NYWGF RESEARCH

Funding for fiscal year: 2025-2026

SECTION 1:

Project title: Reduced susceptibility to powdery mildew by precision gene editing

Principal Investigator with contact info: Adam Bogdanove, ajb7@cornell.edu, 607 255-7831

Co-PI Collaborators with contact info:

Lance Cadle-Davidson, Lance.CadleDavidson@usda.gov, (315) 787-2442

Hilal Betul Kaya, Hilalbetul.kaya@cbu.edu.tr, +90 236 2012461

Collaborators:

Michelle Heck
Research Molecular Biologist
USDA-ARS Emerging Pests and Pathogens Research Unit
and Adjunct Professor, Plant Pathology and Plant-Microbe Biology Section
School of Integrative Plant Science
Cornell University
michelle.cilia@usda.gov
(607) 254-5453

Madeline Oravec
Assistant Professor
Horticulture Section, Cornell AgriTech
School of Integrative Plant Science
Cornell University
mwo23@cornell.edu
(518) 221-9869

Gan-Yuan Zhong
Research Leader
USDA-ARS Grape Genetics Research Unit
Plant Genetic Resources Unit
Cornell AgriTech
ganyuan.zhong@usda.gov
(315) 787-2482

New Research ☒ **Continued Research** ☐

Amount Funded **$** 35,000

SECTION 2:

Project Summary Impact Statement:
Powdery mildew is a major constraint in grape production, requiring frequent fungicide applications that are becoming less effective due to pathogen resistance. This project aimed to develop a more durable disease management strategy by targeting grapevine susceptibility genes (*VvPLL3* and *VvPLL13*) using precision gene editing approaches that do not introduce foreign DNA. Disrupting these genes has the potential to reduce disease susceptibility while maintaining normal vine growth, offering a sustainable alternative to both fungicides and traditional resistance genes. If successful, this approach could provide long-lasting resistance and enable the development of improved grape cultivars without altering desirable varietal traits. More broadly, this work establishes a foundation for applying susceptibility gene editing strategies across grapevine varieties.

Objectives:

1. Obtain Chardonnay vines with knockouts of one, the other, or both of two powdery mildew susceptibility genes, *VvPLL3* and *VvPLL13*.
    a. Continue meristem transformation and screening, increasing the number of inoculations.
    b. Pursue rootstock editing, i.e., transgenic rootstock-mediated delivery of mobile RNAs, for transgene-free editing in the scion.
    c. Adapt Symbiont Technology, i.e. transgenic galls, to deliver mobile RNAs and achieve transgene-free editing in vines.
2. Pending success of Objective 1 during the project period, determine grapevine powdery mildew susceptibility of the edited vines relative to one another and to unedited Chardonnay.
3. Also pending success of Objective 1, test growth and development of the vines in a greenhouse setting.

An overview of the methods and workflow, is provided in Figure 1. As Objective 1 did not yield the desired edits withing the project period, Objectives 2 and 3, illustrated in Figure 2, could not be not pursued.

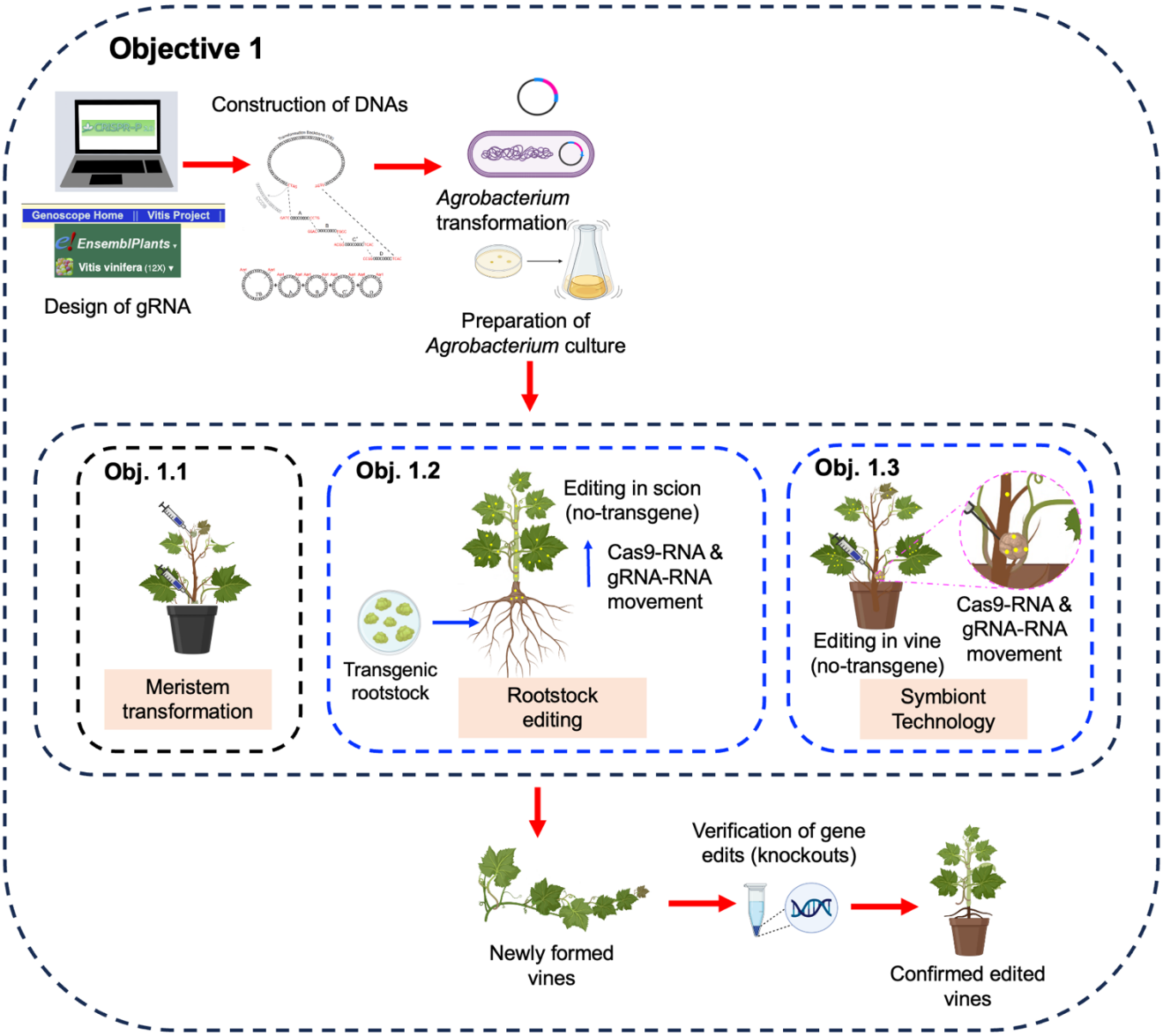


Figure 1. **Schematic of the project workflow, including the proposed additional delivery methods**. Objective 1 involved the design of gRNAs for the target genes using online tools, construction of the DNAs required for CRISPR editing reagents, transformation of Agrobacterium, delivery into plants, and verification of edited plants. Meristem transformation (Obj. 1.1) continued with an increased number of inoculations. For the additional delivery methods (Obj. 1.2 and 1.3, outlined in dotted blue), new DNAs were assembled to generate RNAs with sequences added for movement from rootstock to scion (rootstock editing) and from symbionts to vines (Symbiont Technology). Whether gene editing took place was assayed by PCR and sequencing.

