NYWGF RESEARCH

Funding for fiscal year: 2024-2025

SECTION 1:

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

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New Research ☒ **Continued Research** ☐ (CHECK APPROPRIATE BOX)

Amount Funded **$** 35,000

SECTION 2:

Project Summary Impact Statement:

Powdery mildew (PM) is a major constraint in grape production that requires frequent fungicide application. However, fungicide options are decreasing, and fungicides often become ineffective as the pathogen mutates to become insensitive. Genetic resistance (R) to the disease is another important component of management. However, R genes, like fungicides, tend to become ineffective over time as the pathogen mutates to avoid being recognized and triggering the plant's resistance response.

Plant-pathogen interaction studies have identified a variety of plant genes that, unlike R genes, play important positive-acting roles in disease development, often encoding factors a pathogen relies on to establish infection. When such 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 (*PLL3* and *PLL13*) in grape contribute to the development of PM. This project seeks to knock out these 'susceptibility' (S) genes completely, using non-GMO, precision gene-editing approaches.

PLL genes play various roles in plants, including in cell wall formation and remodeling during growth, development, and wound repair, and softening of fruit as it ripens (Uluisik and Seymour, 2020). There are at least 16 pectate lyase and pectate lyase-like genes in grape, suggesting some redundancy and/or functional overlap (Ma et al., 2023). Reducing the expression of *PLL3* or *PLL13* resulted in less PM, without any effect on vegetative growth or development of the vines (Cadle-Davidson, unpublished).

By knocking out *PLL3* and *PLL13* completely using gene editing, this project will set the stage to determine whether the complete loss of these genes results in even better PM control without affecting vine growth and development, including grape yield and quality. This would be a game changer for the industry. The project will also set the stage for new mechanistic understanding about the disease, specifically the extent to which the *PLL3* and *PLL13* genes contribute to susceptibility. Vines with one, the other, or both knocked out will tell us whether the genes act independently, cooperatively, or redundantly.

An advantage of the S gene knockout approach over introgression of an R gene is that pathogen mutation to make up for the loss of an S gene is less likely than pathogen mutation to evade an R gene, because the latter is loss of a feature while the former is gain of an entirely new function. This means that an S gene knockout can be expected to be more durable than an introduced R gene (Zaidi et al., 2018).

A marked advantage of the gene editing approaches used is that the techniques leave no foreign DNA behind. The edited vines will be non-GMO clones with only the precise and specific changes at the PLL genes. The vines could be commercially grown or used for breeding new resistant grape cultivars. Further studies will be needed to assess field performance of the vines, but if successful, this project will be a major step forward in PM management and serve as proof of principle for using the approach in other grape varieties and for other genes and traits, while leaving all other characteristics of those varieties

unchanged.

Original Objectives:

1. Obtain Chardonnay vines with knockouts of one, the other, or both of two powdery mildew susceptibility genes, *PLL3* and *PLL13*.
2. Determine grapevine powdery mildew susceptibility of the edited vines relative to one another and to unedited Chardonnay.
3. Assess growth and development of the vines in a greenhouse setting.

Materials & Methods:

The methods and workflow originally proposed for this project and followed initially are shown in Figure 1 and detailed below. During the execution of the project, it became clear that higher throughput was needed, and additional approaches came to light. We therefore updated and extended our aims and our Materials and Methods. Those changes are presented in the 'Results/Outcomes/Next Steps' section.

