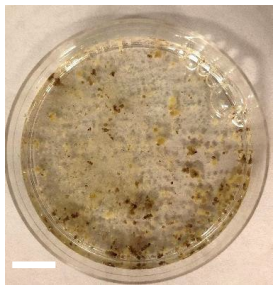
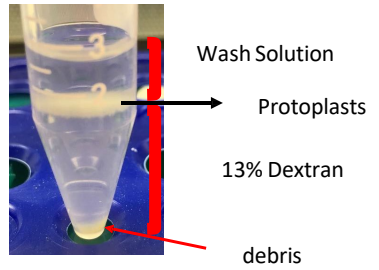
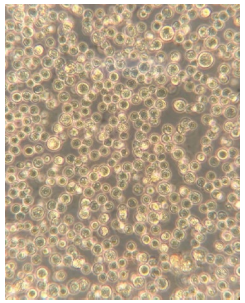
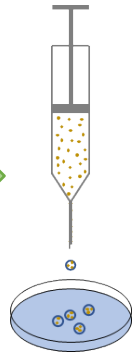
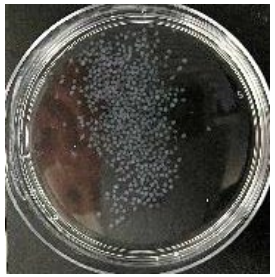




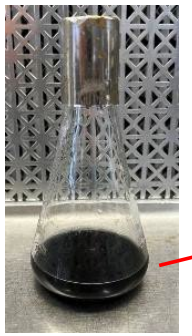
Immature anthers Embryogenic culture Enzymatic digestion Protoplast purification

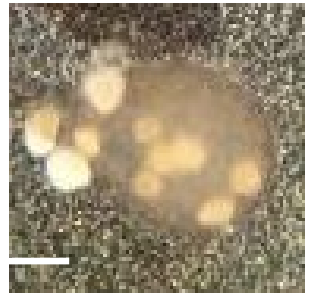




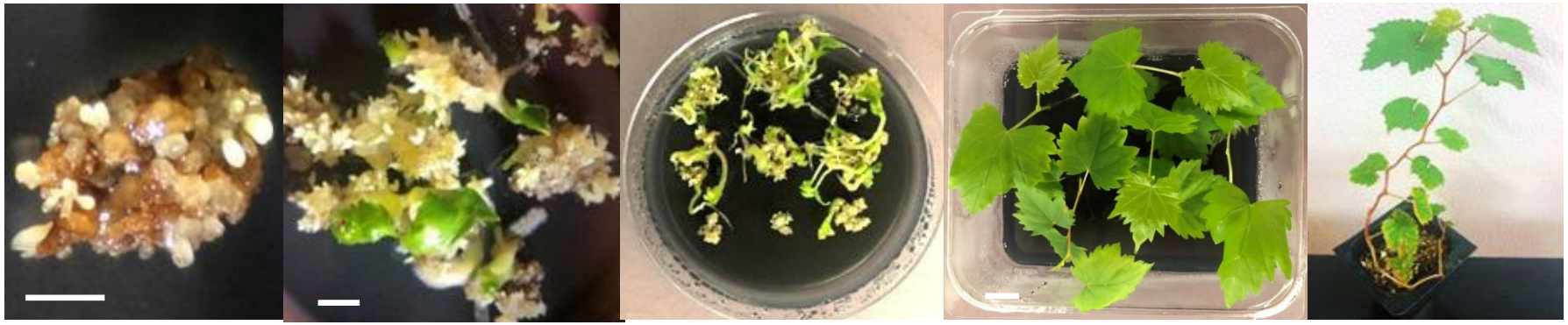


Encapsulate protoplasts in calcium alginate beads.





