Alteration of Arabidopsis thaliana gene using CRISPR/Cas9 to establish a thigmonastic defence mechanism for conservation of biodiversity

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ABSTRACT

Thigmonasty is defined as the nastic response of a plant or fungus to mechanical stimulation such as touch or vibrations. These thigmonastic movements are used as a defense in some plants. A common example of a thigmonastic plant would be the *Mimosa pudica* which has sensitive leaves which would rapidly close in response to any mechanical stimulation including direct touch, vibration, electrical and thermal stimuli. This would significantly reduce the surface area available to herbivores and may appear less attractive and appealing to them. Also, it may physically dislodge small herbivores such as insects that may have been feeding on it. Studies suggest that the reason for this thigmonastic response is the presence of proton pumps. Recently, the simplest, most precise and versatile genome editing tool, CRISPR/Cas9 was developed. With the increased predictability of genetic manipulation enabled by CRISPR/Cas9, foreign genes could be introduced to a plant with ease. In this research work, a foreign gene coding for a proton pump, Plasma membrane ATPase 1 (PMA1) from *Saccharomyces cerevisiae*, was introduced into the chosen plant, *Arabidopsis thaliana*, in a region of high gene expression. A series of tests were then conducted on the transgenic plant to examine the level of expression of the foreign gene and measure the magnitude of its thigmonastic response. A change in turgor pressure in the leaves would suggest that the transfection of PMA1 into the *Arabidopsis thaliana* plant was successful and that a thigmonastic response was successfully introduced to it as an effective defence mechanism against herbivory.

1. INTRODUCTION & LITERATURE REVIEW

One in five of the world's plant species is threatened with extinction putting supplies of food, medicines, materials and gene sources at risk (KewScience, 2016). The estimated rate of ongoing extinction (18–26 extinctions per million species years (E/MSY)) is up to 500 times the background extinction rate for plants (0.05–0.35E/MSY13). However, previous studies have suggested current extinction rates closer to 1,000 times the normal turnover rate with others predicting that rates will soon be 10,000 times faster (Humphreys, Govaerts, Ficinski, Lughadha, & Vorontsova, 2019). Thus, this will undoubtedly pose as a serious issue in the future.

Plants have a wide range of evolved adaptations which improve their survival and reproduction by reducing the impact of herbivores. Within the topic of plant adaptation, one prominent field of research is plant gnosophysiology which is defined as the ability of plants to sense and respond to the environment by adjusting their morphology, physiology, and phenotype accordingly. This research will encompass plant gnosophysiology, more specifically electrophysiology and its effects on a thigmonastic response. The general cause of a thigmonastic response is the generation of strong electrochemical gradients which results in the passive transport of ions across plasma membranes and the concomitant water movement

driven by osmosis (Simons, 1981; Fromm & Eschrich, 1988). In the context of our given example in the abstract, this will lead to a loss of turgor from extensor cells at the pulvini, which are specialized motor organs located at the bases of the leaflets and petioles. When these extensors lose turgor, the flexor cells stretch, resulting in a touch-induced folding leaf movement. These electrochemical gradients enabling rapid ion movements are set up by proton pumps (Roblin, 1982), and *Mimosa* motor cells have an abundance of H^+ ATPase proteins (Fleurat-Lessard et al., 1997), which will accommodate for the required high pump activity for this thigmonastic response. The osmotically driven cellular water loss is extensive, with up to a 25% volume change, and rapid, occurring within 1 s (Fleurat-Lessard et al., 1997b). Such a fast and dramatic loss of water from pulvinar cells is likely facilitated through transporters such as aquaporins or solute-water cotransporters (Fleurat-Lessard et al., 1997a,b; Morillon et al., 2001; Moshelion et al., 2002). As explained earlier, this will result in lower predation pressure, serving as an effective defence mechanism against herbivory, thus increasing survival and reproduction rates, resulting in a decreased chance of being endangered.

