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Organellar DNA Polymerases Gamma I and II

in Arabidopsis thaliana

Jeffrey Michael Brammer, Jr.

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Organellar DNA Polymerases Gamma I and II in *Arabidopsis thaliana*

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Department of Microbiology and Molecular Biology

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Plants have two organelles outside the nucleus which carry their own DNA, mitochondria and chloroplasts. These organelles are descendants of bacteria that were engulfed by their host according to the endosymbiotic theory. Over time, DNA has been exchanged between these organelles and the nucleus. Two polymerases, DNA Polymerases Gamma I and II, are encoded in the nucleus and remain under nuclear control, but are transported into the mitochondria and chloroplasts.

DNA polymerases gamma I and II are two organelle polymerases which have been studied through sequence analysis and shown to localize to both mitochondria and chloroplasts. Little has been done to characterize the activities of these polymerases. Work in tobacco showed the homology of these polymerases to each other and to DNA Polymerase I in bacteria. They have been characterized as being part of the DNA Polymerase A family of polymerases.

In my research I have studied the effect of T-DNA insertions within the DNA Polymerase Gamma I and II genes. Since these DNA Polymerases are targeted to the mitochondria and chloroplasts, I studied the effect of knocking out these genes. A plant heterozygous for an insert in DNA Polymerase Gamma I grows slightly slower than wild type plants with an approximately 20% reduction in mitochondrial and chloroplast DNA copy number. A plant homozygous for an insert in this same gene has a drastic phenotype with stunted plants that grow to around 1 inch tall, with floral stems, and have an approximately 50-55% reduction in mitochondrial and chloroplast DNA copy number. Wild type plants can grow to a height of 12-18 inches with floral stems as a comparison. A plant heterozygous for an insert in the DNA Polymerase Gamma II gene grows slightly slower than wild type plants and has an approximately 15% reduction in mitochondrial DNA copy number and a 50% reduction in chloroplast DNA copy number. These plants also produce much less seed than do other mutants and wild type plants.

Keywords: DNA polymerase, Arabidopsis, Arabidopsis thaliana, gamma polymerase, mitochondrion, mitochondria, chloroplast, plastid, DNA replication

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Literature Review

Only two eukaryotic cell organelles have been discovered which contain a double membrane. These are the chloroplasts and mitochondria. Both organelles are sites of energy production in the cell and are a result of endosymbiosis (Henze and Martin 2003).

Endosymbiosis is a process by which one cell takes up another cell. The cell that is taken up loses its autonomy and can no longer live outside the host cell. At this point, it has become an organelle. The cell cannot live without the organelle nor the other way around.

DNA Replication

DNA replication is a process whereby cells are able to make copies of their genomes prior to cell division. Replication of nuclear genomes is tied to the cell cycle in order to regulate its copy number which is set to one pair of chromosomes in a diploid cell. With each round of the cell cycle and division of the cell, the nuclear genome is copied or replicated. However, this does not hold true for the organellar genomes of the mitochondria and chloroplasts. These genomes are not tied to the cell cycle and their genome copy numbers can be quite high, especially in chloroplasts early in the development of plant growth (Nielsen et al. 2010).

DNA replication, whether in the nucleus or organelles, involves a common set of proteins such as DNA polymerase, DNA primase, DNA helicase, single stranded binding proteins (SSBs), topoisomerases, a sliding clamp and clamp loader. DNA replication begins at an origin of replication, first by the binding of an origin sequence-specific binding protein, followed by DNA helicase, usually in a region high in AT content. The purpose of the helicase is to unwind