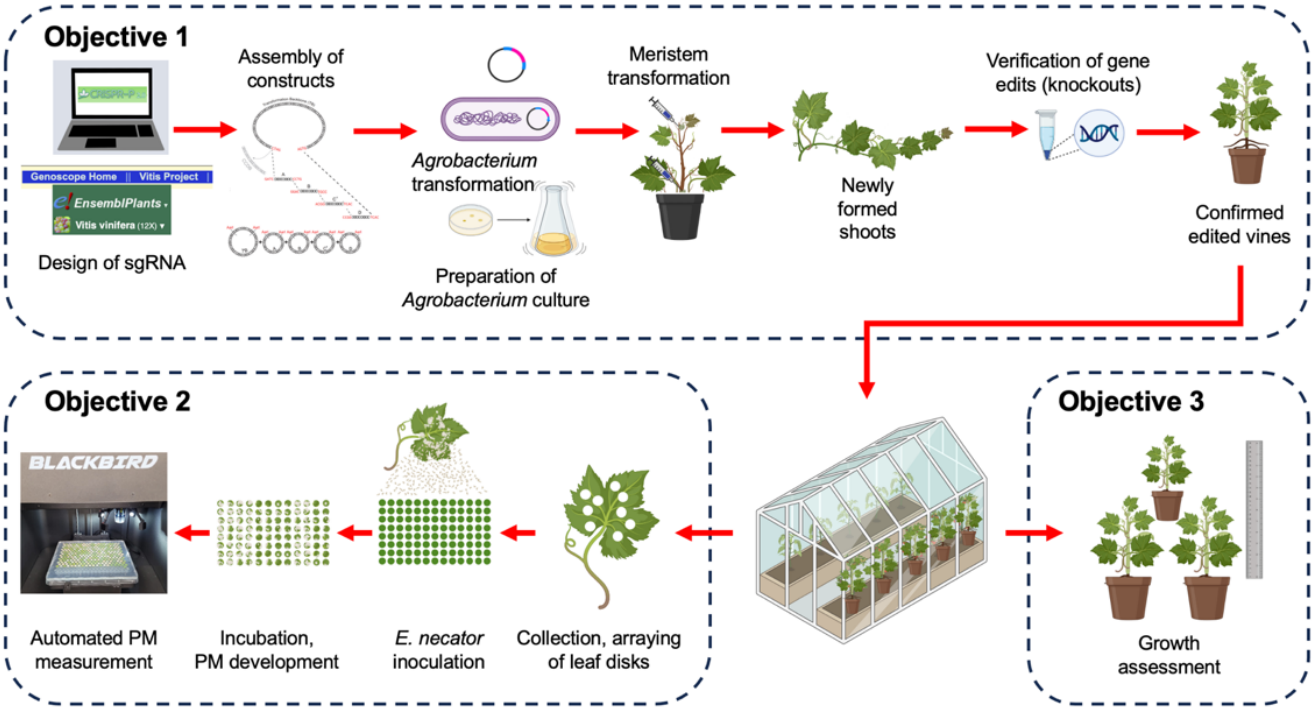

Figure 1. Schematic overview of Materials and Methods originally proposed for this project

Objective 1: Obtain Chardonnay vines with knockouts of one, the other, or both of two powdery mildew susceptibility genes, PLL3 and PLL13.

<u>Design and assembly of DNA plasmid for editing</u>

gRNAs targeting the PLL3 and PLL13 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 as described below.

Verification of 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 *PDS1* and *PLL* 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.

However, no PCR products indicative of successful gene editing were obtained. Due to the lack of detectable edits in the PCR or restriction digestion assays, further characterization of the PCR products, as outlined in the proposal, could not be carried out using Illumina deep sequencing or analysis with TIDE (Brinkman et al., 2014) and CRISPResso2 (Clement et al., 2019).

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

Since no targeted gene edits were detected in the regenerated shoots, we were unable to proceed with the planned experiments to determine the powdery mildew (PM) susceptibility of edited vines relative to unedited Chardonnay. The subsequent steps, including leaf disk inoculation and PM quantification using the Blackbird automated microscopy system, are still planned and will be carried out once sequence-confirmed, edited plant material is available.

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

Similarly, as no sequence-confirmed, non-chimeric shoots with the desired edits were obtained, we were unable to assess the growth and development of edited vines in the greenhouse. Measurements of internode length, leaf dimensions, and plant height at defined time points remain part of the project plan and will be performed following the recovery of suitable edited vines.

Results/Outcomes/Next Steps:

The project to date has focused on implementing meristem transformation, as outlined by Maher et al. (2020), to enable transgene-free (non-GMO) editing of PLL genes. 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.

The procedure involves removing newly formed shoots (Figure 2A), followed by the injection of *Agrobacterium tumefaciens* into the exposed meristematic tissue (Figure 2B). 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.

Newly formed shoots from the injection site (Figure 2C) are screened to identify successful edits and to confirm the absence of transgene integration. Due to the low frequency of editing events, a high-throughput screening process is required to identify modified individuals.