Protoplasts cultured with grape feeder cells



Plant regeneration from protoplast-derived callus (Thompson Seedless)

White wine grapes

Red wine grapes

Dwarf rapid flowering

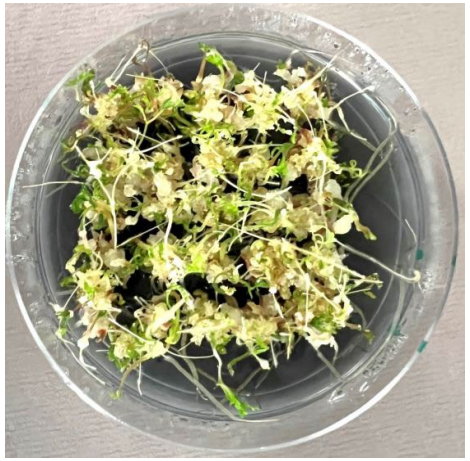
Colombard

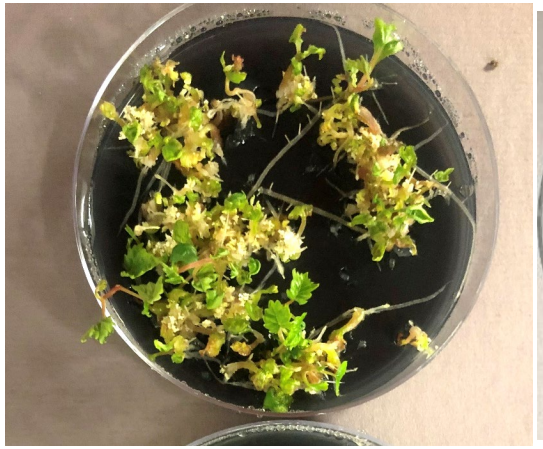
Chardonnay

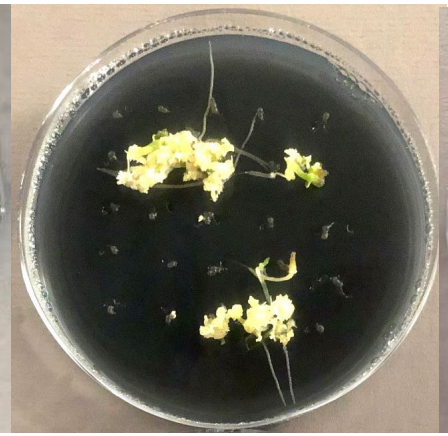
Merlot

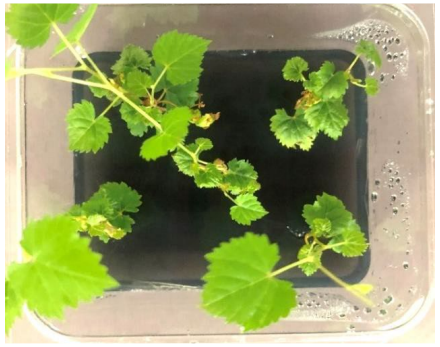
Cabernet Sauvignon

Pixie

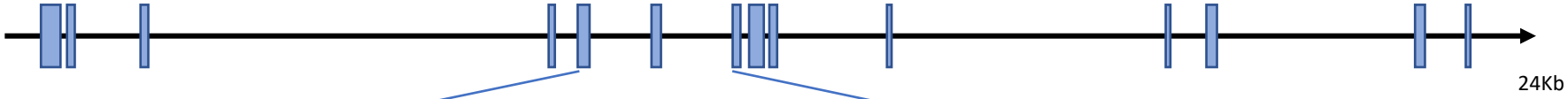








Editing the phytoene desaturase (PDS) gene using CRISPR plasmid



Site 1

PAM

```
Wt          CCAGGG-GAATTCAGCCGATTTGA

#21157-1    CCAGGG-GAATTCAGCCGATTTGA
#21157-2    CCAGGG-GAATTCAGCCGATTTGA

#21014-1    CCAGGG---ATTCAGCCGATTTGA
#21014-2    CCAGGGTGAATTCAGCCGATTTGA
```