As mentioned previously, CRISPR/Cas9 was recently developed and is currently the simplest, most precise and versatile genome editing tool typically used for scientific and industrial purposes. CRISPR/Cas9 was developed based on the function of clustered regularly interspaced short palindromic repeats (CRISPR) which is a family of DNA sequences derived from DNA fragments from viruses that have previously infected a prokaryote. CRISPR arrays will be first transcribed as a single RNA before subsequent processing into shorter CRISPR RNAs (crRNAs), which direct the nucleolytic activity of certain Cas enzymes to degrade target viral nucleic acids during subsequent infections as part of its microbial CRISPR adaptive immune system (Hsu, Lander, & Zhang, 2014). CRISPR/Cas9, on the other hand, has been developed to function slightly differently, being heavily utilized as a genome engineering tool to induce site-directed double-strand breaks in DNA. These breaks can lead to gene inactivation or the introduction of heterologous genes through non-homologous end joining and homologous recombination respectively in many laboratory model organisms (Bibikova et al., 2001, 2003). CRISPR/Cas9 consists of 3 main components which are: CRISPR associated protein 9 (Cas9), Guide RNA (gRNA), and Template DNA. Cas9 is an RNA-guided DNA endonuclease enzyme which serves to recognize protospacer adjacent motifs (PAM) and cleave target DNA sites (Cong et al., 2013; Mali et al., 2013a). The guide RNA is a specific RNA sequence that recognizes the target DNA region of interest and directs the Cas nuclease there for editing. The gRNA is made up of two parts: CRISPR RNA (crRNA), a 17-20 nucleotide sequence complementary to the target DNA, and a trans-activating crRNA (tracrRNA), which serves as a binding scaffold for the Cas nuclease. The effectiveness of the gRNA is determined based on its on-target and off-target activity which represents the specificity of the cleavage site (Zhang, Tee, Wang, Huang, & Yang, 2015). The template DNA is a DNA repair template which contains the desired sequence, typically the gene-of-interest which will enable the gene editing to be more specific and have a higher success rate of utilising homology directed repair (HDR) (Gratz, et al., 2014). The gene editing process are as follows:

The Cas9 protein and the gRNA form a ribonucleoprotein complex through interactions between the gRNA scaffold and surface-exposed positively-charged grooves on Cas9. The Cas9 then undergoes a conformational change upon gRNA binding that shifts the molecule from an inactive, non-DNA binding conformation into an active DNA-binding conformation. Cas9 will only cleave a given locus if the gRNA spacer sequence shares sufficient homology with the target DNA. Mismatches between the target sequence in the 3′ seed sequence completely abolish target cleavage, whereas mismatches toward the 5′ end distal to the PAM often still permit target cleavage. gRNA targeting sequence in this project has perfect homology to the target DNA with no homology elsewhere in the genome which increased its on-target and off-target scores (Capecchi, 1989), resulting in a highly specific Cas9 cleavage site and more accurate data collection (Tycko, Myer, & Hsu, 2016). Cas9 later undergoes a second conformational change upon target binding that positions the nuclease domains, called RuvC and HNH, to cleave opposite strands of the target DNA (Nishimasu et al. 2014; Gorecka et al. 2013). The end result of Cas9-mediated DNA cleavage is a double-strand break (DSB) within the target DNA (∼3-4 nucleotides upstream of the PAM sequence). Homology directed repair then anneals template DNA and the ends of the double-stranded breaks of the target DNA together (Ceccaldi et al., 2016, Pâques and Haber, 1999).

Therefore, this project successfully introduced a thigmonastic response to the *Arabidopsis thaliana* plant that will be triggered by the generation of an electrochemical gradient on touch (Mousavi, Nguyen, Farmer, & Kellenberger, 2014) with the use of the CRISPR/Cas9 gene editing technology, allowing it to be used as an effective defence mechanism against herbivory. This method of increasing the survivability of plant species was aimed to be cost-efficient in the long run as other methods including preservative banks and man-made protections prove to be unfeasible in situ and is in large demand of materials and space. It was also aimed to preserve the aesthetics and wonder of nature (Engelmann, 2010). With this, the chances of the plant species being endangered will be lowered.

2. PROPOSED SOLUTION

In this project, the main goal is to successfully introduce a thigmonastic response to the *Arabidopsis thaliana* plant with the use of the CRISPR/Cas9 gene editing technology. However, the SRC did not have the required resources so there was a need to design the CRISPR components and extract the gene of interest manually from *Saccharomyces cerevisiae.* Other resources that were bought will be listed below.

Brief Overview of Procedures

- Designing of CRISPR components
- CRISPR/Cas9 gene editing process
- Test for recombinant DNA
- Test for changes in turgor pressure on touch

The project will be carried out as shown in the overview above, in which the relevant data sets will be collected to be used for the evaluation of the effectiveness of the solution design.

3. MATERIALS AND METHODS

Materials required for this project include

- SpCas9 EQR variant
- Synthesized gRNA
- *Arabidopsis thaliana* plant
- *Saccharomyces cerevisiae*
- Yeast extract peptone dextrose (YEPD)
- 10 mM dNTP mixture
- 5 U/µl Taq polymerase
- 50 mM MgCl2
- 10 µM PCR primers
- 0.2 M lithium chloride
- 1% SDS solution
- 100 % ethanol
- 70 % ethanol
- Deionised water
- Tris
- NaCl
- 1 M HCl
- Glacial acetic acid
- 0.5 M EDTA
- Silicone oil
- Phyto Agar
- Agarose powder

Equipment required for this project include

- Autoclave
- Incubator
- Laminar flow hood
- Centrifuge
- Vortex mixer
- Thermal cycler
- UV/Vis Spectrophotometer
- 6-well plates
- Electroporation cuvette
- Casting tray
- Electrophoresis system
- Electrophoresis comb
- Electrodes
- Pressure chamber

3.1 Designing of CRISPR components

3.1.1 Source for genes not serving an important function in the plant

Currently, there is no public list of genes and its respective functions designed specifically for the ease of gene selection for gene editing. Lab protocols especially for gene editing is typically not opened for public view. Hence, there is a need to manually interrogate identified genes based on gene lists found in online gene banks such as The Arabidopsis Information Resource (TAIR) to be used for evaluation of its effectiveness.