Figure 2. Meristem transformation. A. Removal and disposal of newly formed shoots on the vine. B. *Agrobacterium tumefaciens*, carrying the DNA for the gene editing machinery, is injected into the wound for DNA transfer into the plant cells. C. Newly formed shoots grow from the injection sites and can then be screened for the desired edit.

Over the course of the project, the necessary DNA constructs for the *PDS1* and *PLL* genes were generated, introduced into *Agrobacterium tumefaciens*, and confirmed by whole plasmid sequencing. *PDS1* was selected as the initial target so that editing efficiency could be readily quantified to estimate throughput needed for later editing of the *PLL* genes. 1,035 meristem inoculations were carried out using the construct targeting the *PDS1* gene, and these resulted in new shoots at 234 sites. Of these, 2 shoots exhibited photobleaching, 1 partial and 1 complete (Figure 3), suggesting successful editing. However, PCR analysis could not be performed on these shoots before they deteriorated, so this conclusion is tentative.

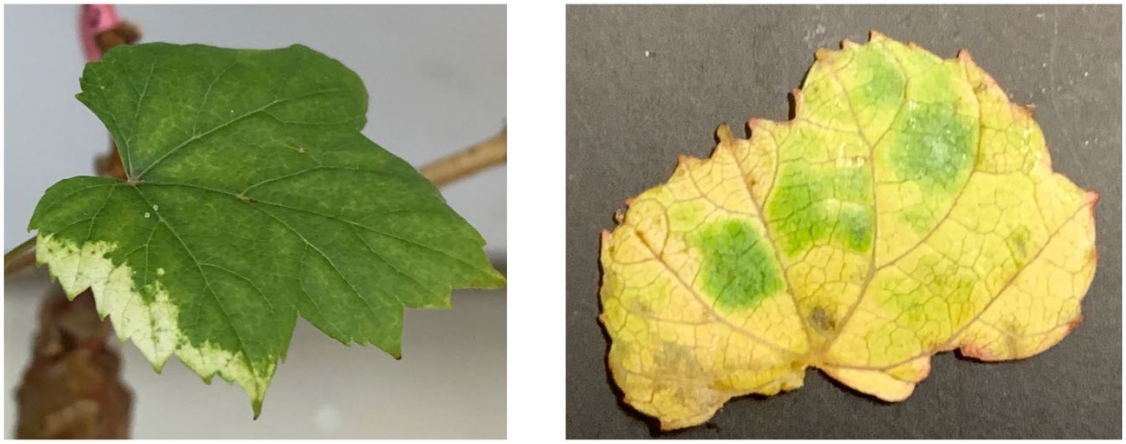

Figure 3. Meristem transformation targeting the PDS1 gene. 2 of 234 new shoots following 1035 inoculations became partially or fully white.

The low number of photobleached shoots following more than 1,000 inoculations indicates that high-throughput screening will be necessary to achieve successful edits of the *PLL* genes. And, we did not obtain molecular confirmation of the *PDS1* edit in the two photobleached leaves. We therefore chose to conduct and screen more meristem transformations targeting the *PDS1* and *PLL* genes. Furthermore, during the project, two additional novel approaches came to light – rootstock editing and symbiont technology (described below). To increase the overall likelihood of success, we chose to pursue these approaches alongside meristem transformation. Thus, we updated our objectives as follows:

Updated Objectives:

1. Obtain Chardonnay vines with knockouts of one, the other, or both of two powdery mildew susceptibility genes, *PLL3* and *PLL13*.
    a. Continue meristem transformation and screening, increasing the number of inoculations.
    b. *NEW* - Pursue rootstock editing, i.e., transgenic rootstock-mediated delivery of mobile RNAs, for transgene-free editing in the scion.
    c. *NEW* - 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 updated methods and workflow, including the new objectives, is provided in Figure 4. If Objective 1 is successful, Objectives 2 and 3, illustrated in Figure 5, will be carried out.

