Project Title: A mobile genome editing system using dicistronic CRISPR/Cas9-tRNA (CC9-tR) for grapevine and other perennial crops (Non-transgenic genome editing system for grapevine) - Year 2.

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1. **Summary**: It is predicted that 50-81% of the growing acreage for perennial crops such as grapevine will fall out of production due to weather changes in the USA by 2040. This urgently demands the breeding of varieties more resilient to periods of environmental stress, without losing yield and consumer perceived quality traits. Perennialism imposes protracted and costly breeding programs (25-30 years) due to lengthy evaluation periods in each breeding step. Moreover, the of use wild species to introgress stress resistance traits into domestic gene pools, often results in poorer quality properties. Genome Editing (GE), particularly the use of CRISPR/Cas9 transgenic constructs, has revolutionised our ability to modify the genome in a targeted manner. This has resulted in the discovery of genes in non-model species, broadened our knowledge of the genetic basis of yield, stress/disease tolerance and fruit quality in perennial crops, and accelerated the breeding programs of many crops in a targeted manner. However, the implementation of GE in grapevine and other perennial crops faces multiple bottlenecks including: Somaclonal variation; lack of efficient *in-vitro* culture and transformation methods in recalcitrant perennial crops; need of sexual crosses for removal of GE and reporter/selection genes; and industry reluctance to genetic modification.

We propose a genome editing system based on the delivery of DNA-free editing constructs from genetically modified donor tissues grafted to tissues of the cultivar/species of interest. Our proposed GE method capitalizes on a recently discovered ability of certain tRNAs to act as signal for systemic movement of dicistronic RNAs. Additionally, we hypothesize that the addition of an RNA methylation signal in the newly designed transcripts will increase their mobility. To test this hypothesis, we have generated 7 seven different constructs and transformed *Arabidopsis thaliana* for a rapid evaluation of their mobility.

In brief, the main results generated during 2024 include:

1. Grapevine genotypes can effectively be transformed both *in-vitro* and *in-vivo* with constructs expressing mobile dicistronic transcripts.
2. Dicistronic transcripts are translated into functional proteins both in the transgenic and the non-transgenic tissues of the plant.
3. Grapevine cuttings with roots transformed with constructs expressing mobile dicistronic Gene Editing genes targeting the grapevine phytoene desaturase (PDS) gene present the expected phenotype in non-transformed leaf tissue. ***Indicating that mobile Gene Editing is possible in grapevine.***

2. **Annual or Final Report**: This is a California Grape Rootstock Research Foundation (CGRRF) annual report.

3. **Project Title**: A mobile genome editing system using dicistronic CRISPR/Cas9-tRNA (CC9-tR) for grapevine and other perennial crops (Non-transgenic genome editing system for grapevine) Year 2. **Proposal number:** 2023-2653.

4. **Principal Investigator/Cooperator(s)**:

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Cooperators:

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5. **Objective(s) and Experiments Conducted to Meet Stated Objective(s)**:

Objective 1 - Validate the mobility of native and artificial dicistronic mRNA:tRNAs in model plants and grapevine, was divided into two different phases:

Phase II - <u>Validation of mobility, identification of sink-acting cells, and intracellular localization of artificial dicistronic constructs</u>

1. **Validation of a transgenic dicistronic transcript mobility in *in-vitro* grown grapevines.**

To assess the mobility of a transgenic dicistronic transcript, *Agrobacterium rhizogenes* strain Arqua-1 was transformed with a plasmid containing a dicistronic ΔGUS:tRNA construct (Zhang et al., 2016). *In-vitro* grown *Cabernet Sauvignon* (CabSav) plantlets were micropropagated and transformed using a green cutting approach. Briefly, single-node cuttings were immersed in 0.5X MS liquid medium supplemented with 100 µM acetosyringone and inoculated with the transformed *A. rhizogenes*, ensuring that the node or any newly emerging tissue were never in contact with the inoculation medium. The cuttings were then transferred to solid MS medium containing cefotaxime and timentin to eliminate *Agrobacterium*, and incubation continued until root production. Successfully transformed roots were identified based on growth from the inoculation site, whereas newly emerging vegetative shoots were considered non-transformed.

To validate transformation efficiency and assess dicistronic ΔGUS:tRNA transcript mobility, GUS staining was performed on three different tissue types: callus **(Fig. 1A-B)**, root **(Fig. 1C-D)**, and leaf tissue **(Fig. 1E-F)**. Light microscopy analysis confirmed GUS-positive staining in all three tissues, indicating:

1. Successful *in-vitro* transformation of grapevine using *A. rhizogenes* Arqua-1.
2. More importantly, that transgenic dicistronic transcripts are mobile and functionally translated into proteins in grapevine.

