The Role of the *APE1* Gene in the Development of *Arabidopsis thaliana*

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ABSTRACT

Acclimation to the photosynthetic environment is among the most important components of development as plants adjust to changing light conditions. Through alterations to thylakoid arrangement and composition, plant physiology adjusts photosynthetic parameters to protect light-capturing machinery and make photosynthesis more effective. Among the genes responsible for these alterations is the *APE1* gene, first studied as one of several acclimation to the photosynthetic environment (*ape*) mutants with varying photosynthetic parameters. Limited change to maximum photosynthetic capacity occurs in *ape* mutants, but more notable changes to the efficiency of photosystems I and II have been recorded. This study sought to further ascertain the role of the *APE1* gene using the CRISPR-Cas9 complex to induce a loss-of-function mutation. I measured mutants for max photosynthetic capacity (Pmax) in addition to various parameters pertaining to photosystem II efficiency (ϕPSII) and photosystem I efficiency (ϕPSI) to determine how the phenotype of the mutant lines is affected. I determined reductions in both ϕPSII and ϕPSI in the mutant lines as compared to the wildtype. Difference in parameters for fluorescence (F_V and F_m) indicated the most likely site of deficiency was somewhere in the electron transport chain between PSII and PSI. Lab closures due to the COVID-19 outbreak prevented the final stage of data collection via immunoblot analysis to determine the exact nature of the mutation. Completion of this data collection represents the future work to be done on the *APE1* gene.

KEYWORDS

acclimation to the photosynthetic environment, P700 absorption, chlorophyll fluorescence, thylakoid composition, photosystem

INTRODUCTION

Facing the broader implications of anthropogenic climate forcings within the last century, we are left with the possibility of applying a technological "fix" to ease our current predicament (Scheben et al. 2016). Methods to offset current emissions, while admirable in what they hope to achieve, are unable to address the present issue of excess greenhouse gases present in the atmosphere and continuing pollution (Cushing et al. 2016). Concerns surrounding the increasing concentrations of greenhouse gases in the atmosphere affect agriculture directly through the imposition of both higher temperatures and reduced rainfall on crops around the world (Deschenes and Greenstone 2007). This is compounded by a rapidly growing population with an expanding middle class that presents greater demand for resources (Kharas and Gertz 2010). Within this context, the genetic engineering and other technological advances in the breeding of plants offer a chance to develop plants that are able to better handle these adverse conditions and better supply food for a growing population. The development of CRISPR-Cas9 technology as a means of modifying the genome offers an especially promising opportunity to create plants that are better adapted to a changing global atmosphere (Ma et al. 2017). Creating plants that are both more drought tolerant and more photosynthetically productive could have enormous implications for the survival of agriculture on a planet beset by rising temperatures and reduced rainfall. Achieving this goal starts with information regarding the specifics of photosynthesis as it relates to changes in the environment.

The plant's response to variation in light levels is crucial to development, competition with other plants, and effective use of available resources. This response occurs on a genetic and phenotypic level. "Acclimation to the photosynthetic environment" occurs either to increase the rate at which light is utilized by the system (Walters and Horton 1995) or to dissipate excess light input that may be harmful to the plant's machinery (Muller et al. 2001). Previous work has detailed alterations in chloroplast composition that results from growing in different light conditions, showing changes to Rubisco concentrations, light harvesting complexes (LHC) and photosystem composition (Walters and Horton 1995; Bailey et al. 2001). Less work has been done to investigate the changes that occur in the photosynthetic machinery when observing the dynamic response to changing light levels different from what the plant has been grown in.

Work by Walters et al. (2003) first identified the *APE* (Acclimation to the Photosynthetic Environment) genes that encode for proteins that may regulate the plant's response to changes in

available light. This was done typically by growing the plants in low-light conditions before a switch to high light and then measurement of the plant's response in these high light conditions (Walters et al. 1995; Niyogi et al. 1997). One of these mutants, "*ape1*", had a mutation in a gene specific and universal to photosynthetic organisms, but the function of the protein this gene encodes was unknown (Karpowicz et al. 2011). Takeaways from the function of this gene in the model organism *Arabidopsis thaliana* could thus be relevant to the broader development of all plants. Unfortunately, the original mutant line of *ape1* mutants was lost due to improper storage of the seeds, making the further study of the role of the *APE1* gene using a loss of function mutant impossible until recently. CRISPR-Cas9 and its ability to create site-specific mutations has been utilized extensively by plant biologists seeking to determine the function of specific genes (Tsutsui and Higashiyama 2017; Shen et al. 2017). Through the use of the CRISPR-Cas9 complex to induce a loss-of-function mutation in the *APE1* gene, I was able to recreate the *ape1* mutants to determine how this gene affects the development of the plant's response to change in light availability.