the DNA into single strands instead of the normal double stranded structure. This allows the DNA polymerase to bind to the origin of replication. SSBs must bind the single stranded DNA in order to keep it from re-annealing with the complimentary strand. DNA replication usually proceeds bi-directionally, although this is not true for all organisms. In prokaryotes, it is Pol III which is the main DNA polymerase involved in DNA replication. It is responsible for replication of both the leading and lagging strands. In eukaryotes, Pol δ is responsible for the lagging strand synthesis and Pol ϵ is responsible for the leading strand. However, in order for DNA replication to initiate, in both prokaryotes and eukaryotes, an RNA primer must be synthesized at the site where replication is to start. DNA polymerases cannot extend a nucleotide chain without a 3' end on which to add the next nucleotide. DNA primase, an RNA polymerase, synthesizes the primer once on the leading strand and very often on the lagging strand at approximately every 200-500 nucleotides. DNA synthesis, in this fashion, proceeds in the same direction as a whole, but the polymerases are still able to move 5' to 3' on the individual strands. The sliding clamp comes into play as DNA synthesis moves along. Its function is to improve processivity or the ability of the DNA polymerase to stay on the DNA strand and disengage. Without the sliding clamp, the DNA polymerase would fall off the strand periodically and would have to then find its way back to the correct site to re-initiate replication. This would make the process of replicating a genome extremely time consuming and inefficient and could lead to a slow response in instances when new cells are needed quickly. The clamp loader is a protein complex which loads the sliding clamp onto the DNA, having an ability to utilize ATP as an energy source. Another important protein within this process of DNA replication is Pol I in prokaryotes. Its responsibility is to remove the RNA primers made by DNA primase and fill the gaps in with dNTPs instead of rNTPs.

One model which explains how these proteins work together is the trombone model and seems to be accurate in its description of how DNA is replicated. All of these proteins are not free floating and disconnected, but with the exception of SSBs, these proteins are actually bound together in a complex known as the holoenzyme, which keeps necessary components of DNA replication together in close proximity.

While DNA replication in the mitochondria and chloroplasts possibly differs in its mechanisms, it still utilizes these same types of proteins in order to replicate the DNA. In understanding DNA replication within the nucleus, we begin to understand DNA replication in the organelles as well.

Mitochondria

Most eukaryotic cells have mitochondria, which provide the cell with a source of energy in the form of ATP, adenosine triphosphate. ATP is created via the glycolysis and citric acid cycles within the cell. Glycolysis uses glucose as the first substrate whereas the citric acid cycle uses pyruvate, which is the end product of glycolysis. Six molecules of ATP are created from one molecule of glucose during glycolysis in aerobic respiration. The citric acid cycle produces 30 molecules of ATP, 15 for each molecule of pyruvate leading to 36 molecules of ATP generated from a single molecule of glucose. As stated above, mitochondria have evolved after a process of endosymbiosis, where, most likely, prokaryotic cells of the alpha-proteobacterium lineage evolved to become more and more dependent on eukaryotic cells and were eventually engulfed within those cells and became organelles (Andersson 2002). As mitochondria evolved, genes were exchanged between the mitochondrion and the nucleus (Blanchard and Lynch 2000).

As mitochondria are energy producers within the cell, it is essential that they are properly maintained. Mitochondrial DNA must be replicated by the appropriate machinery. Mitochondria have multiple copies of their DNA, unlike the nuclear genome. This DNA, like nuclear DNA, must be replicated by DNA polymerases. In tobacco, these polymerases were shown to be encoded in the nucleus and then imported into the mitochondria (Ono et al. 2007).

Chloroplasts

Like the mitochondria, chloroplasts have multiple copies of their genomes and DNA polymerases are needed in order to replicate this DNA. The main function the chloroplasts have in energy production is to produce sugars and other carbohydrates which will go to the mitochondria or to the cytoplasm and are used in matrices within the cell and for plant growth. They are also sources of energy production through the process of photosynthesis, a process whereby energy from sunlight is stored in the form of ATP, the usual energy storage molecule within the cell. This is minor in comparison with the amount of ATP generated by the mitochondria.. The energy from sunlight is converted into energy in the form of chemical bonds through processes such as the Calvin cycle and other redox reactions (Lee et al. 2006).