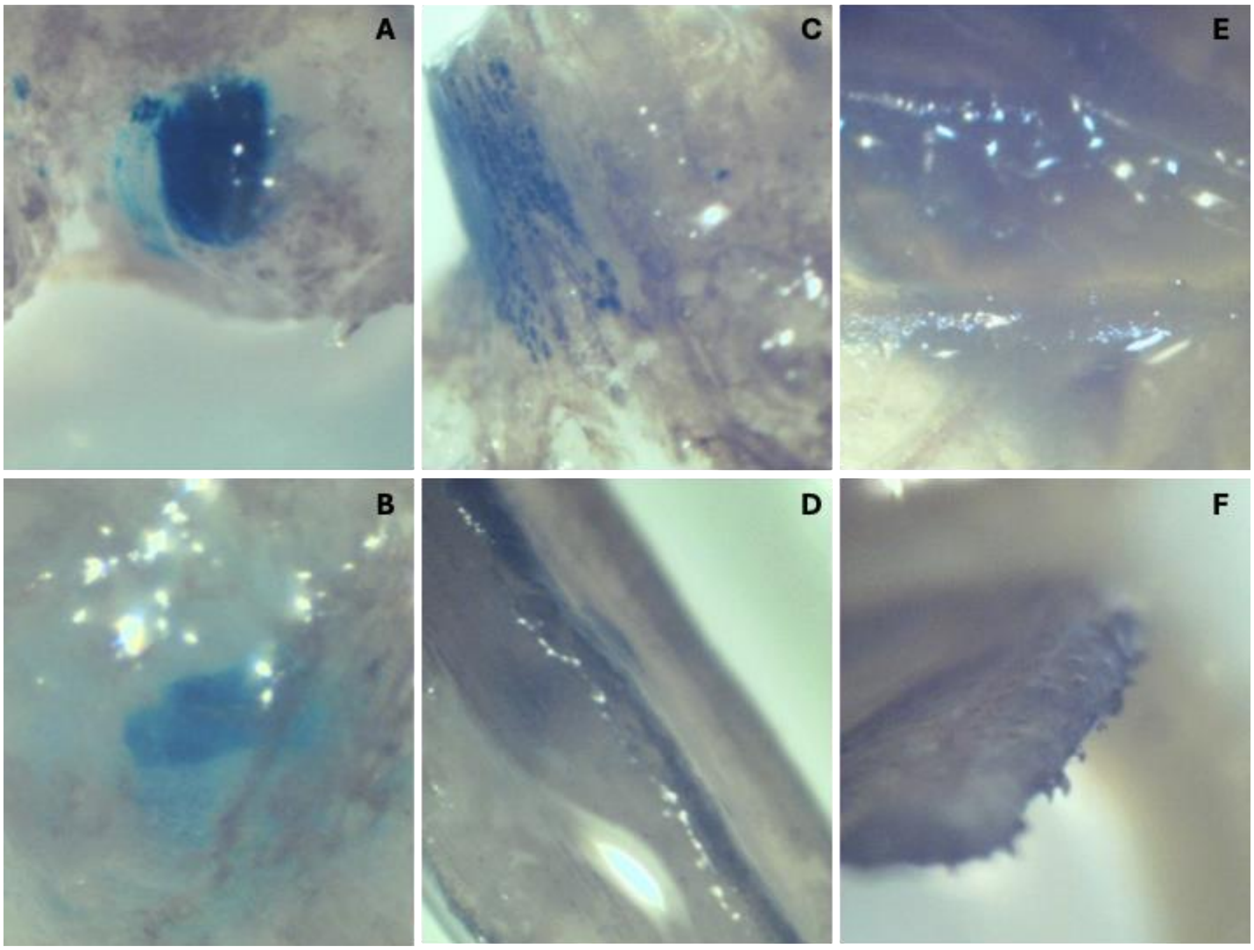

Fig 1. Dicistronic ΔGUS:tRNA transcripts are mobile from transgenic roots to wild-type shoots in in-vitro maintained grapevine plants: *Light microscopy images of GUS stained* ***A-B)*** *ΔGUS:tRNA^{+} transgenic callus,* ***C-D)*** *ΔGUS:tRNA^{+} transgenic roots,* ***E-F)*** *ΔGUS:tRNA^{-} wild-type leaves.*

On going work: Total RNA will be extracted from the apical meristems of the WT component of the grafts. Movement from transgenic to WT tissues of GUS-tRNA fusion transcripts will be tested using RT-PCR. Primers will be designed to amplify fragments spanning the tRNA and the mRNA of the reporter gene. Translation of the reporter gene will be tested and quantified both in planta and in protein extracts.

Objective 2 - Design polycistronic CC9-tR constructs with all the necessary machinery for a functional mobile GE system.

Objective 3 - Confirm the mobility and translation of CC9-tR constructs in model plants and in grapevine.

Phase III - <u>Design and validation of mobility and translation of polycistronic CC9-tR constructs</u>

1. Design and assembly of novel CRISPR/Cas9 mobile constructs with potentially enhanced mobility in grapevine. Plasmids were designed to contain all the necessary genes for gene editing (Fig. 2), including two guiding RNAs targeting the phytoene desaturase (PDS) gene, and zCas9 gene. Because PDS deficiency leads to a visible bleaching phenotype, the PDS gene has been selected as a target gene for CRISPR/Cas9-mediated genome editing in many plant studies. *V. vinifera* is a diploid plant, and bi-allelic mutations in the PDS gene change the leaf color from green to white. All three plasmids only differed in the addition of TLS mobility signals to each of the transcripts (Fig 2). Three *A. rhizogenes* K599 lines where transfected with the built plasmids.