Because acclimation to the photosynthetic environment results in differences to photosynthesis, tools that measure photosynthetic rates through chlorophyll fluorescence are used frequently to determine acclimation responses (Gray et al. 2003; Varotto et al. 2000). Chlorophyll fluorescence measurements are performed using Pulse Amplitude Modulated fluorometers, which use pulses of bright light applied to the leaf and measure the amount of light that is then fluoresced by chlorophyll. Thus, the fluorometer is able to determine how much light is absorbed versus the fraction of light that is utilized by the plant through comparison of the steady-state fluorescence level (F_s) and fluorescence when the reaction centers are closed (F_m) (Brooks and Niyogi 2011). These measurements are used to determine both maximum and operating efficiency of photosystem II (ϕPSII), which can be used to assess if there is a defect in photosystem II (Genty et al., 1990). Similarly, integrity and efficiency of photosystem I can be determined through measurements of P700 absorption, which corresponds to the oxidation state of photosystem I's primary electron donor (Klughammer and Schreiber 1998). This provides information on the interactions between the photosystems driven by electron carriers plastoquinone, cytochrome *b/f*, and plastocyanin (Schreiber, Klughammer, and Neubaur 1988). Together these measurements of photosystem II (PSII) and photosystem I (PSI) efficiency and integrity can be used to determine the exact nature of the defect in the *ape1* mutant.

The goal of this study is to determine the role of the *ape1* gene in the development of the model organism, *Arabidopsis thaliana*. Knockout of the gene and subsequent alterations to phenotype will show how the plants develop and photosynthesize differently when the protein this gene codes for is absent. Levels of photosynthetic efficiency measured through the operation of both photosystems will help to demonstrate the differences in the mutated plants and allow me to determine with more precision the function of the *ape1* gene.

METHODS

Use of CRISPR-Cas9 to create *Arabidopsis* mutants

To create the loss of function mutants in the *APE1* gene, I utilized the CRISPR-Cas9 method of genome engineering as described by Doudna and Charpentier (2014). Sequence specificity of the CRISPR-Cas9 complex is encoded by designing a guide RNA sequence (sgRNA) that binds to the complementary point in the genome encoding a key region of *APE1*. The Cas9 enzyme binds to the sgRNA and performs a "cut" of the double stranded DNA at the location where the designer sgRNA binds. Following this, the cell's endogenous machinery repairs the cut, often resulting in a "loss of function" mutation that eliminates the ability of the gene to code for its specific protein by either creating an early stop codon (nonsense mutation) or changing the reading frame by insertions or deletions (missense mutations). After determining the appropriate design for the sgRNA sequence in consultation with the *Arabidopsis thaliana* genome page TAIR, I obtained pKAMA-ITACHI plasmid primer vectors as described by Tsutsui and Higashiyama (2016). I mixed this vector with competent *Eschericia coli* , allowing the bacteria to grow on plates containing 30mg of gentamicin and 100mg spectinomycin, for which the plasmid DNA contains a resistance. Thus, only those bacteria containing the transformed gene survived for further cultivation. Samples of the bacteria were taken for PCR and subsequent sequencing analysis to determine if these cultures were transformed successfully. Taking the bacteria that grew on the plates and growing them further, I then inoculated competent *Agrobacterium* with the bacteria containing the transformed gene for entry into the plant genome following protocol by Hofgen and Willmitzer (1988). After cultivating the *Agrobacterium* further, I prepared a "floral dip" solution in accordance with Clough and Bent's procedure (1998). I dipped previously cultivated, wild-type *Arabidopsis thaliana* flowers into this mix to allow *Agrobacterium* entry into the undeveloped

cells in the ovaries of the flowers. Ideally, seeds cultivated from these plants contain the "edited" genome with no expression of the *APE1* gene. After allowing flowers to mature and produce seeds, I collected the seeds and sowed them on plates containing 100mg of the herbicide hygromycin, for which the plasmid DNA contains a resistance gene against. Thus, only those seeds containing the plasmid are able to germinate successfully on the plates. I then took these successfully germinated seeds and planted them in 3x3inch pots containing a mix of organic compost and commercial soil for further growth. I grew these plants for 6 weeks before collecting tissue samples for PCR and genetic analysis once again to assure successful transformation of plasmid DNA. All work for this process was completed in the lab and growth chambers of the Niyogi Lab at UC Berkeley.