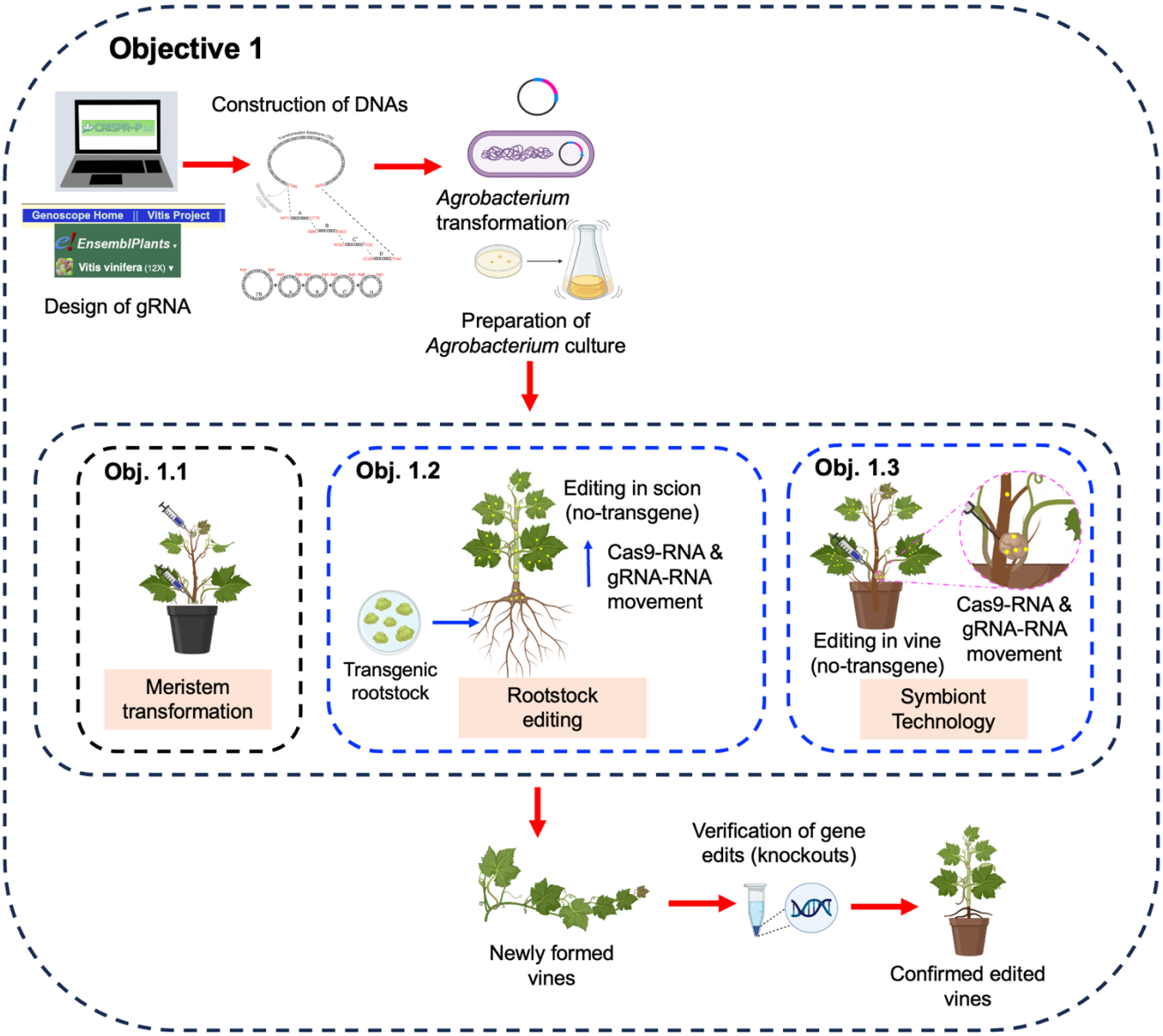

Figure 4. **Schematic of the project workflow, including the proposed additional delivery methods**. Objective 1 involves design of gRNAs for the target genes using online tools, construction of the DNAs needed for the CRISPR editing reagents, transformation of *Agrobacterium*, delivery into plants, and verification of edited plants. Meristem transformation (Obj. 1.1) will continue with an increased number of inoculations. For the additional delivery methods (Obj. 1.2 and 1.3, outlined in dotted blue), new DNAs will be assembled to generate RNAs with a sequence added for movement from rootstock to scion (rootstock editing) and from symbionts to vines (Symbiont Technology). Gene editing will be verified by PCR and sequencing.