Line 1 was transfected using plasmid PTsSSI (Fig. 2A). All transcripts expressed by this plasmid contain TLS mobility signals (i.e., both guiding RNAs, and the zCas9 transcripts). Therefore, a mutant PDS phenotype would be expected both in transformed and non-transformed vegetative tissue. Additionally, all three transcripts are expected to be found in both transformed and non-transformed tissues (Fig. 2A).

Line 2 was transfected using plasmid PTsSSI-one TLS (Fig. 2B). Only guiding RNAs transcripts expressed by this plasmid contain TLS mobility signals. A mutant PDS phenotype would be expected in transformed vegetative tissue but not in the non-transformed vegetative tissue. All three transcripts are expected to be found in the transformed tissues, but only the guiding RNAs transcripts are expected in non-transformed tissues (Fig. 2B).

Line 3 was transfected using plasmid PHSE401 SS2 (Fig. 2C). None of the transcripts expressed by this plasmid contain TLS mobility signals. A mutant PDS phenotype would be expected in transformed vegetative tissue but not in the non-transformed vegetative tissue. All three transcripts are expected to be found in the transformed tissues, but not none of transcripts are expected in non-transformed tissues (Fig. 2C).

Efficient transfection was validated using selective medium and PCR using primers specific for each construct.

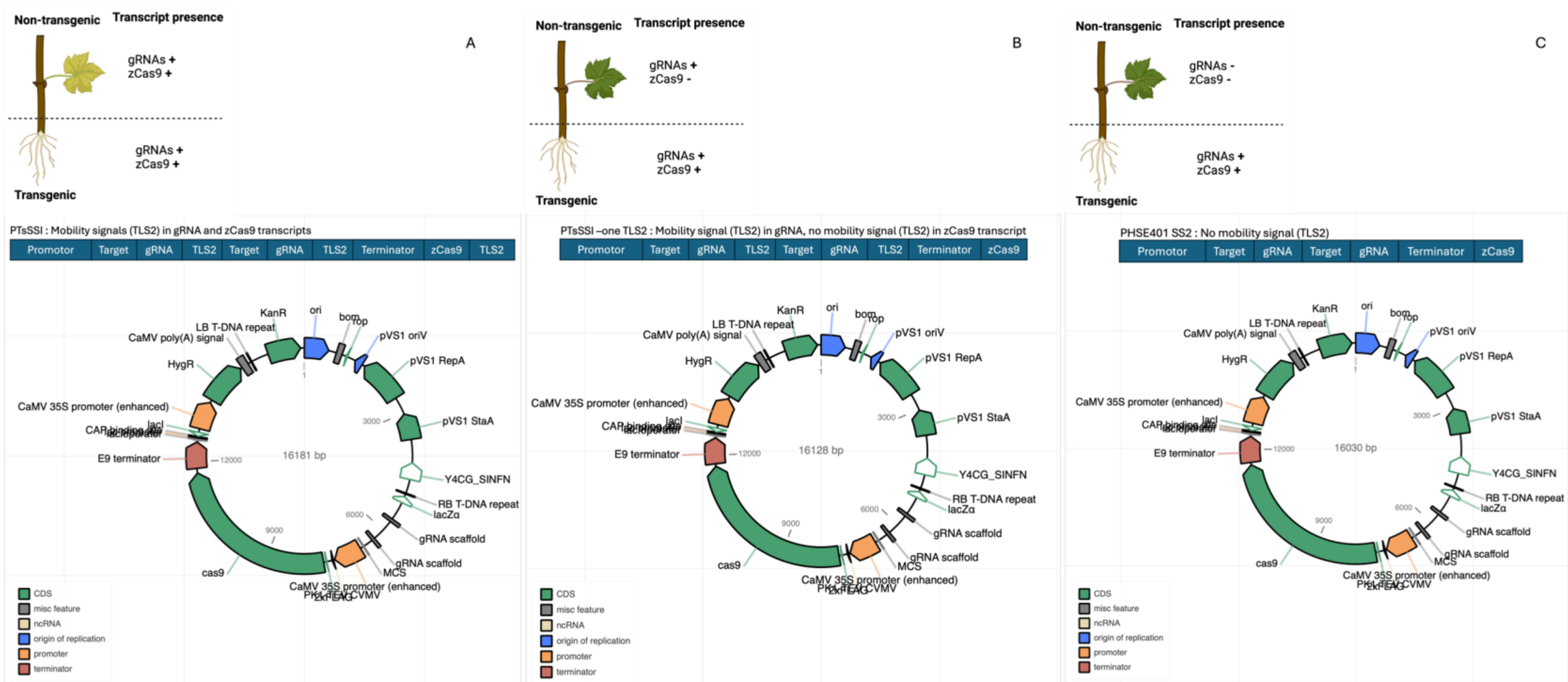

Fig. 2: Diagram and maps of plasmids used for A. rhizogenes K599 transfection: *From top to bottom each panel shows: Expected phenotype of transformed grapevine cuttings, and which transcripts will be present (+) or absent from root and leaf tissue.* ***A)*** *PTsSSI plasmid All transcripts expressed by this plasmid contain TLS mobility signals.* ***B)*** *PTsSSI-one TLS plasmid. Only guiding RNAs transcripts expressed by this plasmid contain TLS mobility signals.* ***C)*** *PHSE401 SS2 plasmid. None of the transcripts expressed by this plasmid contain TLS mobility signals.*

2. **Development of a novel method of genetic transformation in grapevine that do not require *in-vitro* culture.