Phenotyping photosynthesis in *APE1* mutants by P700 absorption and chlorophyll fluorescence

I utilized measurements of both P700 absorption and chlorophyll fluorescence to assess the difference in photosynthetic capacity in the *Arabidopsis* mutants. P700 absorption was measured by a WALZ Dual-PAM fluorometer (Meurer et al. 1996). These measurements are used to calculate photosystem I efficiency in addition to activity in the electron transport chain between photosystems. Redox kinetics were determined based on in-vivo absorbance changes at 830 nm, using measurements of ΔA and ΔAmax to calculate ϕPSI (Harbinson and Woodward 1986). ΔA was calculated after the actinic light was turned off, allowing for full P700 reduction, while ΔAmax was measured after 2 minutes of exposure to the far-red light at 720 nm. Measurements were taken over three rounds of light exposure cycles to ensure uniformity. ϕPSI was calculated according to work by Harbinson and Woodward (1986) using the equation $\phi PSI = 1 - \Delta A/\Delta Amax$, which is roughly equal to the fraction of non-oxidised P700/total P700 (Meurer et al. 1996).

I used measures of chlorophyll fluorescence to further determine the "operating efficiency" of Photosystem II (ϕPSII). This measurement represents linear electron transport in the plant's photosynthetic machinery and shows the quantity of electrons available to the Calvin-Benson cycle for the plant's dark reactions. A Hansatech Fluorometer supplied by the Niyogi Lab at UC Berkeley was used to take these measurements. By exposing leaf samples to a series of strong and weak light pulses, the machine generates a graph showing the re-emission of light from the leaf's

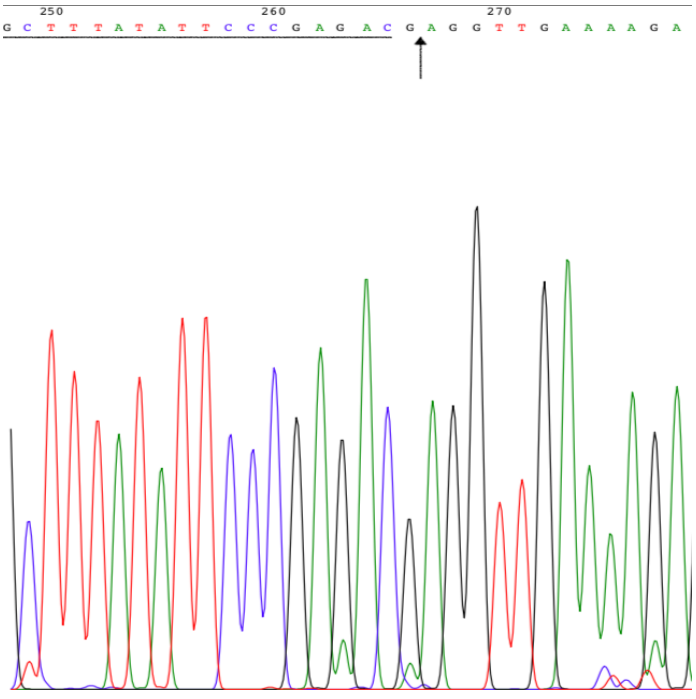
chlorophyll as "fluorescence". Electrons not emitted as fluorescence instead continue to the photosystem for Photosynthetic Electron Transport (PET), with the excess moving to "non-photochemical quenching" (NPQ) which is not utilized by the system. Measurements and subsequent calculations were found following the methods and equations described by Brooks and Niyogi (2011). The actinic light (roughly 2,000 mmol photons/m2/s) was turned on after 20 seconds after exposure to a saturating pulse, measuring maximum fluorescence when all reaction centers are closed (F_m). Following this, saturating pulses continued every 300 seconds to determine the maximum fluorescence after light adaption (F'_m) and steady-state fluorescence level (F_s). These measurements were used to calculate maximum photochemical efficiency of PSII [$F_v/F_m = (F_m - F_o)/F_m$] and operating efficiency of photosystem II [$\phi PSI = (F'_m - F_s)/F'_m$]. All data collection for both machines was carried out in the Niyogi Lab at UC Berkeley.