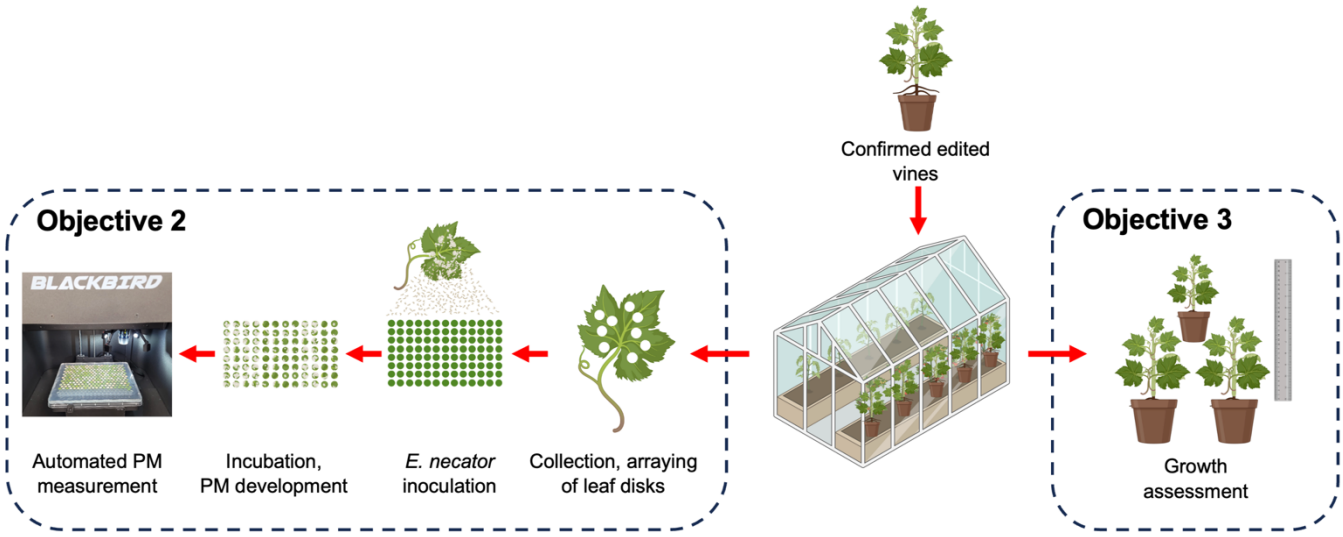


Figure 2. Workflow for assessing powdery mildew susceptibility and growth of the vines in the greenhouse. Objective 2 was to evaluate the PM susceptibility of edited vines compared to unedited ones. It was to use leaf disk inoculation and an automated image analysis system called Blackbird to quantify fungal infection. Objective 3 was to examine the growth and development of the edited vines, measuring key parameters such as internode length, leaf size, and overall vine height at different stages post-transplantation.

Activities & Methods:

Objective 1: Obtain Chardonnay vines with knockouts of one, the other, or both of two powdery mildew susceptibility genes, *VvPLL3* and *VvPLL13*.

a. <u>Continue meristem transformation and screening, increasing the number of inoculations.</u>

Design and assembly of DNA plasmid for editing
gRNAs targeting the VvPLL3 and VvPLL13 and genes were designed based on the *Vitis vinifera* genome sequence PN40024.v4, available in EnsemblPlants (http://plants.ensembl.org), using the CRISPR-P software tool (Lei et al., 2014). The gRNAs were selected for their specificity to minimize potential off-target effects across the genome. Constructs were assembled using the Golden Gate method (Cermak et al., 2017) into the 'transfer DNA' (tDNA) region of a plasmid designed for *Agrobacterium*-mediated plant transformation. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 via the freeze-thaw method, and bacterial cultures were prepared following the protocol described by Maher et al. (2020). To readily track editing efficiency during optimization of the editing approach, we also designed a gRNA for the phytoene desaturase gene *PDS1*. Phytoene desaturase is a key enzyme in the carotenoid biosynthesis pathway (Fraser et al., 1994). Since carotenoids are essential for pigmentation in plants, disruption of *PDS1* causes an albino or variegated phenotype, allowing visual detection of edits instead of a more time-consuming molecular test.

Plant material, meristem transformation, and regeneration
Chardonnay cuttings were initially grown at the USDA-ARS facility in Geneva, NY, and

subsequently transferred to the greenhouse at Cornell University in Ithaca, NY. Side shoots (meristematic tissue) were removed from the cuttings, and the resulting wound sites were inoculated with *Agrobacterium tumefaciens* strain GV3101 using syringes fitted with 31 G needles. The inoculated plants were maintained in a greenhouse under a 16-hour photoperiod, with day/night temperatures set at 25°C/17°C. Cuttings were regularly monitored for shoot re-formation at the injection sites, and newly developed tissues at these sites were collected for further analysis.

Assay to detect knockout edits and absence of tDNA, and propagation of edited vines
Genomic DNA was isolated from the leaves of newly formed shoots following the protocol of Lodhi et al. (1994). The *VvPDS1* and *VvPLL* gene target sites were amplified by PCR using a High-Fidelity DNA polymerase. PCR products were initially assayed by restriction enzyme digestion (Shan et al., 2014), and a separate PCR reaction was conducted to check for the presence of the tDNA used to deliver the gRNAs and Cas9.

b. <u>Pursue rootstock editing, i.e., transgenic rootstock-mediated delivery of mobile RNAs, for transgene-free editing in the scion.</u>

Design and assembly of DNA constructs for rootstock editing
gRNAs targeting the *VvPLL3, VvPLL13*, and *VvPDS1* genes were designed based on the Vitis vinifera genome sequence PN40024.v4, available in EnsemblPlants, using the CRISPR-P software tool (Lei et al., 2014). For each target gene, two gRNAs were selected from different exons to promote complete gene knockout. To facilitate movement from rootstock to scion, tRNA-like sequences were added to both the gRNAs and the 3′ end of Cas9. The resulting binary plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105 via the freeze-thaw method, and bacterial cultures were prepared following the protocol described by Nasti et al. (Nasti et al., 2021). A schematic illustration of the rootstock editing constructs was shown in Figure 3.

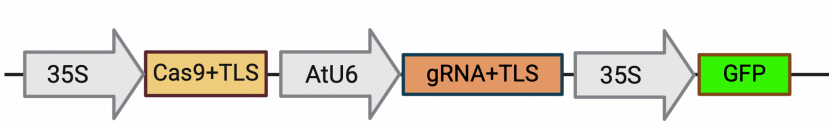


Figure 3. The construct includes a 35S promoter driving expression of Cas9 fused with a tRNA-like sequence (TLS), followed by an AtU6 promoter controlling gRNA-TLS expression. A second 35S promoter drives GFP as a reporter for transformation efficiency.