** A grapevine transformation system that does not require *in-vitro* culture or aseptic conditions was implemented using two different source materials. First, a seedling-based system was tested (Fig. 3). Briefly, seeds were treated using 5000ppm GA as described by Bolt et al 2023. This approach allows for accelerated grapevine seed germination without the need for a protracted stratification treatment. The root of the stablished seedlings was then removed using a scalpel, and the lower end of the hypocotyl was immersed into *Agrobacterium rhizogenes* K599 cultures transformed with the three plasmids described above. Resultant cuttings were then transferred to pots filled with vermiculate and allowed to root. Secondary roots generated from cuttings will then be used as rootstocks to be grafted with wild type (WT) scions to test the mobility of our constructs. However, due to the low rate of cuttings producing secondary roots (Table 1), the approach was abandoned.

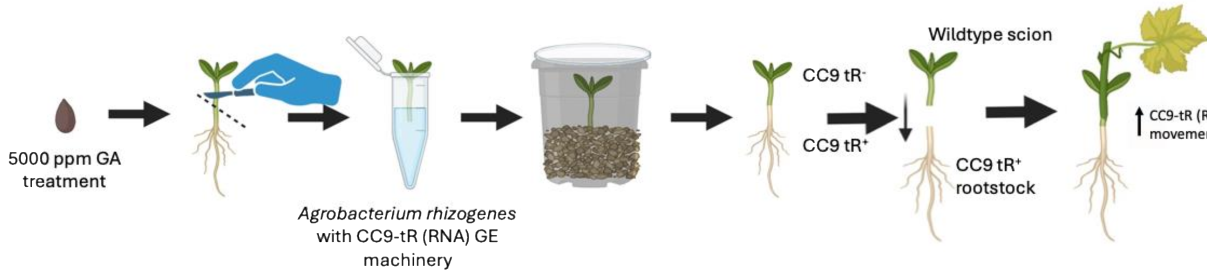

Fig. 3: Proposed methodology for grapevine genetic modification in-vivo using seedlings as transformation material.

Then, a hardwood cutting system was tested. In brief, cuttings containing two dormant nodes were generated from vines kept in pots under greenhouse conditions (Fig. 4). A blade was used to generate cuts on the lower part of the cutting (including the bottom node). Then, cuttings were immersed to the bottom node into *Agrobacterium rhizogenes* K599 cultures transformed with the three plasmids described above. In this case, a high percentage of transformation events resulted in the production of secondary roots (Table 1). Those below the immersed area were expected to be transformed with the constructs described above. Cuttings were then placed in vermiculite in a mist tunnel at 24°C and with supplementary light to break dormancy. The number of plants showing vegetative growth was recorded.

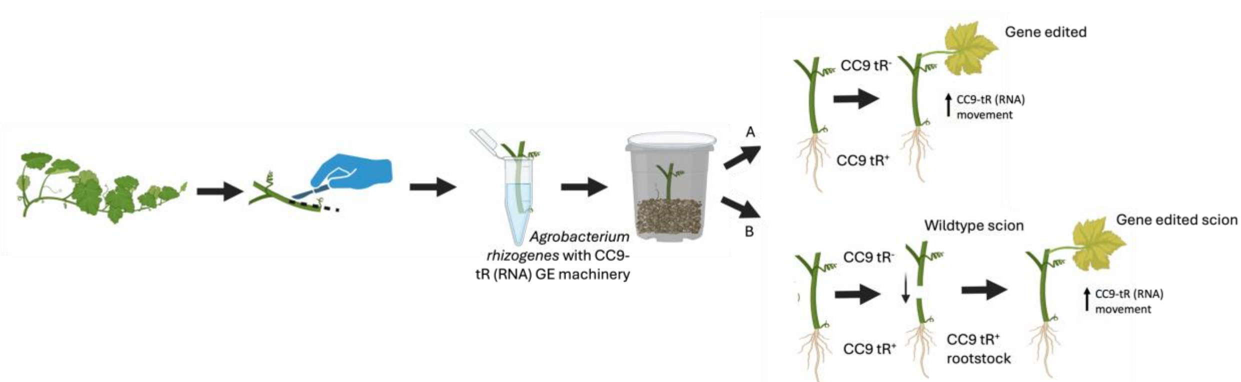

Fig. 4: Proposed methodology for grapevine genetic modification in-vivo using hardwood cuttings as transformation material. *After A. rhizogenes transformation and cutting rooting, the mobility of the inserted dicistronic transcripts is tested using two systems.* ***A)*** *Edited phenotype*

will be monitored in vegetative growth generated from the non-transformed node of the cutting; and ***B)*** *transformed roots from transformed cuttings, positive for the mobile gene editing machinery (CC9tR$^+$), will be grafted to wild type scions, negative for the mobile gene editing machinery (CC9tR$^-$), and monitored for the edited phenotype.*