RESULTS

Mutants characterization and genotyping

The CRISPR-Cas9 complex used for this experiment targeted BP 263 of the 861 BP coding region of the *APE1* gene (Figure 1A). Following the transformation of the candidate plans via the "floral dip" method following the protocol outlined by Clough and Bent (1998), ~40% of the seeds gathered germinated successfully on plates containing hygromycin herbicide. Successfully germinated seedlings were genotyped following 3-4 weeks of growth in to further verify the transformation, showing a variety of mutations created (Figure 1B). These included single BP insertions (*ape1-1*, *ape1-2*), a more extreme 22 BP insertion (*ape1-3*) and a 14 BP deletion (*ape1-4*). All mutations cause a "frameshift" moving all subsequent amino acids out of frame and causing a different transcription.

A.



B.

Wild type	GGCTTTATATTCCCGAGACGAGGTTGAAAAGAGAGGTTTTAAAG CTCGATGTCGTCGGAAGAGCTGCTGATTCCACGAGCTCTTCAGT	
ape1-1	GGCTTTATATTCCCGAGGACGAGGTTGAAAAGAGAGGTTTTAAA GCTCGATGTCGTCGGAAGAGCTGCTGATTCCACGAGCTCTTCAG	+1
ape1-2	GGCTTTATATTCCCGAGTACGAGGTTGAAAAGAGAGGTTTTAAAG CTCGATGTCGTCGGAAGAGCTGCTGATTCCACGAGCTCTTCAN	+1
ape1-3	GGCTTTATATAAAGCCATCTCTGCCATCTCTCTTTGACAACGAGG TTGAAAAGAGAGGTTTTAAAGCTCGATGTCGTCGGAAGAGCTGC TGATTCCACGAGCTCTTCA	+22
ape1-4	GGCTTTATATTCCCG---------- AGAGAGGTTTTAAAGCTCGATGTCGTCGGAAGAGCTGCTGATTC CACGAGCTCTTCAGG	-14

Figure 1 The *ape1* gene knockout. (A) Sequencing data for *ape1* gene. The underlined portion indicates primer sequence. The arrow indicates approximate site of cleavage. (B) Mutation patterns of *ape1* mutant alleles created by CAS9/sgRNA complex. The red characters indicate mutation sites.

Mutant phenotyping to contrast photosynthetic capabilities with wild type subjects

Maximum Photosystem II Efficiency (F_V/F_m) was lower than the wild type measurement of 0.83 (mmol photons/m^2/s) in all lines (Figure 2). However, all mutants remained close to the control values, with the sharpest reduction seen in line *ape1-2* at 0.69 mmol photons/m^2/s, though this varied between replicants. These measurements are consistent with previous findings (Walters et al., 2003). PSII "operating efficiency" (ϕ II) was affected to varying degrees in the mutant lines, with a greater reduction on average than the measurements of F_V/F_m (Figure 2). These reductions compared to the wild type data were all ~0.43 mmol photons/m^2/s. The greatest reduction was in the *ape1-3* line with a ϕ II of 0.36 mmol photons/m^2/s. Measurements of average efficiency of Photosystem I (ϕ I) were also consistently lower than that of the wild type (Figure 3). Line *ape1-4a* had the greatest reduction with a ϕ I of 0.05 mmol photons/m^2/s. Lines *ape1-1, ape1-2b,* and *ape1-4b* had ϕ I values close to 0.45 mmol photons/m^2/s, representing less of a reduction from wild type data. Line *ape1-2a* showed a negative ϕ I value of -0.29 mmol photons/m^2/s. In this case, the values for ΔA/ΔAmax were greater than 1, making calculation for ϕ I negative (Meurer et al.

1996). Sample size was reduced due to lab closures in mid-March meaning that data displayed below is incomplete, making it hard to draw conclusions with certainty.