As with mitochondria, chloroplasts are believed to have evolved through an endosymbiotic process whereby early cyanobacteria became engulfed within other cells, allowing those cells to use photosynthesis to harvest energy (Vesteg et al. 2009). Cyanobacteria are photosynthetic bacteria and have many genes in common with the chloroplast genome with an average sequence conservation of approximately 60% (Sato et al. 1999).

Chloroplast and Mitochondrial DNA Replication

Chloroplasts and mitochondria are a result of endosymbiosis, a condition where one cell took up or engulfed another cell in a symbiotic relationship. Most of the mitochondrial and chloroplast genes were eventually transferred to the nucleus and became part of the nuclear genome (Crimi and Rigolio 2008). Mitochondria in *Arabidopsis thaliana* are much different than their animal counterparts. It differs largely in size and non-coding regions. The chloroplast and mitochondrial genomes are large and have large amounts of non-coding sequence. The *Arabidopsis* chloroplast genome is over 154 kb in length, comprising 87 genes while the mitochondrial genome is over 366 kb, and comprises 57 genes which are only 10% of the mitochondrial genome. Unlike animal mitochondria, plant mitochondrial and chloroplast genomes have large amounts of introns and larger amounts intergenic space. Animal mitochondria have a size of 15-17 kb and no introns, as opposed to the much larger size of plant organellar genomes (Unsel et al. 1997).

DNA replication in plant mitochondria and chloroplasts seems to closely resemble the replication of DNA within bacteria (Holt 2009). DNA replication has been studied in the chloroplasts in *Arabidopsis* (Kunnimalaiyaan and Nielsen 1997), but has not been well studied for the mitochondria. Many assumptions have been made in the past as to how replication occurs in these organelles and what the structure of the genomes entails. DNA polymerases gamma I and II have not been studied except to determine their localization within the cell. Little has been done to study their activity during replication or role of the two proteins in plant development. In tobacco, homologs to DNA Polymerases Gamma I and II have been studied and seen to localize to both the mitochondria and chloroplasts and act in DNA replication.

DNA replication in the chloroplasts and mitochondria of plants is at first glance very similar to nuclear DNA replication. However, while the enzymes may be similar, the mechanisms can be very different. Both the chloroplast and mitochondrial genome map as circular molecules, however, in reality this is not always the case (Woloszynska 2009). Three mechanisms have been shown to be possible for the replication of chloroplast DNA including rolling circle, double D-loop and recombination dependent replication. Different mechanisms may be used at different stages of development (Nielsen et al. 2010). Recombination dependent replication has also been shown to be a possible mode of replication within mitochondria (Woloszynska 2009). Although the mechanisms for replication within the mitochondria and chloroplasts are poorly understood, there is growing knowledge on the subject.

Transfer of Organellar Genes to the Nucleus

According to the theory of endosymbiosis, the mitochondria and chloroplasts were once free living organisms which were engulfed by other single celled organisms. At one time, these progenitors of mitochondria and chloroplasts must have contained many more than 57 and 87 genes, respectively, in order to survive.

Some organellar genes have moved and inserted into the nuclear genome, then becoming an integral part of the cell. The rate at which genes leave the organelles is affected by the fact that most of these DNA sequences will never insert and integrate and those that do integrate may do so within a nuclear gene, causing it to become non-functional. In order for the organellar gene to successfully integrate itself into the nuclear genome and produce a function gene product, it must leave the organelle and be taken up by the nuclear DNA but not in a location which would