Callus transformation and regeneration
Roostock editing constructs were used to transform ‘Chardonnay’ calli prepared by Dr. Gan-Yuan Zhong’s lab (USDA-ARS, Cornell AgriTech). Embryogenic calli were used for transformation following the protocol described by Yang et al., (Yang et al., 2024). After approximately three months of incubation on selection media, somatic embryos were obtained. Transformation was confirmed via fluorescence microscopy, as the constructs included GFP. Calli were transferred from embryo induction medium to embryo maturation medium, followed by shoot induction. Plates were transferred every three weeks onto fresh media. Regenerated shoots were obtained and subsequently transferred to rooting medium.

Assay to detect knockout edits
Genomic DNA was isolated from callus tissue during culture. Target regions were amplified by PCR using gene-specific primers. Amplicons were sent to paired-end Illumina sequencing (2x150 bp) at the Cornell University Biotechnology Resource Center (BRC) Sequencing Facility. Sequencing reads were analyzed using CRISPResso2 (Clement et al., 2019). Editing efficiencies were calculated within a quantification window spanning 10 bp upstream and 10 bp downstream of the predicted Cas9 cleavage site. To account for background noise, editing frequencies were corrected by subtracting values obtained from non-transformed control samples. Two gRNAs were evaluated for each target gene (*VvPDS1, VvPLL3*, and *VvPLL13*).

c. <u>Adapt Symbiont Technology, i.e. transgenic galls, to deliver mobile RNAs and achieve transgene-free editing in vines.</u>

Preparation of constructs for Symbiont Technology
Genome editing reagents including Cas9+TLS and gRNAs+TLS used in rootstock editing were adapted into pSym vectors developed by Heck et al. (2026). The pSym plasmid encodes the PGR cassette genes ipt, IaaM, IaaH and gene 5 under their native promoters from *Agrobacterium tumefaciens* strain C58 together with a gene of interest under the control of an enhanced double 35S promoter. Kanamycin resistance genes are encoded both within the T-DNA (plant) and the vector backbone (bacteria) (Heck et al., 2026). The pSYM vectors, pSTC177 (targeting VvPDS1), pSTC205 (targeting *VvPLL3*), and pSTC206 (targeting *VvPLL13*) were used for symbiont inoculations.

Symbiont Inoculations in grapevine
Grapevine (Vitis vinifera) cultivars ‘Thompson Seedless’ and ‘Chardonnay’ were used for Symbiont inoculations. *Agrobacterium tumefaciens* cultures harboring the respective constructs were grown overnight in LB medium supplemented with appropriate antibiotics at 28 °C. The following day, cultures were diluted to an OD_{600} of 0.1 in 10 mL LB medium containing antibiotics and 100 µM acetosyringone, and incubated for 3 h at 28 °C. Cells were then pelleted and washed at room temperature with induction buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.4–5.6, 400 µM acetosyringone). The bacterial suspension was adjusted to a final OD_{600} of 1.0 and incubated at room temperature for 2 h prior to inoculation.
Wounding was performed using a biopsy punch, and *Agrobacterium* suspensions were inoculated at volumes of 4 µL or 8 µL per site. Following inoculation, inoculation sites wrapped with parafilm. After symbionts were formed on the grapevines, the samples were taken from symbionts and upper and lower sites of the symbionts to confirm movement. Total RNA was extracted using Qiagen RNA extraction kit and cDNA was synthesized from 1 µg of total RNA using the QuantaBio script cDNA supermix and then used for qPCR with iTaq Universal SYBR Green Supermix (Biorad) following the manufacturer’s protocols.

Objective 2: Determine grapevine powdery mildew susceptibility of the edited vines relative to one another and to unedited Chardonnay.

Objective 3: Assess growth and development of the vines in a greenhouse setting

Since no targeted gene edits were obtained using meristem transformation, rootstock editing

and Symbiont Technology, we were unable to proceed with the planned experiments to determine the powdery mildew (PM) susceptibility of edited vines relative to unedited Chardonnay. Similarly, we were unable to assess the growth and development of edited vines in the greenhouse.

Results/Outcomes/Next Steps:

This section reports in full detail the results obtained under this project. For a concise summary of results and future directions for this project, please see SECTION 3.

Maher et al. (2020) demonstrated that developmental regulators can be applied directly in planta to stimulate de novo meristem formation on soil-grown plants. This approach was selected for its speed, reduced labor requirements, and its avoidance of the random mutations often introduced by tissue culture-based transformation methods. Meristem transformation involves removing newly formed shoots, followed by the injection of *Agrobacterium tumefaciens* into the exposed meristematic tissue. The *Agrobacterium* delivers a DNA construct encoding CRISPR components, which are transiently expressed to produce Cas9 and a guide RNA (gRNA). The Cas9 protein is directed by the gRNA to induce targeted double-strand breaks in the target gene. The editing occurs during the cell's natural repair process and does not involve permanent integration of foreign DNA, as the CRISPR construct degrades over time.

For meristem transformation, the necessary DNA constructs for the *VvPDS1* and *VvPLL* genes were generated, introduced into *Agrobacterium tumefaciens*, and confirmed by whole plasmid sequencing. *VvPDS1* was selected as the initial target so that editing efficiency could be readily quantified to estimate throughput needed for later editing of the *VvPLL* genes. A total of 1,529 meristem inoculations were carried out using the construct targeting the *VvPDS1* gene, and these resulted in new shoots at 350 sites (23% regeneration rate). Of these, two shoots exhibited photobleaching, 1 partial and 1 complete (Figure 4), suggesting possible editing. These leaves were first assessed for luciferase activity by detaching and incubating in luciferin, and no signal was detected. Unfortunately, following this assay, the tissues were no longer suitable for nucleic acid extraction, so molecular confirmation of transgenesis or edits could not be performed. In some other regenerated shoots, abnormal growth was observed. However, we did not detect the IPT transgene by PCR or observe luciferase bioluminescence (by subsequently detaching the leaves), suggesting that the abnormal growth may have been due to damage during inoculation. We also obtained one gall, which failed to transition to shoots, potentially due to IPT expression.

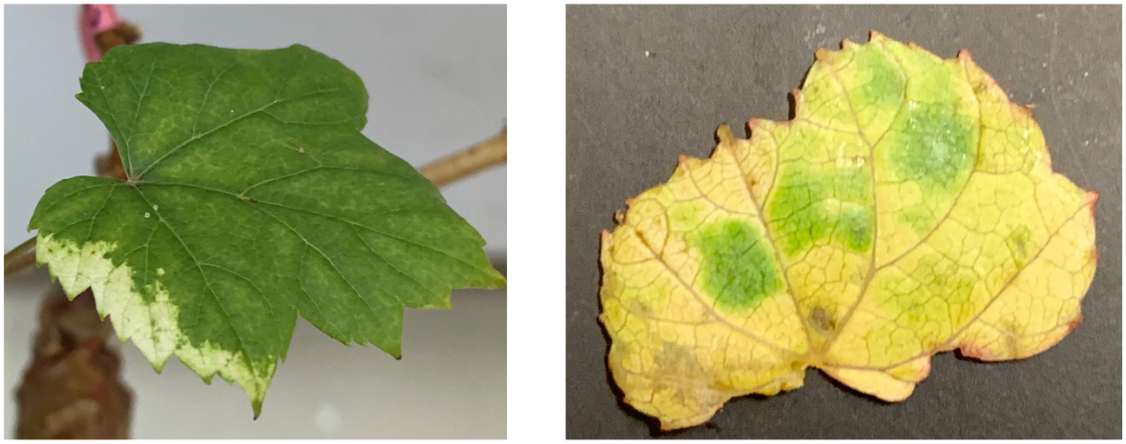


Figure 4. Meristem transformation targeting the PDS1 gene. 2 of 350 new shoots following 1,529 inoculations became partially or fully white.