In this project, since the plan is to introduce the gene coding for plasma membrane ATPase 1 (PMA1) from *Saccharomyces cerevisiae* into the *Arabidopsis thaliana,* it is a must to prevent compromising any plant function. So, it was ensured that the identified genes did not have metabolic functions. This is because once the gene is cleaved, it will be rendered functionless hence this selected cleavage site should be on a gene that is functionless. Some identified genes did not even serve any known metabolic or phenotypic function, thus these identified genes were favourable in our selection.

3.1.2 Check expression levels of target gene

For the best results, the chosen cleavage site should be on a target gene that is very highly expressed in its leaves. With a high level of gene expression, more protein of interest, PMA1, will be synthesized by the plant which increases the expression of its function as a proton pump, enabling rapid ion movements across the plant's plasma membrane. With the aid of some online software, expression levels for specific areas of the plant was researched on and compared accordingly to evaluate the effectiveness of the cleavage site.

With increased gene expression, the magnitude of its thigmonastic response will be higher as the gene of interest is enabled to have a greater effect on the plant. This will reduce the time required for the drooping of leaves and allow for a more efficient extent of the transient thigmonastic response. This will also provide us with clearer results for data analysis, allowing us to be able to effectively pinpoint complications that we face.

3.1.3 Find all possible target sequence upstream of the PAM sequence

The species of Cas9 ordered was the *SpCas9 EQR variant* which has the PAM sequence of 3' NGAG. As mentioned earlier, the guide RNA is a specific 20-nucleotide RNA sequence that recognizes the target DNA region of interest and directs the Cas nuclease there for editing. With the aid of the internet and online genetic engineering designing software, Benchling, we were able to efficiently source for all the possible target sequence upstream of the 3' NGAG PAM sequence.

3.1.4 Analyse the effectiveness of the gRNA sequence

There are 4 main factors that determine the effectiveness of the gRNA sequence: position of cleavage site, on-target activity, off-target activity and mutations. Regarding its position, a closer theoretical cleavage site to the 5' end of the target gene will allow the gene editing process to occur at a faster rate which substantially reduces the degradation of CRISPR/Cas9 components, thus increasing the chances of the gene edit being perfect, allowing us to obtain more accurate and reliable results. As mentioned previously, on-target and off-target activities represent the specificity of the cleavage site. On-target activity is defined as Cas9 cleavage at a desired location specified by a gRNA target sequence while off-target activity is defined as Cas9 cleavage at undesired locations due to gRNA targeting sequence with sufficient homology to recruit Cas9 to unintended genomic locations. Thus, gRNA must be specific to the target sequence. Specific gRNAs will have low off-target activity which is more favourable since off-target activity may lead to inaccurate interpretation of experimental results. So, the on-target and off-target scores were calculated based on algorithms developed by researchers (Doench & Hsu, 2016) and compared with one another to evaluate the best gRNA candidate out of the hundreds of others gRNA sequences identified. Finally, due to a double-strand break by the Cas9 protein and the insertion of our gene of interest, there is a possibility of mutations caused by a frame shift. Therefore, our gRNA sequences were checked for possible frame shift mutations which is factored into the evaluation of their individual effectiveness.

3.1.5 BLAST the target sequence

Using the Basic Local Alignment Search Tool (BLAST) provided by the National Center of Biotechnology Information (NCBI), the entire *Arabidopsis thaliana* genome was sourced for a genetic sequence that shared some homology with our chosen target sequence. With this, we manged to obtain a lot of relevant information about our target sequence, including its expected value, homology identities, gaps and the strand. These data were then collected and compared with one another to evaluate which target sequence candidate was the most effective for the best DNA cleaving activity during the CRISPR gene edit.

3.2 PCR amplification of gene of interest

3.2.1 Primer design

To design the 2 primers required for PCR, we applied the formula to calculate each primer's ideal annealing temperature. These primers were then reconfigured for the process of PCR to be more effective. After designing, the primers were then checked against online softwares to confirm its efficiency.