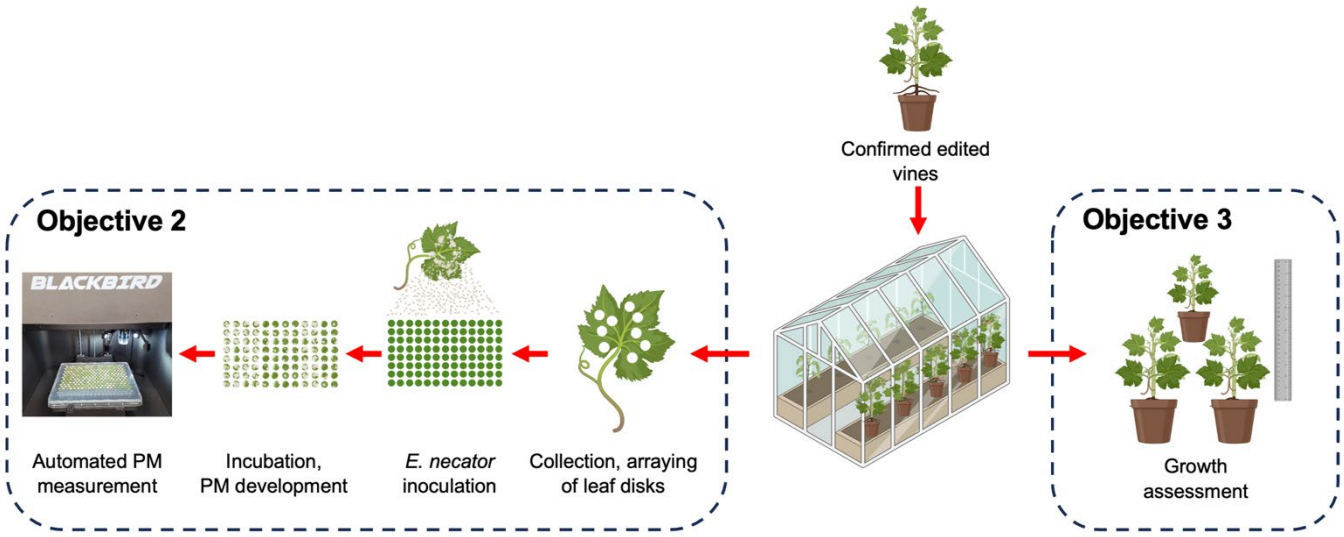

Figure 5. Workflow for assessing powdery mildew susceptibility and growth of the vines in the greenhouse. Objective 2 is to evaluate the PM susceptibility of edited vines compared to unedited ones. It will use leaf disk inoculation and an automated image analysis system called Blackbird to quantify fungal infection. Objective 3 examines 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.

We will continue meristem inoculations as originally detailed, aiming for at least 1000 inoculations more per *PLL* construct.

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 *PDS1* and *PLL* 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. We are currently monitoring the embryos for shoot and root regeneration. These ongoing efforts represent a critical step toward establishing rootstock editing in grapevine. Success in this area would support the broader goal of enabling non-GMO editing solutions for cultivars that are difficult to transform. Figure 6 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 provide a foundation for monitoring shoot and root regeneration in subsequent stages of rootstock editing.