Over the course of the project, we reached out to another group which also has been implementing meristem transformation in grapevine. Two research groups (Cornell University and Albany, California USDA Station) that have independently pursued the approach rigorously but without success. Both groups used the IPT gene driven by the 35S promoter, as reported by Maher et al. (2020) In summary, meristem transformation using the IPT gene failed to produce transformed or edited shoots to a practicable degree in grapevine under the experimental conditions used in the study. Including a previous thesis (Spicer 2023), this outcome has been consistent across multiple cultivars, laboratories, and experimental conditions.

While the tissue culture-free transformation approach remains promising, it seems clear that achieving tissue-culture free genome editing in soil-grown grapevine will require concerted, species- and cultivar-specific optimization. We reported the methods used, including modifications to previously published protocols, along with the negative results and future considerations. These findings were published in PhytoFrontiers (Kaya et al., 2026).

The rootstock editing approach involves creating a transgenic rootstock capable of producing and delivering two mobile RNAs into a non-GMO scion. The RNAs are made mobile by including a sequence that forms a tRNA-like structure (TLS). Once in the scion, one RNA generates the Cas9 protein and the other serves as the gRNA, allowing editing there without the introduction of foreign DNA (the RNAs are eventually degraded). Rootstock editing has been demonstrated in *Arabidopsis* and *Brassica rapa*, resulting in stable, transgene-free edited plants (Yang et al., 2023). The approach offers a significant advantage by enabling editing in transformation-recalcitrant cultivars using a rootstock donor that can be more easily transformed.

To pursue this approach, constructs targeting the *VvPDS1* and *VvPLL* genes were designed, assembled, sequence-verified, and introduced into *Agrobacterium tumefaciens*. These were then used to transform ‘Chardonnay’ calli prepared by Dr. Gan-Yuan Zhong’s lab (USDA-ARS, Cornell AgriTech). Dr. Zhong has been a vital collaborator in this project, contributing extensive expertise in grapevine tissue culture and transformation systems, and leading efforts to optimize callus generation and support downstream regeneration.

Following three months of incubation on selection media, some somatic embryos were obtained. We included a gene for green fluorescent protein (GFP) in the t-DNA, which enabled confirmation of transformation via fluorescence microscopy. Figure 5 shows the callus transformation workflow and experimental results, including embryo development and GFP expression confirmation after three months of incubation on selection media. These results demonstrate the successful transformation of grapevine calli and provided a foundation for monitoring shoot and root regeneration in subsequent stages of rootstock editing.

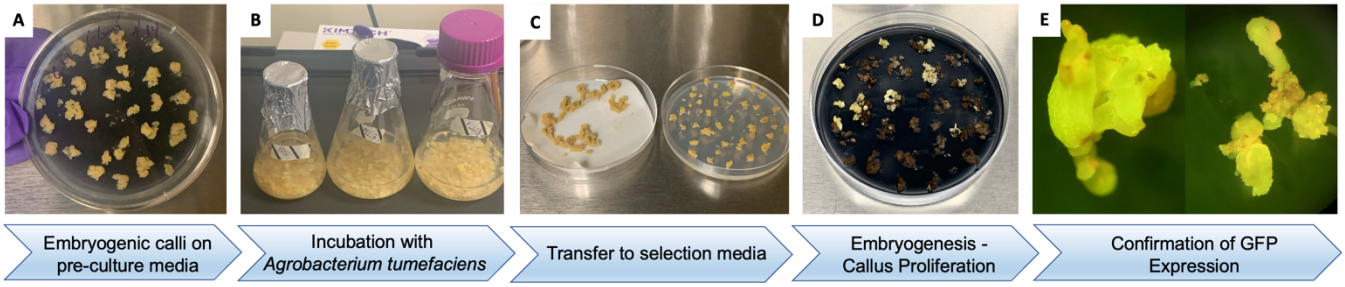


Figure 5. Schematic overview of the *Agrobacterium*-mediated transformation process in embryogenic calli. A. Embryogenic calli were first established on pre-culture media. B. Calli were incubated with *Agrobacterium tumefaciens* for co-cultivation. C. Following infection, calli were transferred to selection media. D. Successful embryogenesis and callus proliferation were observed under selection. E. GFP expression was confirmed in transformed tissues using fluorescence microscopy, indicating successful genetic transformation.

Editing efficiencies of CRISPR/Cas9 constructs targeting *VvPDS1, VvPLL3*, and *VvPLL13* were evaluated (Figure 6). Editing efficiencies for two gRNAs per target gene were assessed using biological replicates pooled across transformation experiments (n=6 per gRNA).

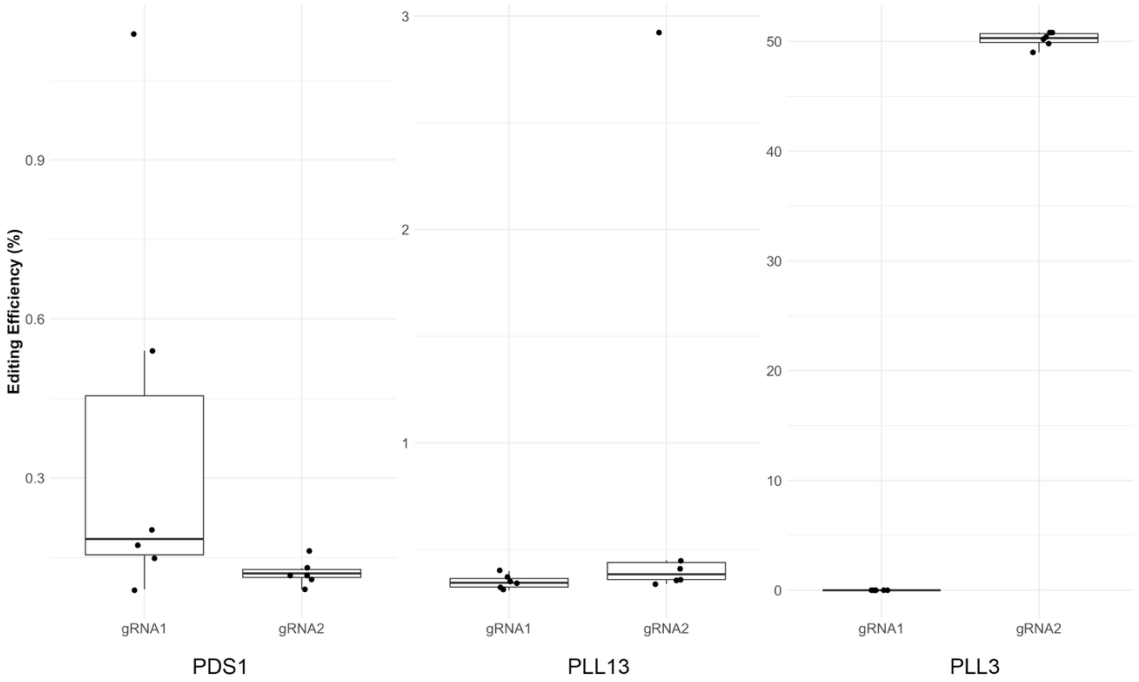


Figure 6. CRISPR/Cas9 editing efficiency across target genes and gRNAs. Editing efficiency (%) for each gRNA targeting *VvPDS1*, *VvPLL3*, and *VvPLL13* is shown. Each point represents an individual biological replicate pooled across independent transformation days. Boxplots indicate the distribution of editing efficiencies, with the center line representing the median and the box denoting the interquartile range. Data are grouped by target gene to allow comparison between gRNAs within each locus.