Formula: Tm= 64.9 +41*(yG+zC-16.4)/(wA+xT+yG+zC)

Notes:

- Primers should the length of 18-24 bases
- 40-60% G/C content
- Start and end with 1-2 G/C pairs
- Melting temperature (Tm) of 50-60°C
- Primer pairs should have a Tm within 5°C of each other
- Primer pairs should not have complementary regions

3.2.2 Preparation of PCR buffer for amplification of gene of interest from yeast

PCR buffer composition (10X): 200 mM Tris-HCl (pH 8.4), 500 mM KCl

Mix the following:

- 10µl 10X PCR buffer
- 2µl dNTP mixture (10mM)
- 3µl MgCl2 (50mM)
- 5µl Primer mix (10µM each)
- 1-20µl Template DNA
- 0.5µl Taq DNA Polymerase (5U/µl)
- 100µl Autoclaved distilled water

3.2.3 Extraction of Saccharomyces cerevisiae DNA

1. Pick one yeast colony from the plate or spin down 100-200 µl of liquid yeast culture (OD600=0.4). Suspend cells in 100 µl of 200mM LiCl, 1 % SDS solution.
2. Incubate for 5 minutes at 70°C.
3. Add 300µl of 96-100 % ethanol, vortex.
4. Spin down DNA and cell debris at 13000rpm for 10 minutes.
5. Wash pellet with 70 % ethanol
6. Dissolve pellet in 100 µl of H2O and spin down cell debris for 30 seconds at 13000rpm
7. Use 1 µl of supernatant for PCR.

3.2.4 PCR amplification of gene of interest

1. Add all components in the PCR buffer, except for the Taq DNA Polymerase.
2. Mix contents of tube and overlay with 50 µl of mineral or silicone oil.
3. Cap tubes and centrifuge briefly to collect the contents to the bottom.
4. Incubate tubes in a thermal cycler at 94°C for 3 minutes to completely denature the template.
5. After denaturation at 94°C, maintain the reaction at 80°C.
6. Add 0.5 µl of Taq DNA Polymerase (2.5 U) to each reaction. Be certain to add the enzyme beneath the layer of oil.
7. Perform 25-35 cycles of PCR amplification as follows: Denature 94°C for 45 seconds. Anneal 58°C for 30 seconds. Extend 72°C for 1 minute, 30 seconds.
8. Incubate for an additional 10 minutes at 72°C and maintain the reaction at 4°C. The samples can be stored at -20° until use.

3.3 CRISPR/Cas9 gene editing process

3.3.1 Preparation of TBS electroporation buffer

1. Dissolve 6.05 g Tris and 8.76 g NaCl in 800 mL of H_2O.
2. Adjust pH to 7.6 with 1 M HCl.
3. Make volume up to 1 L with high purity distilled or deionized water.
4. Once prepared, TBS is stable at 4°C for 3 months.

3.3.2 Cell Plating

1. Trypsinize (to kill adherent cell) and count cells.
2. Plate cells to achieve 70–80% confluence the next day. Cell densities greater than 80% may reduce electroporation efficiency. For example, plate 2.5–3 × 10^6 cells in a 150 mm dish.
3. Incubate cells at 37 °C in 5% CO2 overnight.

3.3.3 Electroporation

1. Prepare 6-well plates by transferring 2 mL of pre-warmed culture medium to each well required for the number of samples and replicates in the experiment. Pre-incubate/equilibrate by placing at 37 °C in 5% CO2 while preparing samples.
2. Prepare a 100 µM synthetic sgRNA stock solution by adding the appropriate volume of Tris buffer to the sgRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 100 µM.
3. In 1.7 mL tubes, prepare samples to be electroporated. Combine 150 pmol of Cas9 protein with 3 µL (300 pmol) of synthetic sgRNA working solution for a final concentration of 1.5 µM and 3 µM, respectively. This creates the Cas9 ribonucleoprotein (RNP) complex.
4. Incubate at room temperature for 10–15 minutes.
5. Collect 1 × 10^6 cells for each sample. Centrifuge at ~ 500g for 1 minute at room temperature.
6. Aspirate medium from the cell pellet and resuspend in 100 µL of appropriate electroporation buffer.
7. Add 20 µL of template DNA to the suspension
8. Transfer resuspended cells to the 1.7 mL tube containing Cas9 RNP complex. Gently mix components and transfer the entire volume to an electroporation cuvette. Sample should cover the bottom of the cuvette; tap to remove any air bubbles.
9. Electroporate sample at 0.5mA for 10 minutes.
10. Use a transfer pipette to gently layer pre-incubated medium on top of electroporated cells from one well of a 6-well plate. Gently aspirate cells from the bottom of the cuvette and transfer to the well.
11. Incubate cells at 37 °C in 5% CO2 for a total of 48 to 72 hours after electroporation.
12. The cells were then inserted into the leaves of the *Arabidopsis thaliana* plant

3.4 Test for recombinant DNA

3.4.1 Preparation of TAE electrophoresis buffer (50X)

1. Weigh out 242 grams of Tris base.
2. Dissolve it in approximately 750 ml of deionized water.

3. Carefully add 57.1 ml of glacial acid and 100 ml of 0.5 M EDTA (pH 8.0).
4. After that, adjust the solution to a final volume of 1 L. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.