cause a deleterious insertion mutation. All of these factors combine for an extremely low rate of gene transfer (Blanchard and Lynch 2000). However, the process of endosymbiosis occurred around one billion years ago and has had time to transfer most of the organellar genomes to the nucleus. After the organellar gene has transferred to the nuclear genome, it disappears from the organellar genome, a process which is not well understood. There are several hypotheses on why this happens, including a hypothesis that insertions can and will happen over time, that a smaller organellar genome would provide an advantage against other competing organisms, that deleterious mutations would occur in the organellar genome after the transfer and that the driving force is actually the positive mutation as a result of this transfer (Blanchard and Lynch 2000). These genes do transfer and they then disappear from the organellar genomes. The mutation rate in the nuclear genome is lower than in the mitochondria and chloroplasts, providing a driving force for the transfer of genes from the organelles to the nucleus. This gene transfer is very slow at present and seems to have stopped altogether in some organisms. It seems that at the very beginning of the endosymbiotic process there was a large and relatively rapid transfer of genes between the organelles and the nucleus, and this transfer then quickly slowed down and became a process which occurred randomly over time (Kleine et al. 2009). Most transferred DNA is not functional within the nucleus, but becomes what is called nuclear mitochondrial DNAs and nuclear chloroplast DNAs. These sections of DNA are non-functional; however, they provide a platform for evolution of the genome within the nucleus. Most of the DNA that is transferred and successfully integrated and expressed takes on localization signals to target the protein products to the organelle from which the gene originated. However, some of these genes are used by the cell in a novel way, outside the organelle and in purposes that have little to do with maintaining the organelles. In *Arabidopsis* around 18% of nuclear genes or approximately 4500 genes

originated from the chloroplast progenitor and are homologous with cyanobacterial genes. Of these, around half now perform functions outside the organelles (Kleine et.al. 2009).

This transfer of genetic information between organelles and the nucleus is often accomplished through the insertion of single genes. However, there are instances of multi-gene transfer as well as large sections of the genome transferred. A 270 kb fragment of the *Arabidopsis* mitochondrial genome is located on chromosome II in the nucleus (Blanchard and Lynch 2000). This fragment is a duplication and has not disappeared from the mitochondrial genome.

Beyond this, there are further challenges to creating functional gene transfer. If the gene is transferred from the organelle to the nucleus and becomes integrated, its protein product must localize to the correct organelle. Since the original gene in the organelle would not have a transit peptide encoded, the nuclear version of the gene must acquire the code for a correct transit peptide. This does happen, but it is infrequent. Since land plants have appeared, at least eleven mitochondrial genes and one chloroplast gene, which we know of, have made this transition to the nucleus (Blanchard and Lynch 2000). In addition, there are problems with regulation of expression. Mitochondria and chloroplasts are not single copy number organelles, as discussed above, and the nucleus would need to express these organellar transferred genes at a sufficient rate in order for the gene to then disappear from the organelle or become non-functional in the organelle.

Gene transfer is also not one way. Gene transfer can occur from the nucleus to the mitochondria or from the chloroplasts to the mitochondria. For example, in the *Arabidopsis* mitochondrial genome there are fragments of chloroplast DNA, nuclear genes, retroposons and

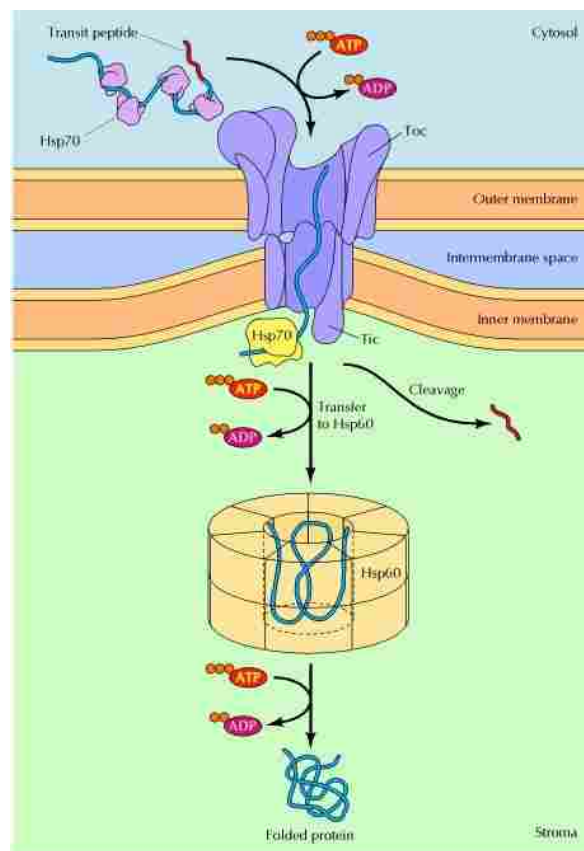
other sequences not found in other mitochondria. The *Arabidopsis* mitochondrial genome contains 12 native tRNAs, but additionally contains 6 chloroplast tRNAs and around 10 nuclear tRNAs (Blanchard 2000). However, nuclear gene transfer to the chloroplasts occurs rarely, if ever, and has not to this point been identified (Kleine et al. 2009).