Low editing efficiencies were observed for *VvPDS1* and *VvPLL13* across all tested gRNAs. In contrast, amplicon sequencing of the *VvPLL3* gRNA2 target site revealed a predominant deletion event. This deletion spans the last nucleotide of codon 85 and the first two nucleotides of codon 86. Although this deletion affects two adjacent codons, it results in the loss of a single serine residue and does not disrupt the reading frame (Figure 7). The remainder of the protein sequence remains unchanged. Allele frequency table for *VvPLL3* gRNA2 was shown in Figure 8.

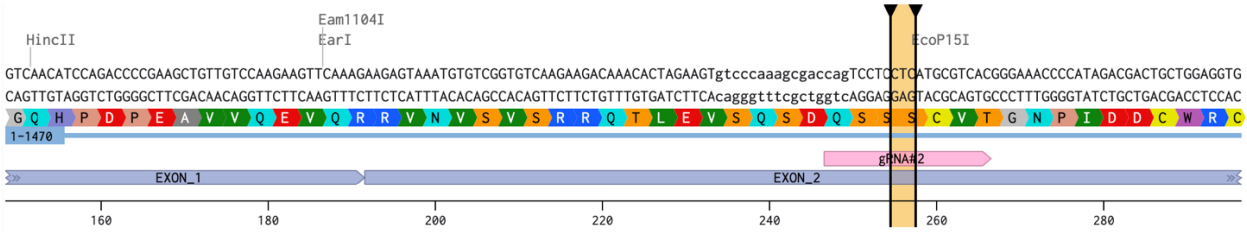


Figure 7. Deletion at the *VvPLL3* gRNA2 target site. The deletion spans codons 85-86 and results in a single amino acid loss.

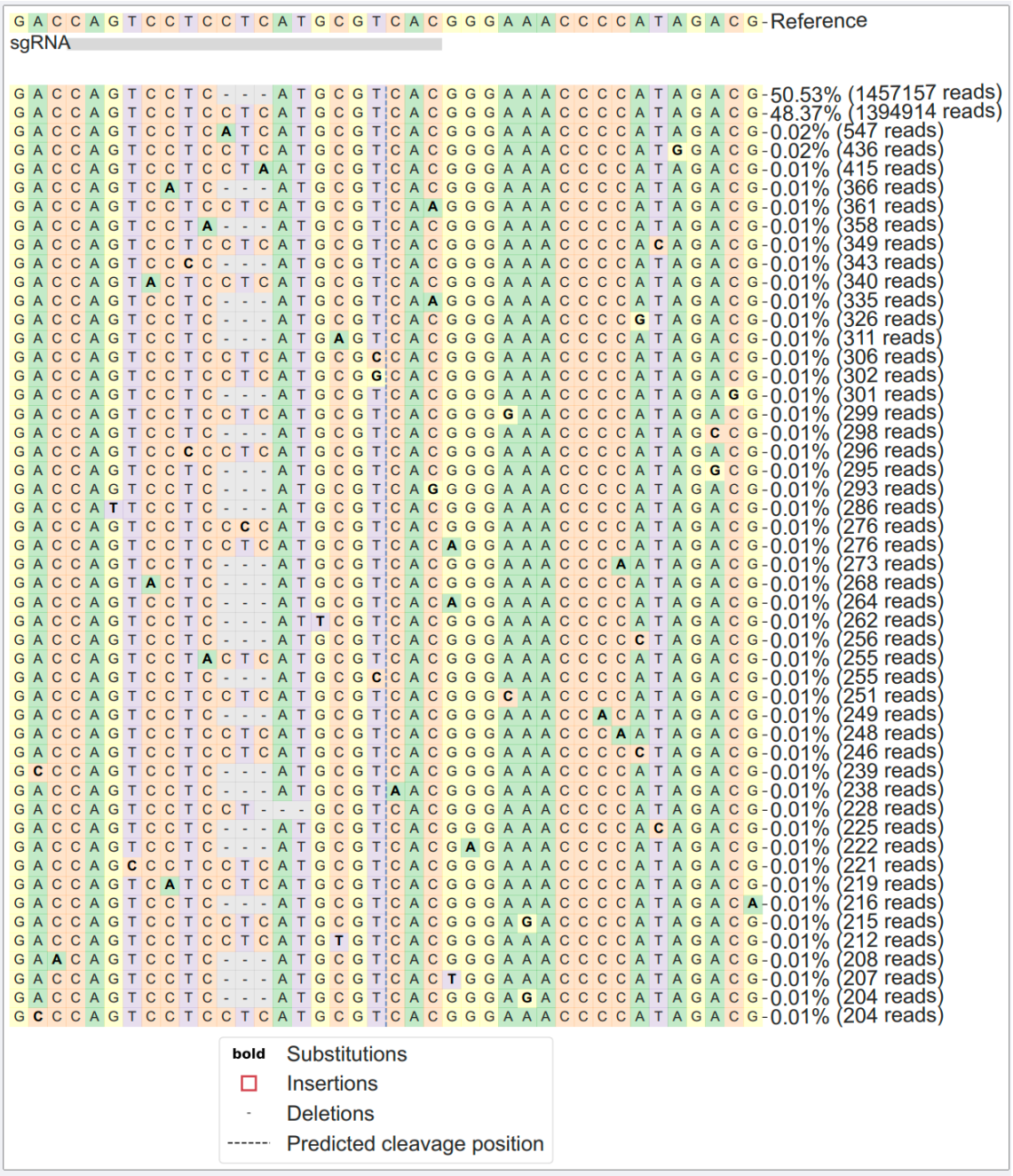


Figure 8. Distribution of edited alleles at the target site. Visualization of allele variants around the predicted cleavage site for the sgRNA CAGTCCTCCTCATGCGTCAC. Horizontal dashed lines indicate deletions, and the vertical dashed line marks the predicted Cas9 cleavage site.

Although regenerated shoots were obtained from transformed callus samples following selection and regeneration (Figure 9), amplicon sequencing analysis indicated that these regenerated lines did not carry the expected edits at the target loci. A 3 bp deletion at the *VvPLL3* gRNA2 target site results in an in-frame deletion, which is unlikely to produce a loss-of-function allele of *VvPLL3* as originally intended.