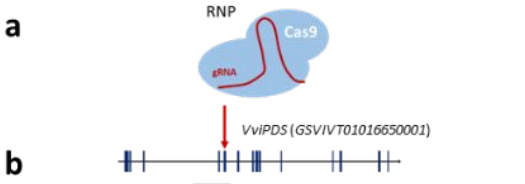
Site 2

PAM

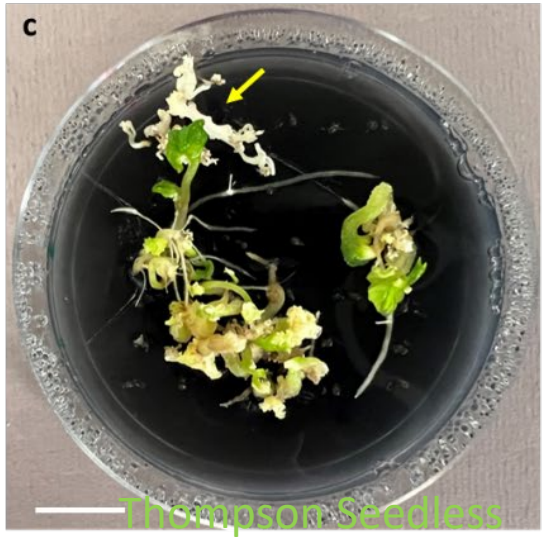
```
Wt          CCTGAT-CGAGTAACAGATGAGGT

#21157-1    CCTGATGCGAGTAACAGATGAGGT
#21157-2    CCTGATVCGAGTAACAGATGAGGT

#21014-1    CCTGAT-CGAGTAACAGATGAGGT
#21014-2    CCTGATTCGAGTAACAGATGAGGT
```

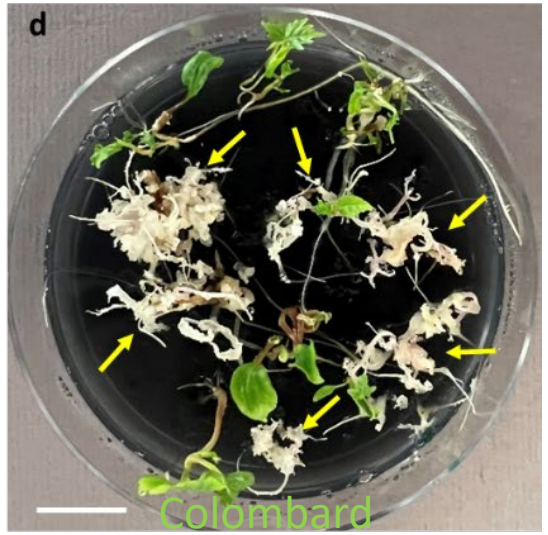
Scheme showing the genomic structure of PDS gene (GSVIVT01016650001). Exons are represented as blue rectangles. Target sites in exon 5 and exon 7, with the sequence of the wild type and edited alleles are shown below. The 200 nt insertion is represented as V in the target sequence of #22157-2 line.

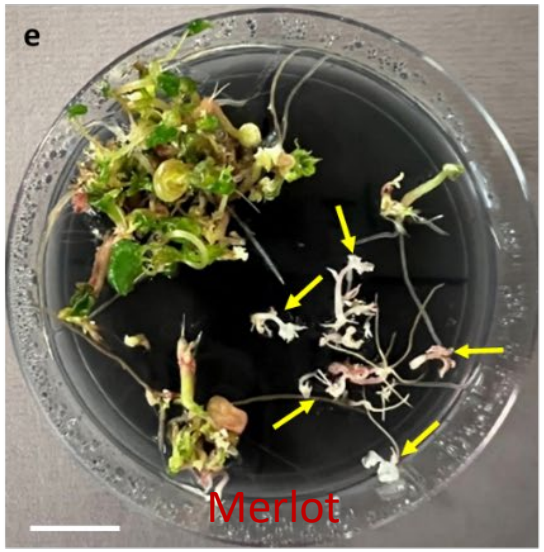
Regeneration of PHYTOENE DESATURASE (*PDS)* -edited grape plants from ribonucleoprotein (RNP) transfected protoplasts