Thirty three percent of cuttings transformed with plasmid PTsSSI (i.e., containing mobility signals in both the guiding RNA transcript, and the zCas9 transcript) showed the expected gene edited PDS gene phenotype (Fig. 4a, Table 1, and Fig 5). As expected, none of the cuttings transformed with plasmids PTsSSI-one TLS (i.e., containing a mobility signal only the guiding RNA transcript, but not in the zCas9 transcript), or with PHSE401 SS2 (i.e., without mobility signal in both transcripts) show evidence of the expected mutant phenotype. These results suggest that this approach can be used to 1) Obtained chimeric plants with genetically modified roots with transformed with the CRISPR/Cas9 machinery, and non-genetically modified vegetative growth, and 2) that the CRISPR/Cas9 is mobile and capable of gene editing on the new growth from the non-genetically modified node.

On going work: We are currently validating these results using real time PCR and real-time RT-PCR to prove the insertion of the Gene Editing machinery in the root tissue, and the presence of Gene Editing transcripts in the leaf tissue.

Table 1: Summary of grapevine transformation/gene editing methods results.

Plasmid	Tissue type	Expected mutant phenotype	Transformed cuttings	Rooted cutting	Vegetative growth	% Rooted	% Vegetative growth	% mutant phenotype
PTsSSI	Seedling	Yes	14	1	0	7%	0%	0%
PHSE401 SS2	Seedling	No	13	3	1	23%	8%	0%
PTsSSI –one TLS2	Seedling	No	12	0	0	0%	0%	0%
PTsSSI	Hard wood	Yes	30	28	6	93%	20%	33%
PHSE401 SS2	Hard wood	No	47	45	35	96%	74%	0%
PTsSSI –one TLS2	Hard wood	No	37	21	28	57%	76%	0%

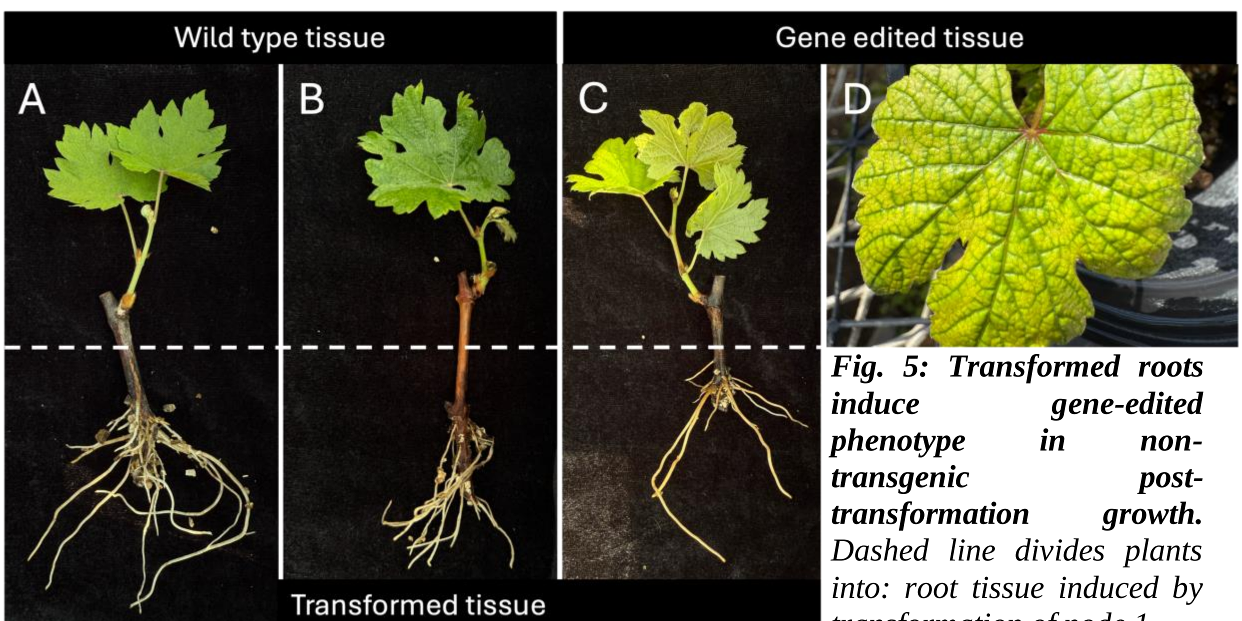

Fig. 5: Transformed roots induce gene-edited phenotype in non-transgenic post-transformation growth. *Dashed line divides plants into: root tissue induced by transformation of node 1*