3.4.2 Gel electrophoresis

1. Place the agarose gel (on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.
2. Fill the electrophoresis apparatus chamber with 10 ml of 50x concentrated buffer and 490 ml of distilled water.
3. Make sure the gel is completely covered with buffer.
4. Proceed to loading the samples (in this case our DNA) and conducting electrophoresis.
5. Load the DNA sample into the well. The amount of sample that should be loaded is 35-38 µl.
6. After the sample is loaded, carefully snap the cover down onto the electrode terminals.
7. Insert the plug of the black wire into the black input of the power source (negative input). Insert the plug of the red wire into the red input of the power source (positive input).
8. Set the power source at the 70 volts and conduct electrophoresis for 1 hour.
9. Check to see that current is flowing properly.
10. After approximately 10 minutes, the coloured dyes will begin to separate.
11. Document the gel results.

3.5 Test for changes in turgor pressure on touch

3.5.1 Scholander bomb test

1. After checking that the pressure chamber and seal are in good condition, clean, and dry, check that the incoming gas will enter close to the bottom. Cover the bottom with a layer of water so that the incoming gas passes through the water. Make a baffle to prevent water from splashing onto the leaf. Line the walls with wet filter paper. Connect a cylinder of compressed air to the gas line.
2. Select the sample, avoiding damaged tissue whenever possible. Excise the leaf with a razor blade, insert it swiftly into the seal in the chamber top, and assemble the chamber. The time from excision to sealing the chamber should be no longer than 10 sec to avoid dehydrating the leaf.
3. After the leaf has been placed in the chamber, apply a small amount of pressure and check for leaks. If air is leaking through a manually sealed unit, slowly tighten the seal until audible leakage stops. Raise the pressure slowly and in small steps.
4. Observe the cut surface of the petiole as pressure is being applied. Increase the pressure until liquid is standing on the cut surface.
5. After liquid appears, reduce the pressure and allow the liquid to be pulled into the leaf until a wet film is all that remains on the cut surface. This is the position of the xylem solution before excising the sample, and it should require a balancing pressure that exactly opposes the tension in the xylem before excision. The meniscus is flat indicating that the water is not constrained by tensions that would otherwise be operating. Adjust the pressure so that the water film remains at the cut surface. For routine measurements, it will suffice to observe the meniscus for only 1 or 2 min. The balancing pressure is the negative of the tension in the xylem.