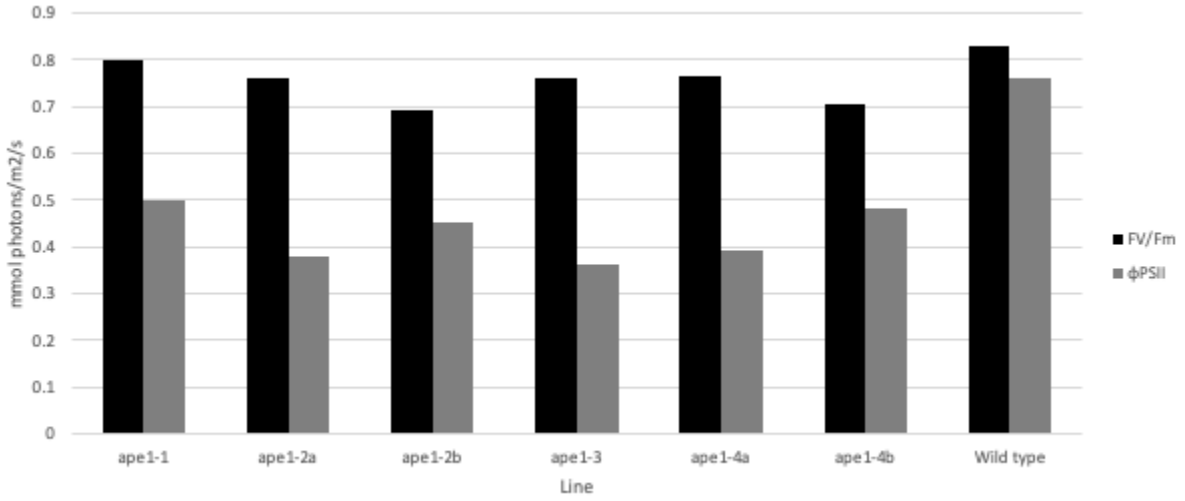


Figure 2 Measurements of maximum and operating efficiency of PSII in mutant lines and wild type. Indicated by black and grey bars, respectively. Denotations "a" and "b" in *ape1-2* and *ape1-4* represent biological replicates of the same line. Measurements were taken after dark adaptation of low-light grown samples according to measurements of F_v and F_m (Brooks and Niyogi 2011).

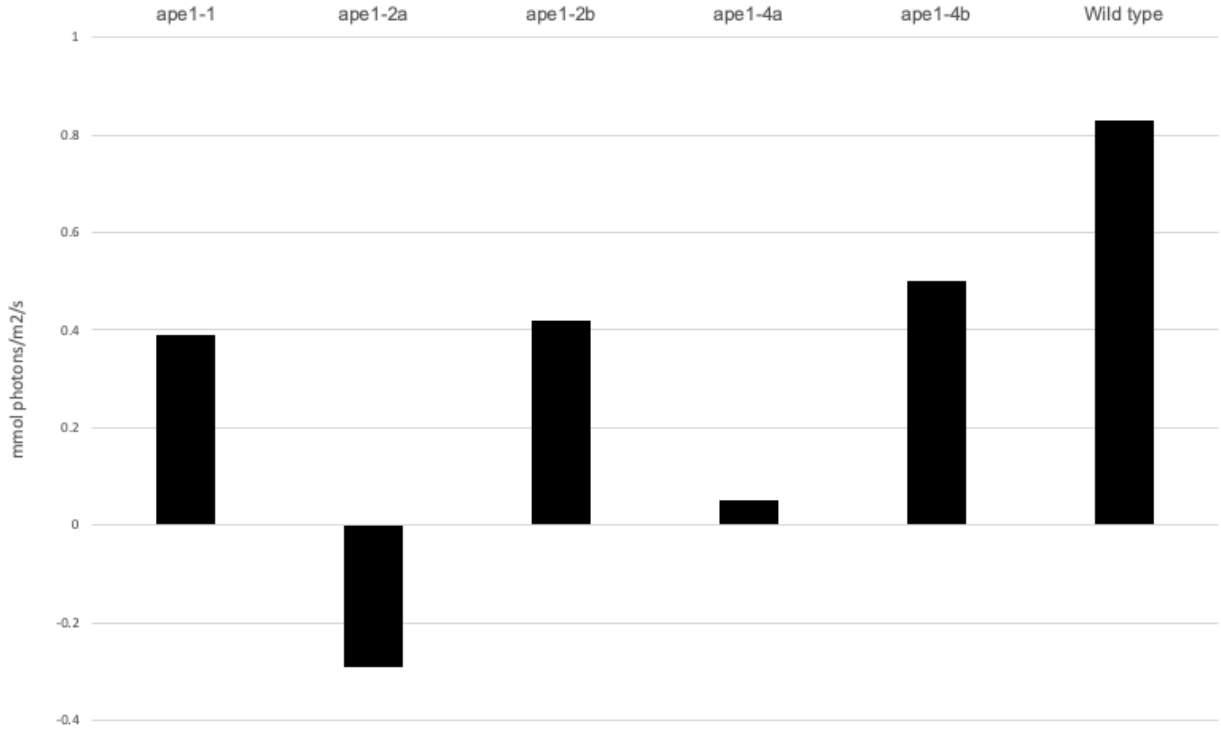


Figure 3 Measurements of average photosystem I efficiency in mutant lines and wild type. Denotations "a" and "b" in *ape1-2* and *ape1-4* represent biological replicates of the same line. Line *ape1-3* was not sampled due to lab

closure. Measurements were taken after dark adaptation of low-light grown samples according to measurements of F_v and F_m (Brooks and Niyogi 2011).