*using A. rhizogenes, and vegetative tissue grown from node 2 after transformation. **A** double negative control cutting transformed with non-mobile gene editing constructs (PHSE401 SS2), **B** single negative control cutting transformed with mobility signal in gRNA and no mobility signal in zCas9 transcripts (PTsSSI –one TLS2), and C cutting transformed with mobile gene editing construct (PTsSSI). **D** Leaf showing expected phenotype after knock-out of PDS gene via gene editing.*

3. Structural characterization of novel CRISPR/Cas9 mobile constructs with potentially enhanced mobility. RNA-fold software was used to predict folding structures of the dicistronic gRNA:TLS and zCas9:TLS transcripts designed to include the enhanced mobility signal for RNA methylation (S2 hereafter) described in 2023 report. In summary, the two main findings are 1) Addition of the S2 signal does not affect the overall predicted folding of dicistronic transcripts (Fig 6), and 2) that the S2 signal folds similarly to a $TLS^{Met\text{-}\Delta DT}$ (Zhang et al 2016) (Fig 6), which has been reported to induce mobility of dicistronic transcripts. This suggests that the S-2 could be improving mobility not only because it is a target for RNA methylation but also, it could act as a de-facto TLS (mobility signal).

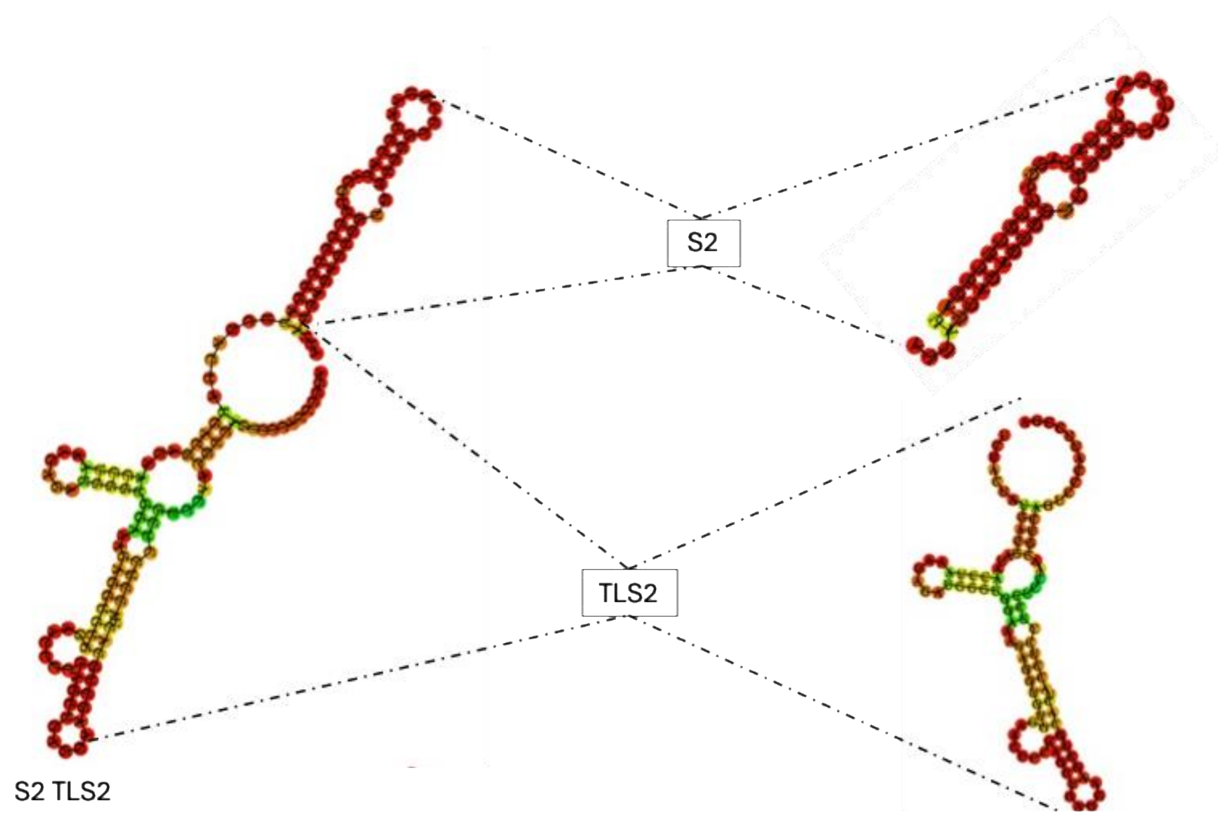

Fig. 6. Predicted folding of dicistronic construct containing and S2 and a TLS sequence. *Predicted folding of transcripts containing both sequences (S2 TLS2) and of the independent sequences (S2) and TLS2, showing identical folds in both conditions.*

