Applying CRISPR-Cas9 Gene Knockout Technology to *Arabidopsis*

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One of the most exciting and emergent technologies in the life sciences is CRISPR-Cas9 or Clustered Regularly Inter-Spaced Palindromic Repeats using the CRISPR associated protein 9. This technology allows researchers to engineer an organism's genome in ways never before possible by introducing specific breaks into the DNA which can be used for gene inactivation (gene knockout) or gene editing (1). This project was aimed at beginning to integrate this cutting edge technology into research at Hampden-Sydney by applying CRISPR gene knockout to *Arabidopsis thaliana.* This is a small flowering plant and well-accepted model organism because of its relatively small genome and the fact that it was the first plant to be sequenced (2). The first step of this project was to select genes of interest from the genome of *Arabidopsis* that will have interesting effects on the plants phenotype that will be easy to determine early in the plants life. Some potential genes of interest include ones related to drought resistance, disease resistance, and reproduction as these are all areas of new and relevant research in plant biology (2). This was done through a literature search and research into prior work done by Hampden-Sydney and Randolph Macon College. Eight plasmids were chosen which had been used in previous experimentation without or with limited success (Fig. 1).

Gene	Gene accession	Class 0.0 gRNA seeds	Comments
pgm	At5g51820	3	Fail to produce starch; phenotype = reduced gravity perception
hy5	At5g11260	2	Phenotype = elongated hypocotyl; might be lethal in knockout
im	At4g22260	2	Phenotype = green/white variegation; has flowering defects, might be delayed
arc3	At1g75010	1	Phenotype = chloroplast division defect; has 7-8x fewer chloroplasts per cell, ones that it has are bigger
wer1	At5g14750	0 (40 class 1.0)	Phenotype = plant makes extra root hairs
gl1	At3g27920	0 (39 class 1.0)	Phenotype = lacks trichomes on leaves (i.e. hairless); unclear if knockout is lethal
tmm-1 (too many mouths)	At1g80080	8	Phenotype = stomata numbers increased, in clusters; pronounced on cotyledons; might display glabrous phenotype, too
scr	At3g54220	2	Phenotype = shorter roots

Fig. 1 The eight genes that were chosen for the project along with their accession numbers and description of the expected phenotype (Obtained from Dr. Nicholas Ruppel)

The plasmids were obtained from Sigma Aldrich and PCR was used for verification of the plasmids. The adult plants were then transformed via floral dip and the seeds were then collected. The dipped seeds were then planted on both hygromycin and control plates and observed for growth. The plants with the plasmid containing *Agrobacterium* incorporated into them are expected to show the phenotypical differences outlined in Figure 1 when compared to the control plants.

Arabidopsis seeds were cold-treated for three days at 4°C in water. The seeds were then planted in soil using a pipette to distribute seeds to each of the corners of the pots. After initial growth appeared, seedlings were thinned by hand to two plants per pot. Plants were grown under growth lights until proper size. The *agrobacteria* colonies containing the plasmids were transferred to conical tubes containing LB broth and were rotated at room temperature for three days or until bacteria growth was present. Plasmids were confirmed by colony PCR. 2uL of template were combined with 2uL of dH_2O, 10uL of master mix, and 3uL each of the forward primer 5'-ATCTCAAAATTCCGGCAGAAC-3' and the reverse primer 5'-CCATCCAATCACTACTTCGTCTC-3'. These were then run at 95° C for 2 minutes for initial denaturation; 30 cycles of 95° C/30 seconds, 60° C/30 seconds, and 72° C/ 30 seconds; 1 cycle of 72° C/5 minutes. The results were then visualized in a 1% agarose gel using EZ Vision and run at 120V. A dipping solution was made using 5% sucrose + 10 mM $MgCl_2$ in sterile distilled water. 45 milliliters of each *Agro* culture was poured it into conical tube and centrifuged at 2500 rpm. The supernatant was poured off and an additional 45 mL of *Agro* culture was poured on top of the pellet. Centrifugation was repeated and the supernatant poured off. A small volume of dipping solution (~10 mL) was poured in and the pellet was resuspended.