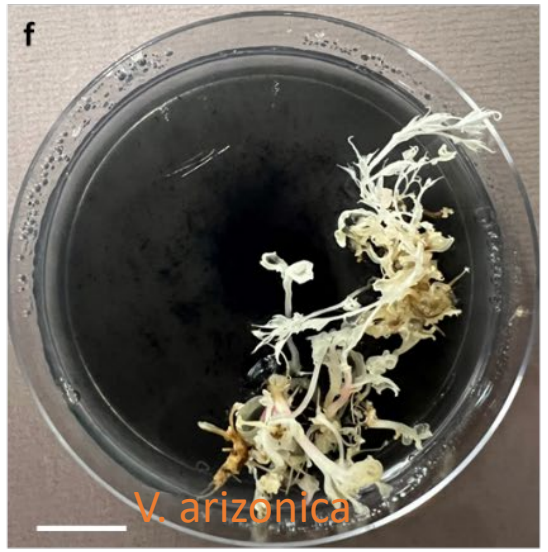


Wt	AGCCAGGG-GAATTCAGCCGATTTGA
Thompson Seedless	
#1	AGCCAGGG---ATTCAGCCGATTTGA
	AGCCAGGG-----TCAGCCGATTTGA
	AGCCAGGGTGAATTCAGCCGATTTGA
Colombard	
#8,32,34	AGCCAGGGTGAATTCAGCCGATTTGA
	AGCCAGGGGGAATTCAGCCGATTTGA
#14-1,14-2	AGCCAGGGAGAATTCAGCCGATTTGA
	AGCCAGGG--AATTCAGCCGATTTGA
#12,24,25	AGCCAGGG---ATTCAGCCGATTTGA
	AGCCAGGG--AATTCAGCCGATTTGA
Merlot	
#1	AGCCAGGG---ATTCAGCCGATTTGA
	----------AGCCGATTTGA
#2-1,2-2	AGCCAGGGTGAATTCAGCCGATTTGA
2-3,2-4	del 130bp
#7-1,7-2	AGCCAGGG------CAGCCGATTTGA
V. arizonica	
#1	AGCCAGGGTGAATTCAGCCGATTTGA
	del 60bp
#2	AGCCAGGG---ATTCAGCCGATTTGA
	del 70bp
#3	AGCCAGGG--AATTCAGCCGATTTGA
	del 50bp