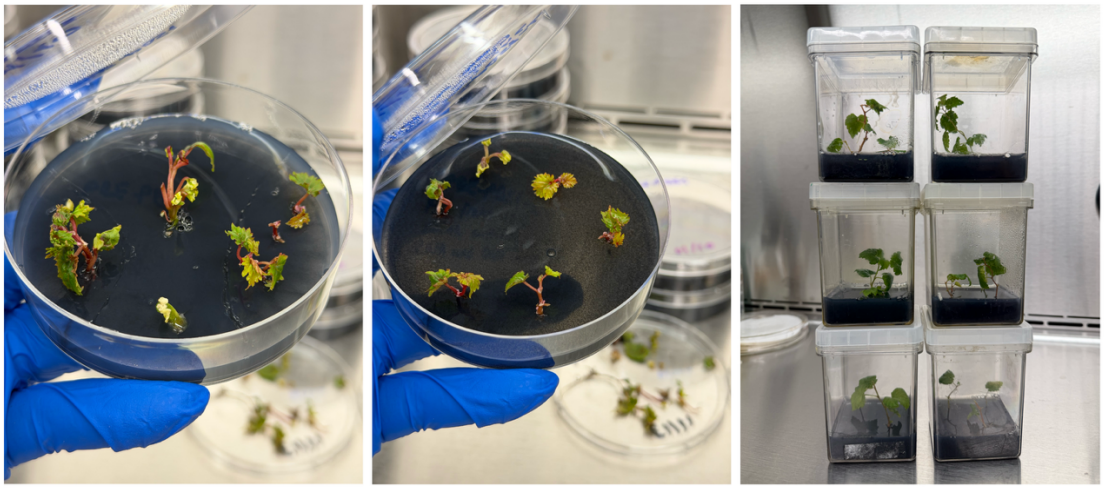


Figure 9. Regeneration of transgenic grapevine shoots.

Symbiont Technology was developed by Dr. Michelle Heck (USDA-ARS, Ithaca) for the production and delivery of biomolecules to control citrus greening disease (Heck et al., 2026). It uses a partially disarmed *Agrobacterium tumefaciens* strain carrying the necessary DNA to induce gall ('symbiont') formation and to generate a molecule capable of moving throughout the tree to treat the infection. In the symbiont system, the DNA transferred by *Agrobacterium* retains the genes required for gall formation, but the genes for biosynthesis of opines, molecules *Agrobacterium* feeds on in nature, are replaced with genes to produce the molecule(s) of interest. This is designed to reduce the physiological burden on the plant and to limit multiplication of *Agrobacterium* that could lead to spontaneous gall formation. Indeed, the Heck lab has demonstrated that citrus trees can support symbiont galls without negative impacts on plant health or yield, that symbionts producing therapeutic molecules can reduce citrus greening symptoms in greenhouse trials, and that additional galls do not form spontaneously.

We reasoned that a symbiont could be engineered to produce the RNAs required for editing, made mobile by TLS sequences. Movement of the RNAs out of the symbiont would be expected to result in gene edits in the surrounding vine tissues allowing propagation of shoots without any foreign DNA, ultimately resulting in edited, non-GMO vines. In collaboration with Dr. Heck and her lab, we inoculated Chardonnay and Thompson Seedless cuttings with the disarmed *Agrobacterium* strain carrying the plasmid pSymbiont containing no extra DNA or DNA for GFP. Robust gall formation was observed in each case and UV images confirmed expression of the fluorescent protein (Figure 10). Constructs for editing the *VvPDS1* and *VvPLL* genes were then cloned into the pSymbiont vector, sequence-verified, and introduced into the *Agrobacterium* strain. In each construct, the GFP gene was included to allow visual confirmation of expression of the transferred DNA in the gall (Figure 11). Gall formation in Chardonnay cuttings (not shown) was slower, and the galls were smaller compared to those observed in Thompson Seedless cuttings.

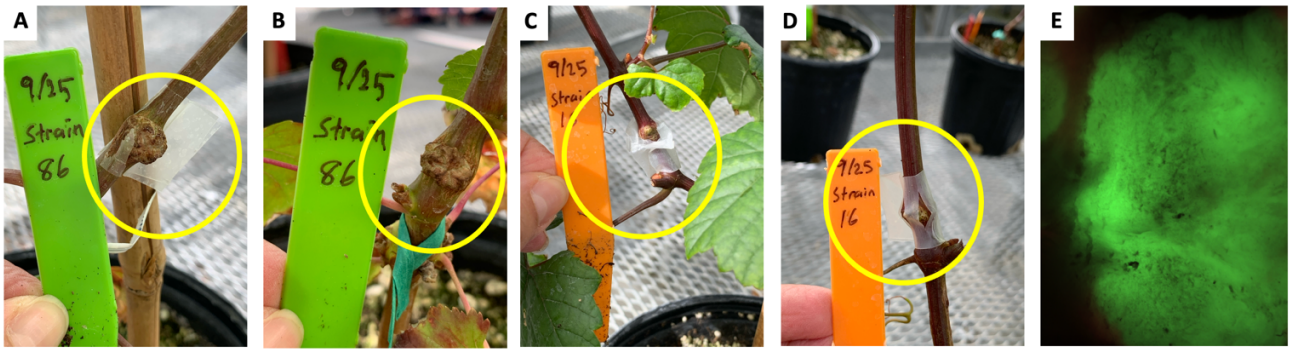


Figure 10. Symbiont formation three weeks following inoculation with different *Agrobacterium* strains. A-B. Results for *Agrobacterium* with no added DNA. C-D. Results for *Agrobacterium* with DNA for green fluorescent protein (pSym-GFP). E. Fluorescent image showing GFP-expressing gall tissue.

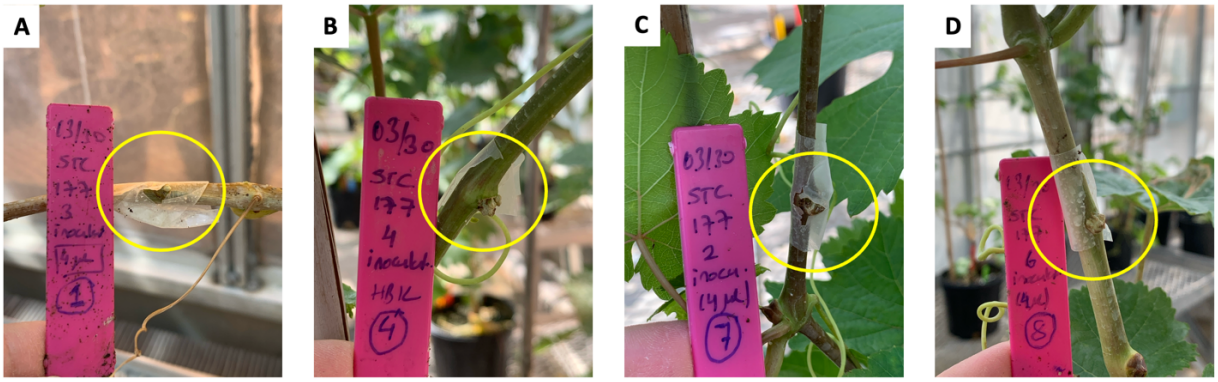
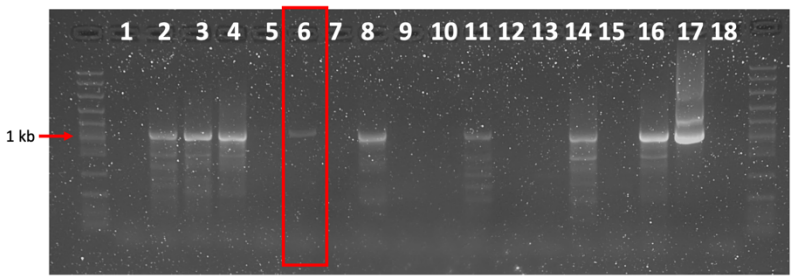


Figure 11. Symbiont formation following inoculation with pSymbiont-PDS1 vector. (A–D) Representative images of gall formation on Thompson Seedless grapevine cuttings.