DISCUSSION

The loss-of-function mutation induced in the *APE1* gene does influence photosynthesis. The quantitative effects on photosynthesis varied considerably between mutant alleles likely as a consequence of the limited number of replicates we were able to perform due to the COVID-19 shutdown. However, all mutant lines indicated a reduced efficiency of PSI and PSII. Reduced efficiency of these two systems suggests that the *ape1* gene is responsible for the regulation of some component of these photosystems or the electron transport chain between them.

PSII average and maximum efficiency

Mutants showing a F_V/F_m (max photosystem II efficiency) under the threshold value of 0.5 likely have a defect with PSII (Meurer et al. 1996). The average F_V/F_m was well above 0.5 for all lines, meaning that the *ape1* mutants likely have a defect in the electron transport chain or PSI. This is also supported by ϕ II measurements for all mutant lines, none of which showed a reduction strong enough to indicate a defective PSII. Work by Walters et al. (2003) on *ape1* mutants shows similar findings regarding the efficiency of PSII, with mutant lines showing a reduced ϕPSII upon transfer from low light to high light conditions. They noted a change in the Chl *a/b* ratios, hypothesizing that *ape1* mutants have a reduced ability to alter thylakoid composition in response to change in the light environment. Changes in thylakoid composition in this instance most likely means degradation of the light harvesting complex of PSII (Yang et al., 1998), which could account for the observed differences in Chl fluorescence. We could not compare our *ape1* mutants to the original *ape1* mutants created by Walters et al. (2003) because seeds from this work are no longer viable. The loss of the original seeds necessitated the use of CRISPR-Cas9 mediated mutagenesis to recreate the mutations in *APE1*.

PSI efficiency

A defective PSI is indicated by a lack of far-red-induced absorbance change that would be captured in the P700 measurements (Meurer et al. 1996). Given that all mutants exhibited a non-

zero ϕPSI, it is more likely that the defect in these lines occurs in the electron transport chain. Line *ape1-2a* exhibited a negative ϕPSI, indicating that the defect could be in PSI. Line *ape1-4a* had a notably reduced ϕPSI with smaller reductions to F_V/F_m suggesting defects in the electron transport chain. Again, sample size was limited due to lab closure, making it difficult to know if these reductions are consistent.

Previous research suggests signaling pathways related to acclimation depend on the state of the plastoquinone pool, an electron receptor in PSII with an essential role in the electron transport chain (Kim et al., 1993). Alterations to the regulation of plastoquinone synthesis or activity could be the cause of the observed reduction in PSI and PSII efficiency, but it is impossible to make this claim with certainty in the absence of data showing verifying structural differences in the mutant lines.

Limitations and Future Directions

To determine the exact effects of these mutations on photosynthetic electron transport and alterations to thylakoid composition, it would be necessary to perform an immunoblot or similar procedure. This would separate the complexes in PSII and PSI and allow us to see which may be missing or altered to know where along the electron transport chain the defect occurs. Unfortunately, lab closures due to COVID-19 prevented the collection of this crucial data. This also impacted the sample size in data collection, making the statistical significance of the data difficult to quantify. Collected F_V/F_m, ϕPSII and ϕPSI data verifies past research and suggests that the *APE1* gene may be responsible for a component of the electron transport chain. Future studies could replicate this manner of data collection, as knowledge of these parameters is essential in determining photosystem defects. This should be completed with data showing structural composition that would verify the missing component responsible for the defect.

Conclusions

Knowledge of the genetic components of photosynthesis is an important first step towards future genetic modifications making plants more photosynthetically efficient. Genetically

modified foods already represent a crucial component of global food supplies, and understanding what genes regulate different processes within the plant is necessary to perform mutagenesis aimed at enhancement. Genes responsible for the plant's acclimation to changing light levels influence the early stages of photosynthesis through light collection and movement of electrons through photosystems. CRISPR-Cas9 induced mutations aimed at enhancing photosynthesis therefore must start with this process. Data collected through this study demonstrating the exact role of the *APE1* gene represents the foundational information for future genetic engineering in plants to enhance photosynthesis and ultimately improve both yield and survivability.

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