6. Release the air in the chamber and remove the leaf. Inspect for damage from pressurizing and sealing.

4. RESULTS AND DISCUSSION

Expression level of the chosen target gene

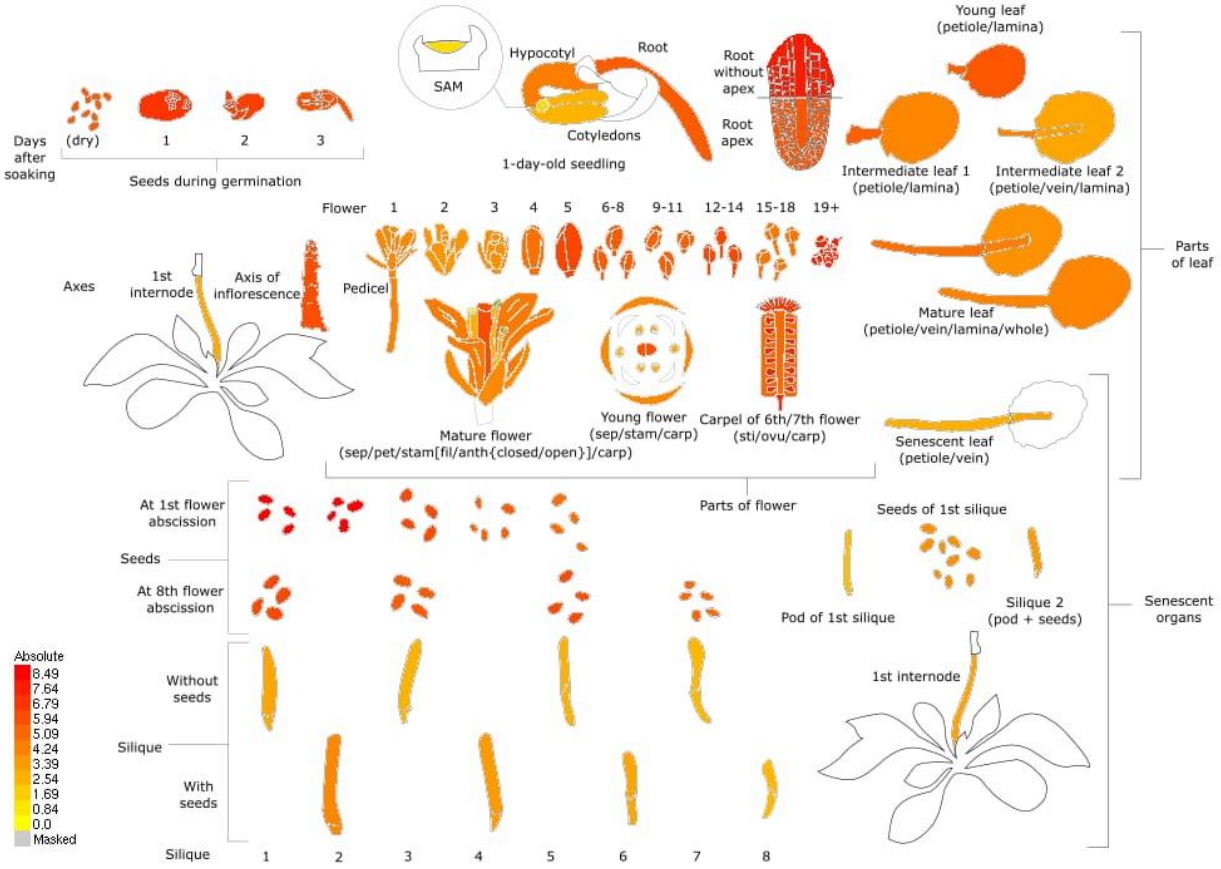


Figure 1: Expression levels of our chosen target gene AT1G11592

Using the identified genes that was successfully interrogated from online gene banks, tests were run to check for its expression levels. The best target gene was chosen on the basis of having the highest expression in leaves which is the region of the plant that we are most interested in. Based on all the results collected, it was determined that the target gene, AT1G11592, was the best.

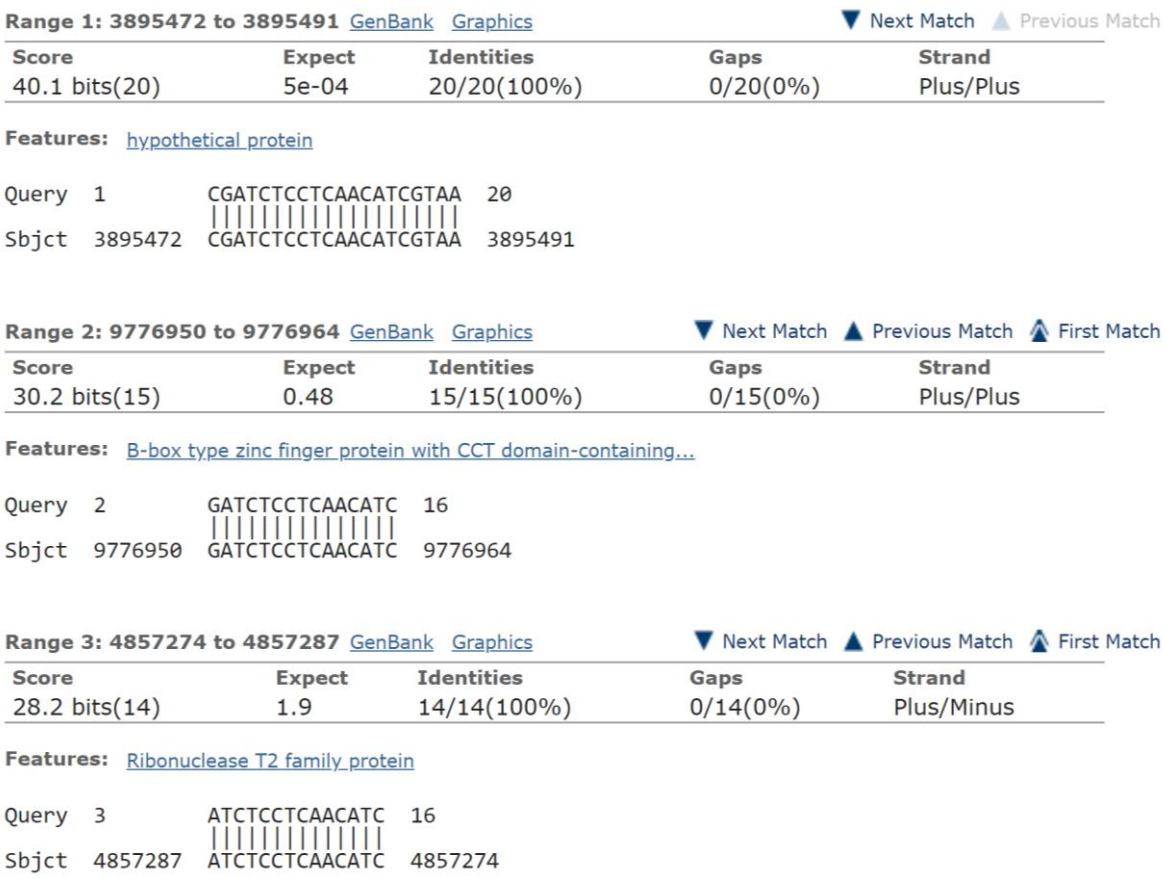
Best target sequence candidate chosen

Position	Sequence	PAM	On-Target Score	Off-Target Score	Strand
3895472 - 3895491	cgatctcctcaacatcgtaa	ggag	67.1	100.0	+