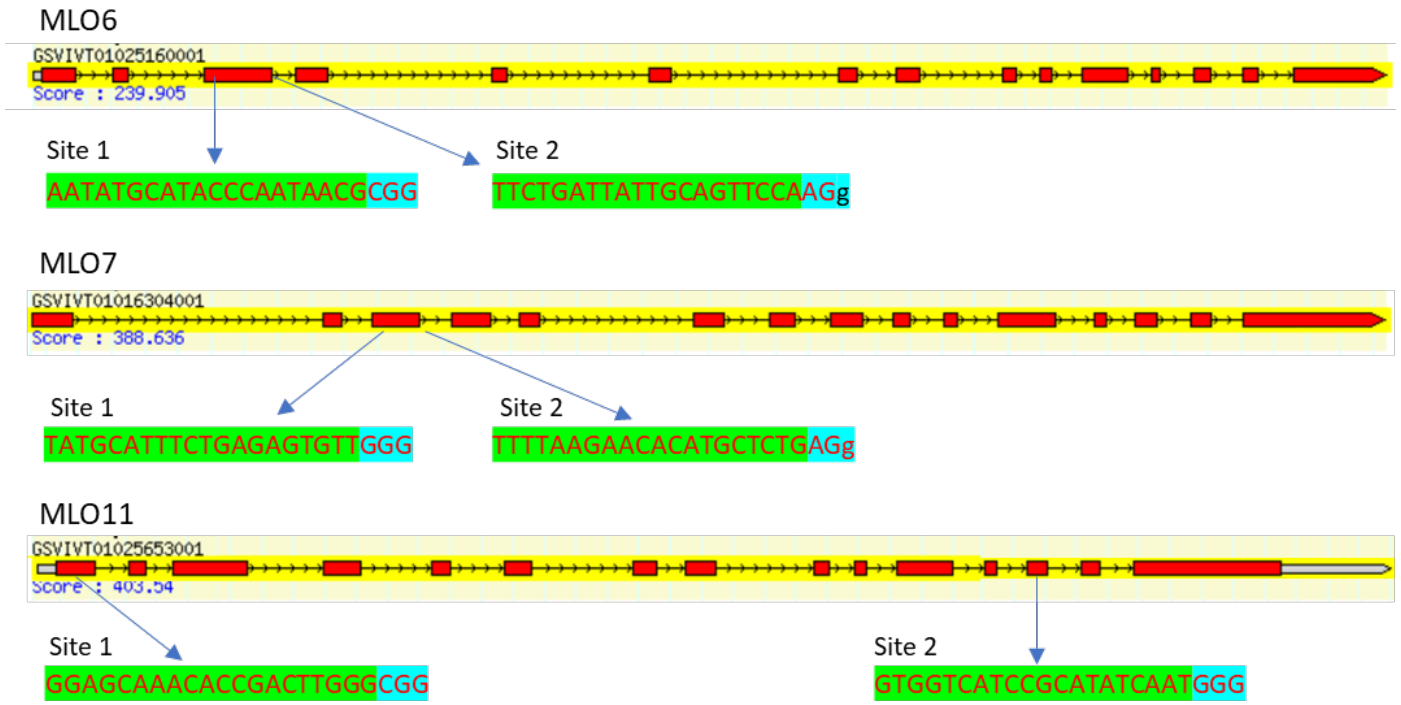








Targeting MLO 6, 7, and 11 with CRISPR-plasmid



Targeted *Mildew resistance locus o (*MLO) genes. Genomic structure of MLO6, MLO7 and MLO11 genes, showing the exons in red, introns as lines and the position of the CRISPR target sites. The sequence of the targeted sites is indicated below.

Thompson Seedless-Edits in MLO using CRISPR plasmid

Genotype of 26 of 170 regenerated Thompson seedless plants (Lines) with edits in MLO genes. The genotype of each target site in MLO6, MLO7 and MLO11 genes is indicated for each plant. Plants with the same genotype were grouped together, as they could potentially be clones. Large del means large deletion removing both targeted sequences. Wild type sequences are indicated in green, heterozygous (het) mutations are indicated in yellow and homozygous (Hom) or biallelic mutations in red.

Genotypes	Line	MLO6		MLO7		MLO11	
		site 1	site 2	site 1	site 2	site 1	site 2
1	40-1	TA>AT het	del2 het	insT hom	insT het	insT hom	insA/del3
	39-2	TA>AT het	del2 het	insT hom	insT het	insT hom	insA/del3
	39-3	TA>AT het	del2 het	insT hom	insT het	insT hom	insA/del3
	39-4	TA>AT het	del2 het	insT hom	insT het	insT hom	insA/del3
2	93-2	del1 het	del6 Het	insT hom	insT/del3	insT hom	insAA
3	39-1	del1 het	wt	insT het	wt	del1 hom	insA hom
4	41-1	del1 het	wt	insT het	wt	insT het	insA/insAA
5	32-1	del1 hom	insT het	insT hom	insT het	insT/del2	insT/insA
	117	del1 hom	insT het	insT hom	insT het	insT/del2	insT/insA
6	112	del1 hom	wt	insT hom	insT/del1	insT/del1	insA/del3
7	3--1	del1 hom	insT hom	insT/large del	large del	insT hom	no read
	103	del1 hom	insT hom	insT/large del	large del	insT hom	insA/del3
8	107	wt	insT het	insT het	del1 het	del1 het	no read
9	48-1	large del	large del	insT/large del	del1/large del	insT het	insT/insA
10	24-1	insA/large del	insT/large del	insT hom	del2/del3	insT hom	insT hom
11	44-1	wt	wt	insT het	wt	wt	insA hom
	45-1	wt	wt	insT het	wt	wt	insA hom
	119	wt	wt	insT het	wt	wt	insA hom
12	23-1	wt	wt	insT hom	wt	del9/del1/insA	insA(25%)
	23-2	wt	wt	insT hom	wt	del9/del1/insA	insA(25%)
13	87-2	wt	wt	wt	wt	insT/TT	insA het
	92-1	wt	wt	wt	wt	insT/TT	Ir (insA het)
14	106	wt	wt	wt	wt	insT het	wt
15	118	wt	wt	wt	wt	wt	insA het
16	29-1	wt	wt	insT het	wt	wt	wt
	29-2	wt	wt	insT het	wt	wt	wt