Tissue samples were collected following inoculation for downstream molecular analysis. RNA was extracted from treated tissues and used for RT-PCR and RT-qPCR to evaluate gene expression and assess the effectiveness of symbiont-mediated delivery. The initial screening of symbiont inoculations is summarized in Figure 12. RT-PCR performed on cDNA using Cas9-specific primers produced the expected amplicons in all gall samples, as well as in one sample collected from tissue adjacent to the gall (lower site).

RT-qPCR analysis of gRNA expression (Figure 11B) showed strong amplification within gall tissues, whereas little to no signal was detected in surrounding non-gall tissues. In particular, gRNA targeting *VvPDS1* (gRNA1) showed weak or undetectable expression outside the gall region (Table 1).

A



B

Sample#	Plant #	Sample - location
1	5	Gall #1 – upper adjacent leaf
2	5	Gall #1 – gall tissue
3	5	Gall #2 – gall tissue
4	5	Gall #3 – gall tissue
5	5	Gall #3 – upper side of gall
6	5	Gall #3 – lower side of gall
7	6	Gall #8 – upper side of gall
8	6	Gall #8 – gall tissue
9	6	Gall #8 – lower side of gall
10	7	Gall #10 – upper side of gall
11	7	Gall #11 – gall tissue
12	7	Gall #12 – lower side of gall
13	5	Gall #14 – upper side of gall
14	5	Gall #14 – gall tissue
15	5	Gall #14 – lower side of gall
16	5	Gall #16 – gall tissue
17		Pre-PDS1 construct (PCR positive control)
18		WT leaf (negative control)

C

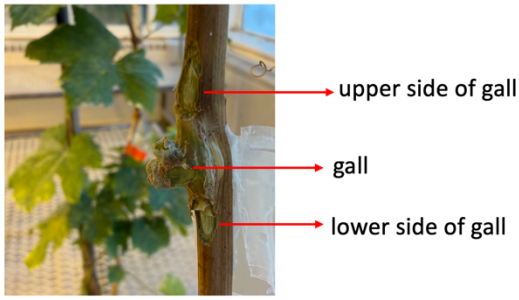


Figure 12. Detection of Cas9 expression in gall and surrounding tissues following symbiont-mediated inoculation. A. RT-PCR analysis of cDNA from RNA samples using Cas9-specific primers. The expected amplicon size is 1.2 kb. Bands corresponding to the expected fragment were detected in gall tissues and in one sample adjacent to the gall (lane 6), B. Description of sampled tissues collected from plants inoculated with the pSTC177 construct targeting VvPDS1, including gall tissue and adjacent upper and lower regions. C. Sampling strategy illustrating the locations of tissue collection.

Table 1. RT-qPCR analysis of gRNA expression across gall and adjacent tissues.

Sample#	Sample - location	Ave Cq for Primers	
		PDS1-gRNA1	PDS1-gRNA2
1	Gall #1 – upper adjacent leaf	ND (non-detected)	34.82
2	Gall #1 – gall tissue	28	29.65
3	Gall #2 – gall tissue	27.65	28.47
4	Gall #3 – gall tissue	26.51	27.71
5	Gall #3 – upper side of gall	37.94	34.22
6	Gall #3 – lower side of gall	37.08	33.35
7	Gall #8 – upper side of gall	38.27	34.63
8	Gall #8 – gall tissue	28	28.44
9	Gall #8 – lower side of gall	39.96	33.19
10	Gall #10 – upper side of gall	ND	36.19
11	Gall #11 – gall tissue	31.86	33.64
12	Gall #12 – lower side of gall	ND	35.21
13	Gall #14 – upper side of gall	ND	34.54
14	Gall #14 – gall tissue	28.82	30.48
15	Gall #14 –lower side of gall	ND	32.98
16	Gall #16 – gall tissue	25.99	28
17	Leaf from WT grapevine-1	34.76	33.43
18	Leaf from WT grapevine-1	ND	33.34

An additional set of samples was collected from the same and independent galls to further evaluate symbiont-mediated delivery. Tissue samples were obtained from gall regions as well as adjacent tissues and analyzed by RT-PCR using Cas9-specific primers (Figure 13). As in the initial screening, strong amplification of the expected fragment was observed in one gall tissue (Sample #22). However, faint bands at the expected size were also detected in several samples collected outside the gall (samples 25–27), suggesting limited spread of expression beyond the gall region. Bands corresponding to the expected size were cut from the gel and sent for Sanger sequencing. Sequence analysis confirmed that all amplicons matched the Cas9 sequence (Figure 14).