4. Generation of *A. thaliana* transgenic lines containing dicistronic constructs with predicted enhanced mobility. 32 *A. thaliana* lines were generated by transforming Col-0 plants with seven different constructs expressing zCas9 from an estradiol-inducible promoter and two NIA1-targeting gRNAs (*gNIA1*) driven by the constitutive Pol-III promoters *U6-26* and *U6-*

29. Three lines per transformation event were selected for further experimentation. T2 seeds from all the 6 out of the seven transformation events were grown in selective media containing 0.5 MS salts, 1% sucrose, and 1% micro agar, and 10ug/mL Hygromycin, 10ug/mL Hygromycin/50ug/mL Kanamycin, and 50 ug/mL Kanamycin to select for transformed plants (Fig. 7). Selection success ranged from 0 to 97% (Table 2). Plants selected for resistance to both antibiotics were kept to generate T3 lines and for further analysis.

On going work: The effect on mobility and editing efficiency of the S2 signal is currently being analyzed. To test this, T3 plants containing the seven constructs will be used to generate reciprocal hypocotyl micrografts with wild type plants using a silicone chip. Then the inducible transgenes will be activated by growing the micrografted plants in new plates (0.5 MS salts, 0% sucrose, 1% micro agar (Duchefa Biochemie), and 5 µM estradiol) and vertically grown in short-day conditions. The presence of the expected gene edited phenotype will be recorded and 21 days after grafting plants will be harvested from plates for RNA and DNA isolation to estimate the abundance of mobile transcripts in transgenic and wild type tissues and the percentage of gene edited cells per tissue using RT-PCR.

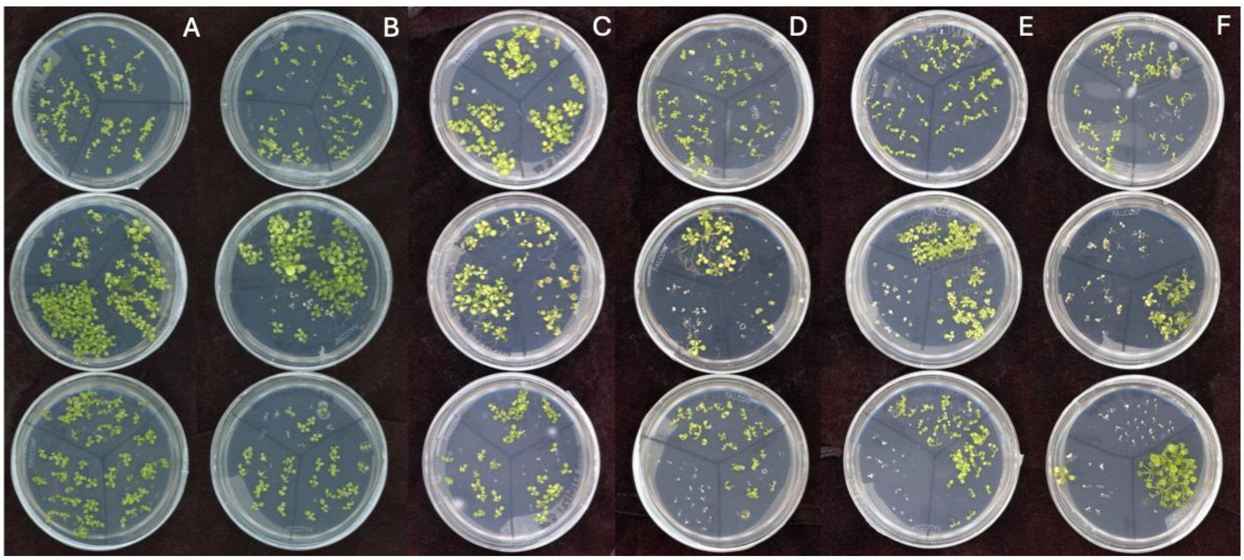

Fig 7: Antibiotic selection of T2 A. thaliana lines transformed with estradiol-inducible Cas9:tRNA and two NIA1-targeting gRNAs (gNIA1:tRNA) driven by the constitutive Pol-III promoters U6-26 and U6-29 with and without S2 sequences. *From top to bottom on the images is 10ug/mL Hygromycin, 10ug/mL Hygromycin/50ug/mL Kanamycin, and 50 ug/mL Kanamycin. A. thaliana lines A) P401-9, P401-11, and P401-15; B) P400-7, P400-9, and P400-20; C) P2191-10, P2191-14, and P2191-4; D) P2190-4, P2190-8, and P2190-10; E) P399-1, P399-4, and P399-6; F) P402-6, P402-8, and P402-16.*

Table 2: Selection of T2 A. thaliana transformed lines with dicistronic gRNA:tRNA and zCas9:tRNA constructs with or without the S2 RNA methylation signal. *Selection success is shown for seeds grown in media containing 10ug/mL Hygromycin, 10ug/mL Hygromycin/50ug/mL Kanamycin, and 50 ug/mL Kanamycin. Mobile transcript design column shows the conceptual diagrams with the relative position of each sequence for each construct. Two or three lines were selected for each construct for analysis. Lines E850-2 and E850-5 had not been tested at the time of this report.*