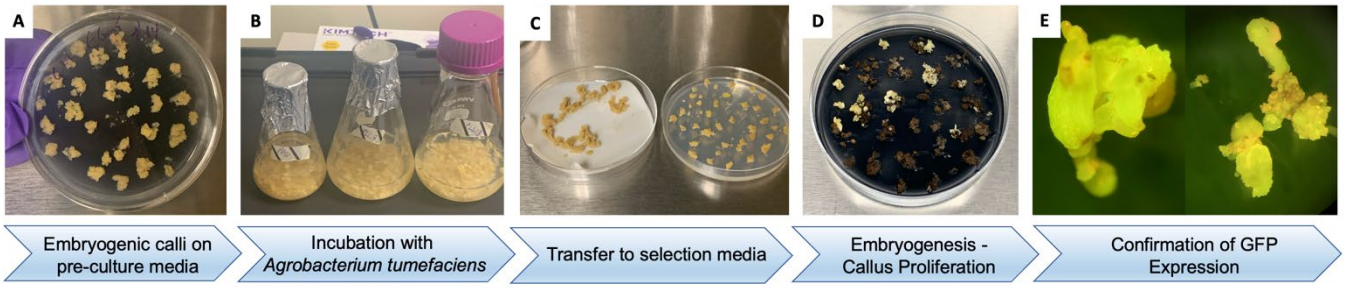

Figure 6. Schematic overview of the *Agrobacterium*-mediated transformation process in embryogenic calli. A. Embryogenic calli were first established on pre-culture media. B. Calli were incubated with *Agrobacterium tumefaciens* for co-cultivation. C. Following infection, calli were transferred to selection 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.

Symbiont Technology was developed by Dr. Michelle Heck (USDA-ARS, Ithaca) for the production and delivery of biomolecules to control citrus greening disease (unpublished). 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 7). Constructs for editing the *PDS1* and *PLL* 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. So far, we have inoculated 10 Chardonnay and Thompson Seedless cuttings with *Agrobacterium* carrying the *PDS1* editing construct, and galls have already formed on some (Figure 8). Gall formation in Chardonnay cuttings (not shown) was slower, and the galls were smaller compared to those observed in Thompson Seedless cuttings. Screening for editing events will be performed next, alongside inoculations using constructs targeting the *PLL* genes.

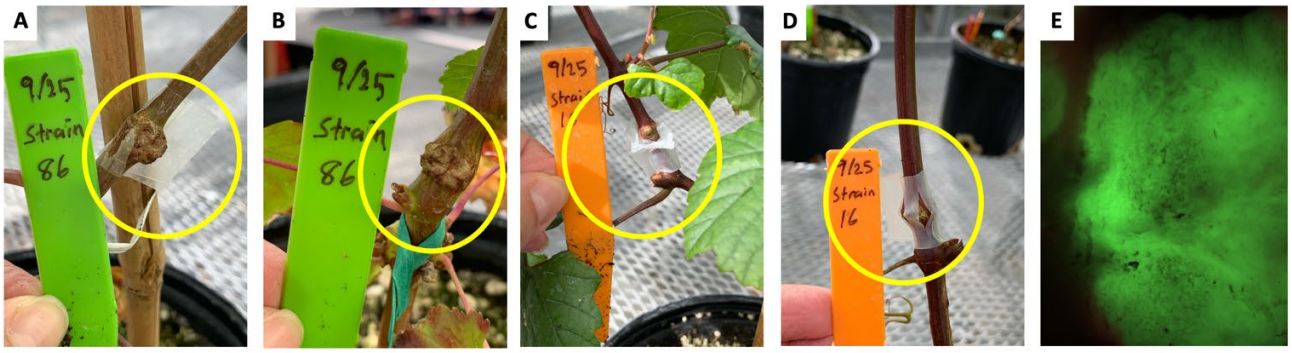

Figure 7. Symbiont formation three weeks following inoculation with different *Agrobacterium* strains. A-B. Results for *Agrobacterium* with no added DNA. C-D. Results for *Agrobacterium* with DNA for green fluorescent protein (pSym-GFP). E. Fluorescent image showing GFP-expressing gall tissue.