Malbec
Edits in MLO using CRISPR plasmid

Genotypes	Line	MLO6		MLO7		MLO11	
		site 1	site 2	site 1	site 2	site 1	site 2
1	24	wt	wt	insT hom	wt	del1/insT	insA het
2	39	wt	wt	wt	wt	insT hom	wt
3	50	wt	wt	wt	wt	insT hom	del3 het

Genotype of 3/22 regenerated Malbec plants (Lines) with edits in MLO genes. The genotype of each target site in MLO6, MLO7 and MLO11 genes is indicated for each plant. Wild type sequences are indicated in green, heterozygous (het) mutations are indicated in yellow and homozygous (Hom) or biallelic mutations in red.

Thompson Seedless- Edits in MLO using RNPs

Genotypes	Line	MLO6		MLO7		MLO11	
		site 1	site 2	site 1	site 2	site 1	site 2
1	2	wt	insT het	wt	insT het	wt	insA hom
2	13	wt	wt	wt	del2 hom	wt	insA hom
	15	wt	wt	wt	del2 hom	wt	insA hom
3	10	wt	wt	wt	wt	wt	insA het
	11	wt	wt	wt	wt	wt	insA het
	12	wt	wt	wt	wt	wt	insA het
4	9	wt	wt	wt	wt	wt	del3/insA
	7	wt	wt	wt	wt	wt	del3/insA

Genotype of 8 of 22 regenerated Thompson seedless plants (Lines) with edits in MLO genes after transfection with RNPs. The genotype of each target site in MLO6, MLO7 and MLO11 genes is indicated for each plant. Note that RNPs only target site 2 in each MLO gene. Wild type sequences are indicated in green, heterozygous (het) mutations are indicated in yellow and homozygous (Hom) or biallelic mutations in red.

Thompson Seedless grapevines containing edits in MLO genes

- Identify areas where significant progress could be made towards developing effective control strategies.
  - Protoplast-mediate genome editing knockout technology can be used to generate grape plants with a diverse combination of edited which can be used as a tool to allow for the identification and validation of putative susceptibility and resistant genes.

- What knowledge gaps barriers need to be overcome?
  - Identifying susceptibility genes that are required for red blotch and leafroll infection.
  - Identifying putative resistance genes for red blotch and leafroll in Vitis species.
  - Develop protoplast-mediated genome editing for those species to allow us to knockout putative resistance genes and then test the regenerated plants for susceptibility.
  - Developing a protoplast-mediated gene knock-in technology for grapevines.
    - A gene knock in platform will allow us to test the efficacy of putative resistance genes by incorporating whole gene cassettes into susceptible lines and screening for resistance.
    - Need to identify genomic safe harbor (GSH) regions for knock-in gene cassettes.

Acknowledgements

- Juan Debernardi- UC Davis
- CDFA Pierce’s Disease and Glassy-winged Sharpshooter Board