Line	10ug/mL Hygromycin			10ug/mL Hygromycin - 50ug/mL Kanamycin			50ug/mL Kanamycin			Mobile transcripts design
	Germinated seeds	# Selection success	% selection	Germinated seeds	# Selection success	% selection	Germinated seeds	# Selection success	% selection	
P401-9	44	35	79.5%	43	39	90.7%	52	44	84.6%	S2 Cas9 TLS2 + NIA1 TLS2 S2
P401-11	35	24	68.6%	25	21	84.0%	37	34	91.9%	
P401-15	19	15	78.9%	26	22	84.6%	32	31	96.9%	
P400-7	30	27	90.0%	24	21	87.5%	22	17	77.3%	S2 Cas9 TLS2 + NIA1 TLS2
P400-9	25	20	80.0%	27	23	85.2%	23	18	78.3%	
P400-20	22	3	13.6%	20	10	50.0%	22	11	50.0%	
P2191-6	15	12	80.0%	16	14	87.5%	17	12	70.6%	Cas9 S2 TLS2 + NIA1 TLS2 S2
P2191-10	22	10	45.5%	23	15	65.2%	17	13	76.5%	
P2191-14	23	11	47.8%	31	27	87.1%	15	11	73.3%	
P2190-4	17	7	41.2%	20	15	75.0%	29	22	75.9%	Cas9 S2 TLS2 + NIA1 TLS2
P2190-8	17	1	5.9%	32	21	65.6%	22	0	0.0%	
P2190-10	14	3	21.4%	14	8	57.1%	16	12	75.0%	
E850-2	-	-	-	-	-	-	-	-	-	Cas9 TLS2 S2 + NIA1 TLS2 S2
E850-5	-	-	-	-	-	-	-	-	-	
P399-1	26	17	65.4%	30	22	73.3%	38	24	63.2%	Cas9 TLS2 S2 + NIA1 TLS2
P399-4	25	0	0.0%	19	13	68.4%	19	0	0.0%	
P399-6	33	27	81.8%	26	24	92.3%	40	36	90.0%	
P402-6	21	0	0.0%	33	30	90.9%	32	0	0.0%	Cas9 TLS2 + NIA1 TLS2
P402-8	15	0	0.0%	17	15	88.2%	12	1	8.3%	
P402-16	12	7	58.3%	20	16	80.0%	13	11	84.6%	

7. **Outside Presentations of Research**: Graduate student Bridget Bolt presented part of these results during the Plant and Soil Science Graduate Program Annual Symposium.

To achieve a widespread dissemination, we will deliver the projects outputs via a range of mechanisms, including local seminars, national and international conferences, updates in industry journals, final report, scientific papers and intellectual property protection. A particular effort will be put into presenting the results of this work during professional meetings such as the International Propagators' Society meeting, The American society of viticulture and enology, etc. Sequencing data will be deposited into online public databases, and thus will be freely available to others. For the dissemination of the results to the general public, media releases will be written with assistance from the Mass Media & Communication team at UKY. In addition, the PI, and collaborators will update their social network platforms regularly to promote publications and achievements of the project. Any public engagements or awards that manifest from the project will be put on the College of Agriculture, Food and Environment UKY website. Finally, we propose the creation of an international repository of CC9-tR rootstocks that can be used by the research and industry communities for the development of gene edited perennial crop commercial lines.

8. **Research Success Statements**: Although the results presented here are only preliminary they clearly indicate that the development of ***that a non-transgenic editing system based on the mobility of dicistronic mRNA:tRNA transcripts is possible in grapevine***. We believe that the results from this innovative proposal will help accelerating the breeding of grapevines, and other perennial crops, by developing, validating and applying a novel Genome Editing system that will generate non-transgenic lines, and circumvent the need for *in-vitro* culture and sexual crosses. This will allow the breeding of varieties more resilient to periods of environmental stress, without losing yield and consumer perceived quality traits. Finally, CC9-tR lines developed during this project will constitute the seed for a repository to be created with further funding. Such collection will be available to future research/breeding programs.

9. **Funds Status**: In November 2023 funding for the second year of proposed research was announced by the California Grape Rootstock Research Foundation. Due to delays in the agreement signing by the University of Kentucky, and to our consequent inability to hire project personnel (Graduate Student Researcher) until the funds were made available by the University of Kentucky, during the first year of the proposal, a six-month extension was requested to accommodate for the delay in availability of funding. To date, a total of $30,470 have been spent from the budgeted $ 69,910 as described on the table below. A new six-month extension has been requested for the 2023-2024 proposal.

	Budgeted 2023-2024	**Cumulative Expenses**
Personnel	$30,170	$17,369
Supplies and expenses	$39,740	$13,101
Total	**$69,910**	**$30,470**