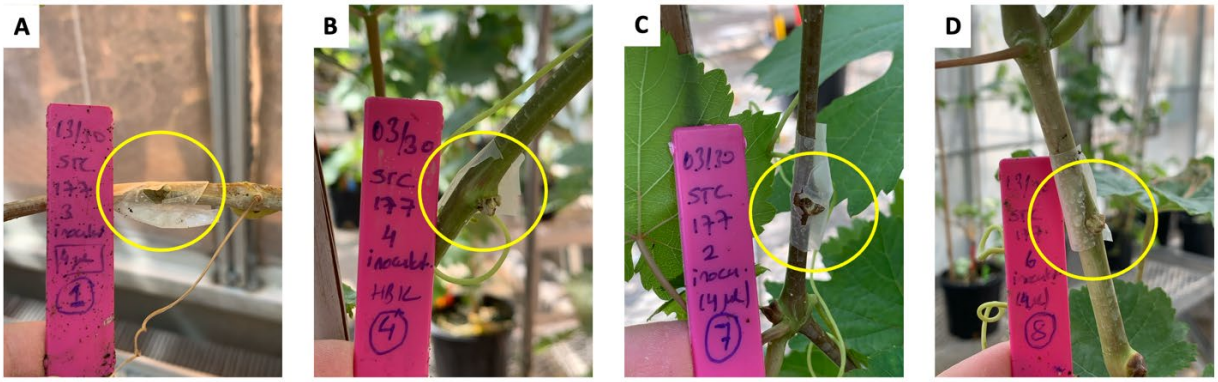

Figure 8. Symbiont formation following inoculation with pSymbiont-PDS1 vector. (A–D) Representative images of gall formation on Thompson Seedless grapevine cuttings.

Technology Transfer Plan: Because it has become clear that higher throughput than originally anticipated is required to obtain edited vines, full characterization of edited vines (necessary prior to use in production or breeding) will be deferred until a future iteration, so a technology transfer plan is not applicable at this time.

Attachments: N/A

SECTION 3: (The goal of this research is to benefit growers and producers across New York State. Result summaries will be shared on the NYWGF website and via email newsletters. To that end, this section should be brief and written in terms understandable for the average grower and producer, as well as consumers and trade interested in our industry.)

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 seeks to knock out these susceptibility genes completely, using non-GMO, precision gene-editing approaches, 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 takes 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, so, if successful, the project will deliver durably PM-resistant clones of an existing cultivar that can be used directly or as breeding parents for future cultivars, a potential game-changer for the industry.

Project Results/next steps: The originally proposed approach, 'meristem transformation,' involves inoculating cut stems with *Agrobacterium* delivering DNA encoding the editing reagents, to obtain edits in shoots that subsequently emerge from those cut stems, without leaving any of the introduced DNA behind. This approach also avoids tissue culture, which can sometimes introduce unintended genetic changes. Over 1,000 inoculations were performed, targeting first a gene that when edited would turn the new growth white, to allow us to determine the overall efficiency of the approach before targeting the susceptibility genes. New shoots emerged from 234 of the inoculated sites. Of these, evidence of editing (white or partially white leaves) was observed in 2. It was thus clear that higher throughput than originally anticipated will be required to obtain edits in the susceptibility genes. Therefore, we modified our original objectives to increase throughput. Specifically, our next steps are to carry out at least 1000 inoculations more per gene target.

Also, during the project, two additional novel approaches came to light – rootstock editing and Symbiont Technology. We have further modified our initial objectives to adopt these two additional approaches, to maximize the likelihood of obtaining edited vines in the next phase of the project. Rootstock editing involves generating a GMO rootstock that produces the editing reagents, engineered to be mobile within the plant. When the editing reagents move across the graft junction into a non-GMO scion, they can act there before they are degraded, allowing isolation and propagation of edited vines with no foreign DNA. Similarly, Symbiont Technology involves movement of the editing reagents from a gall induced on the vine by a partially disarmed *Agrobacterium* strain. The *Agrobacterium* is engineered to deliver the DNA needed to make the reagents in the gall. When the editing reagents move out of the gall, they can act in the surrounding tissue before they are degraded, allowing isolation and propagation of edited vines with no foreign DNA.

Over the past 3 months, we have successfully completed the initial critical tissue culture steps needed to generate GMO Chardonnay producing the mobile editing reagents for use

as a rootstock. Next steps are to regenerate plants from the tissue culture material, for propagation and future grafting experiments. To account for the possibility the reagents will preferentially move downward, we will use the GMO vine separately as scion, grafted to a non-GMO rootstock.

For the Symbiont Technology approach, we have carried out an initial set of inoculations in Thompson Seedless and in Chardonnay. We have observed robust gall formation, and, for galls on Thompson seedless (which have grown faster) we have confirmed expression of the transferred DNA. Next steps are to carry out 500 or more inoculations with editing constructs for each of the gene targets and screen for any successful editing in the vines.

Once edited vines are obtained by any of the methods being pursued, we will test them for PM resistance and characterize their growth and development

Supporting attachments: N/A

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