140 milliliters of dipping solution was then poured into a glass bowl and the re-suspended *Agro* solution was added along with 75 microliters Silwet L-77 and mixed. The floral parts of the *Arabidopsis thaliana* plants were briefly submerged (10 sec) in the bacterial suspension. The pots were placed on their side in a flat and covered with a lid and placed under growth lights. Once the plants had seeded, the seeds were collected and then washed with 70% EtOH for 2 minutes, then 50% bleach for 5-10 minutes, and then washed with sterile dH_2O to completely remove bleach.

To make the growth media, 1.11 grams of MS with Vitamins was added to 1 liter of distilled water along with 1 gram of MES buffer and stirred until in solution. The pH was titrated to 5.6 with potassium hydroxide and then 15 grams of

Phytoblend agar was added and the solution was autoclaved on a liquid setting. The solution was cooled and 1 mL of hygromycin was added to the solution for the non-control plates. The plates were then poured and allowed to solidify overnight. The seeds were evenly distributed onto the labeled plated and placed under growth lights.

The initial growth of the *Arabidopsis* plants went well and 54 viable plants were grown allowing for 6 plants to be dipped for each plasmid plus one set of six as an undipped control. The eight selected plasmids were successfully incorporated into *Agrobacteria* colonies which was confirmed by PCR and electrophoresis. The gel showed single bands around 350 bp for all plasmids which confirmed that the *Agrobacterium* clones contain the CRISPR Cas-9 plasmid (Fig 2).

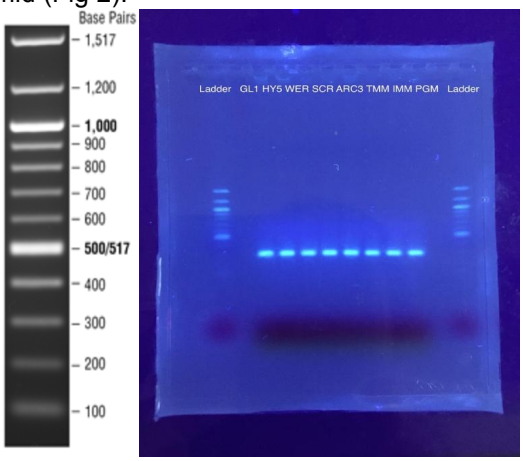

Fig. 2 The agarose gel visualization of the PCR confirmation of the plasmids, bands at ~350bp confirm that the *Agrobacterium* clones contain the CRISPR Cas-9 plasmid.

The plants were dipped into the *Agrobacterium* solution, allowed to seed, and then sterilized and plated on both hygromycin and control plates. After five weeks of growth time only the non-hygromycin control plates showed any significant growth while the hygromycin plates showed no growth at all.

The goal of this project was to transform the phenotypes of *Arabidopsis* via eight different CRISPR plasmids. The initial results of the PCR confirmation were encouraging as they showed successful incorporation of the plasmid into the *Agrobacterium* in all eight cases. However, the project ultimately did not perform as expected due to no non-control plants grew after the floral dip and transformation. This failure of the plants to grow could be due to a number of factors which would cause the resistance to hygromycin to not be incorporated into the seeds and thus preventing growth. This could be a problem with getting the plasmid into the seeds so that the transformation can occur or it could be due to the plasmids themselves being defective. A colleague at Randolph Macon working on the project in tandem also failed to get any growth of the transformed plants using the same plasmids so it appears to be a problem with the plasmids themselves instead of the technique.

Future experimentation will focus on repeating the experiment using different plasmids, ones incorporating BAR selection instead of hygromycin. This will be repeated on a select number of the plasmids and will eliminate the growth media and allow T1 growth simply in soil. At the same time, design will be done on new targets for the GL1 and HY5 plasmids as the previous targets were in introns and the chances of success are better if an exon is targeted. Additionally, research will be done into potential new targets that can be used in addition to the current gene targets that have been selected.

If this method is successful, it can be used to investigate single disruption lines that are being collected as a part of the unPAK (Undergraduate Phenotyping of *Arabidopsis* Knockouts) program whose focus is on understanding the phenotypes and the genome of *Arabidopsis* (3).

REFERENCES

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