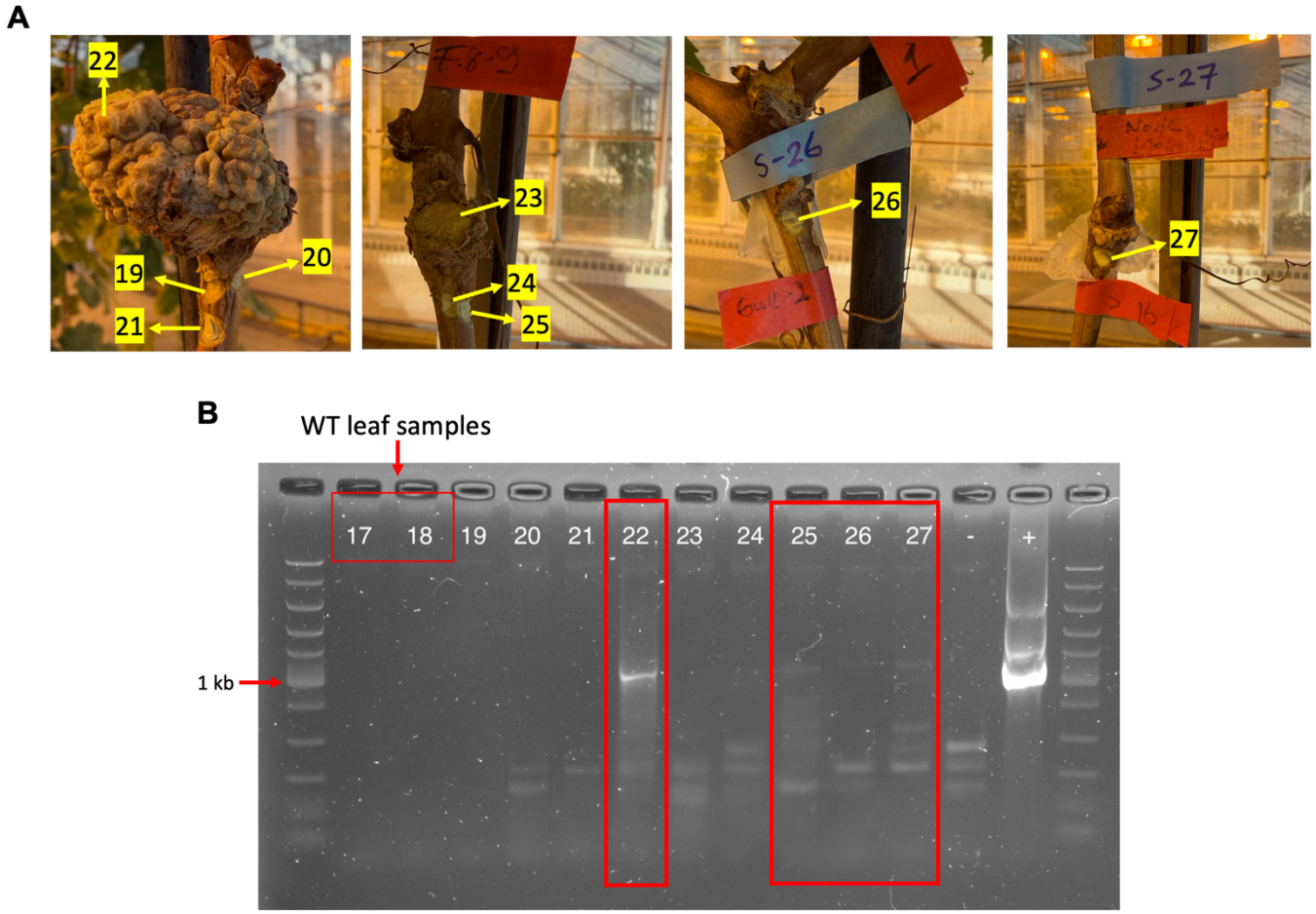


Figure 13. Detection of Cas9 expression in gall and surrounding tissues following symbiont-mediated inoculation. A. Representative images of gall tissues and sampled regions, with numbered sampling points indicated. B. RT-PCR analysis of cDNA using Cas9-specific primers showing the expected 1.2 kb amplicon. Wild-type leaf samples (lanes 17–18) showed no amplification. Positive (+) and negative (–) controls are included.

Figure 14. Confirmation of amplicons by Sanger sequencing. Alignment of Sanger sequencing reads obtained from gel-extracted PCR products with the reference Cas9 sequence, confirming the identity of the amplified fragments.

Technology Transfer Plan: None at this time (edited vines have not been obtained). However, the Cadle-Davidson lab will continue to propagate the transgenic Chardonnay plants for future pursuit of the rootstock editing approach, and under a separately funded project, Co-PI Kaya will join forces with the Heck lab to follow up on the observed movement of RNAs from galls, toward optimizing that approach for future application.

Attachments: N/A

SECTION 3:

Project summary and objectives

Powdery mildew (PM) is a major constraint in grape production for which fungicides and PM resistance genes are limited and subject to defeat as the pathogen mutates. Plant-pathogen interaction studies have identified a variety of plant genes that, rather than providing resistance, impart disease susceptibility, often genes exploited by the pathogen to establish infection. When these genes are disrupted, the plants are less susceptible or completely resistant to a disease; MLO is a naturally occurring example. Prior experiments, using

technology to reduce expression of a gene, established that two pectate lyase-like genes in grape contribute to the development of PM. This project sought to knock out these susceptibility genes completely, using non-GMO, precision gene-editing, setting the stage to determine whether that results in a practical level of PM resistance without affecting other characteristics of the vine.

Importance of research to the NY wine industry

In New York, powdery mildew typically requires 10-15 fungicide applications per year. The pathogen, *Erysiphe necator*, has repeatedly developed resistance to each of the major classes of fungicides, and strains have also arisen that collectively defeat all but 3 of the 15 known PM resistance (R) genes in grape. This project took a new approach, using gene editing technology to knockout grape genes that must be present for disease to develop, so called 'susceptibility' (S) genes. An important advantage of the S gene knockout approach is that it can be more difficult for the pathogen to mutant to make up for the loss of an S gene than to mutate to evade an R gene, so the approach can be more durable. A further advantage is that gene editing creates targeted mutations, of the kind that might occur naturally, without leaving any foreign DNA behind, promising durably PM-resistant clones of an existing cultivar that could be used directly or as breeding parents for future cultivars, a potential game-changer for the industry.

Results and Future Prospects

This project sought to use a transgene-free genome editing approach called ***meristem transformation*** to knock out two powdery mildew susceptibility genes and determine whether the resulting plants develop normally. Meristem transformation relies on transient expression of the editing reagents rather than expression from a stably inserted transgene. A large number of trials were conducted and protocol optimizations attempted. While some visual evidence of editing was obtained, editing could not be confirmed molecularly. Two other research groups were also unable to get this approach to work in grapevine. We published a paper with one of those groups detailing our negative results and suggestions for the future, to help prevent fruitless duplication of effort by others (Kaya et al., 2026). Also, we expanded the project to include two other approaches, rootstock editing and Symbiont Technology.

Rootstock editing relies on a transgenic rootstock to produce mobile RNAs for transient expression of the editing reagents in the scion, which remains transgene free. We generated three sets of transgenic Chardonnay plants for use as rootstock, one each for two of the susceptibility genes and one for a third gene that when edited would result in white leaves. In the transgenics for one of the susceptibility genes, we detected a DNA sequence change at the editing target site using a molecular assay. However, the change was a small mutation that likely does not affect the function of the gene. We speculate that the gene might be essential and that knockout mutations are lethal, preventing us from obtaining any knockout edited plants. This remains to be explored. Also, whether the sequence change occurred spontaneously or due to editing needs to be confirmed. But if confirmed as an edit, the transgenic rootstock will be useful for a future proof of principle experiment to determine whether the same edit can be obtained in a transgene free scion grafted to the transgenic. If so, the approach could be pursued for other, production-relevant target genes

Symbiont Technology relies on a locally induced, transgenic gall ("symbiont") to generate RNAs that will move out of the gall for transgene-free editing in the vine. Though the frequency varied across trials, we were able to obtain galls. We tested a subset for transgene expression, and all were positive. For 10 robust galls, we also assayed tissues next to the gall for evidence of editing but detected none. However, in two cases we detected small amounts of the mobile RNAs at short distances (~1 cm) both above and below the gall. So, while we did not achieve our ultimate goal of an edited vine using this method, we obtained proof of principle for using galls to generate mobile RNAs that can move into a genetically unmodified vine. Future research to make more RNA move into the vine and further away from the gall could set the stage for use of this technology for transgene-free, transient expression of genome editing or other reagents, for example antimicrobial or insecticidal peptides, or use of the RNAs themselves as antivirals.

Supporting attachments: N/A

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