gRNA sequence: GCUAGAGGAGUUGUAGCAUU

Based on the data as shown above, the cleaving site of the best target sequence candidate identified had a great position on the target gene and incredible on-target and off-target scores. The cleaving site is located at the front of the target gene at a position of approximately the first tenth of its full length, taking a shorter distance for the CRISPR components to trace along the gene, hence being more efficient and it reduces degradation of the CRISPR components. Also, on-target scores of above 65 are considered very specific given that most target sequences have on-target scores of below 50. The off-target score of 100 implies that there is only one specific binding site for the Cas9

Blast data of the best target sequence candidate



Range 1: 3895472 to 3895491 GenBank Graphics — Next Match, Previous Match

Score	Expect	Identities	Gaps	Strand
40.1 bits(20)	5e-04	20/20(100%)	0/20(0%)	Plus/Plus

Features: hypothetical protein

```
Query  1        CGATCTCCTCAACATCGTAA  20
                ||||||||||||||||||||
Sbjct  3895472  CGATCTCCTCAACATCGTAA  3895491
```

Range 2: 9776950 to 9776964 GenBank Graphics — Next Match, Previous Match, First Match

Score	Expect	Identities	Gaps	Strand
30.2 bits(15)	0.48	15/15(100%)	0/15(0%)	Plus/Plus

Features: B-box type zinc finger protein with CCT domain-containing...

```
Query  2        GATCTCCTCAACATC  16
                |||||||||||||||
Sbjct  9776950  GATCTCCTCAACATC  9776964
```

Range 3: 4857274 to 4857287 GenBank Graphics — Next Match, Previous Match, First Match

Score	Expect	Identities	Gaps	Strand
28.2 bits(14)	1.9	14/14(100%)	0/14(0%)	Plus/Minus

Features: Ribonuclease T2 family protein

```
Query  3        ATCTCCTCAACATC  16
                ||||||||||||||
Sbjct  4857287  ATCTCCTCAACATC  4857274
```

Based on the data collected, the results showed no other genetic sequence similar to the

target sequence, with the next most similar genetic sequence sharing only 15/20 of the nucleotide identity of the original target sequence. Due to the considerable number of mismatches in the genetic sequence, the gRNA will not cleave it, hence its off-target activities will be much lower which means that the specificity of the gRNA cleavage is significantly high, resulting in an effective gene edit which is also evident as it has the highest blast score.

Best primer candidate chosen

Penalty	Direction	% GC	Tm°C	Location	Primer
0.929	Forward	50.0%	57.1°	3895308 - 3895329	5’ ACAGAAGGGAAGGAATCGGGAA 3’
	Reverse	54.5%	58.1°	3895496 - 3895517	5’ AGTTACCTGGAGCTGTGTCGTC 3’

This primer was chosen as it has the lowest penalty score as compared to other primer candidates. Its annealing temperature is also only 1° apart, making it the perfect candidate for an effective PCR primer set.

Relationship between turgor pressure and time

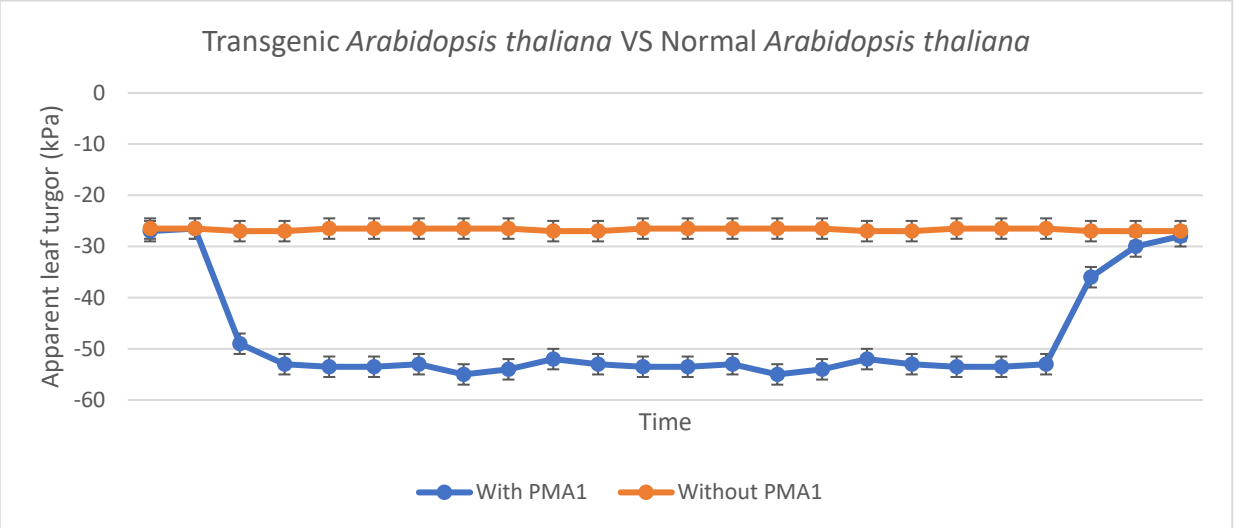


Figure 2: Data obtained from testing changes in turgor pressure

As can be seen from the graph, this is the typical trend observed in transgenic *Arabidopsis thaliana* leaf. On touch, there is a noticeable decline in cell turgor pressure, illustrating the effects of the introduced gene of interest, plasma membrane ATPase 1 (PMA1) from *Saccharomyces cerevisiae*, in the leaves of our transgenic plant. The process of the thigmonastic response leading to the decline in cell turgor pressure is as follows:

1. Mechanical stimuli producing compressive or decompressive mechanical stress on the cell wall deform or alter the tension of the cell membrane, activating mechano-sensory ion channels.
2. These channels transduce the touch stimulus into an electrical signal or receptor potential
3. The receptor potential is a small depolarization brought about by mechano-sensory Ca^{2+} channels at the plasma membrane.
4. Accumulation of ions within the vacuole that lead to motor cell turgor require a large amount of energy.
5. The normal equipment of the vacuole membrane appears to be insufficient to energize these ionic fluxes.
6. Therefore, the concomitant rise of H^+-ATPase to energize ion fluxes, and vacuolar aquaporins, which are responsive for the water fluxes, seems to be a prerequisite to leaf differentiation and movement.
7. Plasma membrane H^+-ATPases (H^+-pumps) are the primary active transporters that translocate protons to the outside of each cell, providing the electrical and chemical energy that drives solute transport.
8. An H^+-ATPase creates the H^+ electrochemical gradient across the plasma membrane.
9. This enzyme produces both a chemical gradient of protons (delta pH) as well as an outside-positive membrane potential gradient, which together comprise the proton motive force.
10. This transmembrane energy is used by the secondary transporter systems and channels for the transport of osmolytes and nutrients.
11. The Ca^{2+} released stimulates Ca^{2+}- activated Cl^- channels.
12. The receptor potential increases in amplitude and/or duration more or less incrementally with increasing magnitude of stimulus until a critical threshold voltage initiates an action potential.
13. The elevated Ca^{2+} activates Cl^- efflux via Ca^{2+}- activated Cl^- channels, further depolarizing the cell and activating outwardly directed voltage-dependent K^+ channels.
14. Under normal conditions, the action potential has a characteristic threshold and form, and propagates from cell to cell.
15. The result is an efflux of Cl^- and K^+, water efflux and transient turgor reduction, and cell contraction.
16. Thus, the leaves start to wilt (and droop due to loss of turgor pressure) and decrease in size as they curl/shrivel up.
17. After the cells have shrunk, they can be refilled with water again by moving the chloride and potassium ions back into the cells (the process reversed), but this is a slower process and requires energy expenditure to reestablish the transmembrane proton gradient and to reimport the released solutes.

Based on the graph, the sudden decline in cell turgor pressure as well as its reestablishment after a period of time prove that there was indeed a thigmonastic response carried out by the transgenic *Arabidopsis thaliana* plant. Hence, the CRISPR/Cas9 edit of the plant was successful.

5. CONCLUSION

The project had successfully genetically edited the *Arabidopsis thaliana* plant using CRISPR/Cas9, introducing a foreign gene that codes for a proton pump, plasma membrane ATPase 1 (PMA1) from *Saccharomyces cerevisiae.* With this, a thigmonastic response is also introduced to the *Arabidopsis thaliana* plant that will be triggered by the generation of an electrochemical gradient on touch, allowing it to be used as an effective defence mechanism against herbivory.

The accuracy of the results can be further improved by genetically editing more plants to test for the data's significance value which can be done in the future if time permits. Also, other genes coding for proton pumps can be tested on the plant, allowing for a comparison of effectiveness between the foreign genes. Some limitations faced during the project include long delivery waiting times and the lack of resources available in the school lab, thus it is recommended to start working on the project earlier.

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