Some genes within the chloroplasts and mitochondria seem to have resisted transfer to the nucleus. These genes have some similarities. Most are very hydrophobic proteins and may have difficulties crossing the organelle membranes were they to be expressed in the nucleus and translated in the cytoplasm. Another similarity is that they are directly involved in energy transduction within the organelles and it is possible that in order to effectively govern this process, these genes are best expressed and regulated in the organelle (Kleine et al. 2009).

Transport of Proteins into the Mitochondria and Chloroplasts

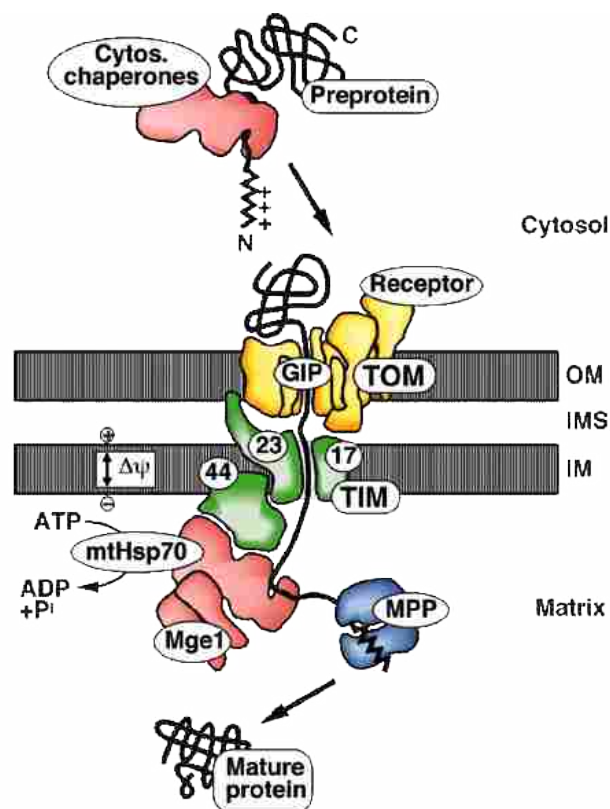
As discussed, most protein products that function in the chloroplasts and mitochondria are encoded in the nucleus. These proteins are expressed in the nucleus and translated in the cytoplasm. These proteins have a transit peptide which will target them to the chloroplast, mitochondria or both. In the chloroplast, the main machinery involved in importation of these proteins utilizes two protein complexes; translocon at the inner envelope membrane of chloroplasts (TIC) and translocon at the outer envelope membrane of the chloroplasts (TOC). The mitochondrion has a similar system utilizing the transporter inner membrane (TIM) complex and the transporter outer membrane (TOM) complex. The transit peptides for both the mitochondria and chloroplast have a net positive charge due to a lack of acidic residues (Balsera et al. 2009).

Chloroplast transit peptides do not have conserved sequences. These transit peptides are not permanent parts of the protein, but are cleaved from the protein by stromal processing peptidase during or after import into the chloroplast. These transit peptides are still not well understood and there seems to be other criteria which would allow them to be localized to the chloroplast. They vary in length from 20-150 amino acids. They have similar features in that they have a positive charge, have several hydroxylated amino acids and they do not form a secondary structure in an aqueous solution. These seem to be the important features that allow them to enter the chloroplast (Balsera et al. 2009).



Cooper, 2000

After translation in the cytoplasm, the chloroplast protein pre-cursors must be localized or transported to the chloroplast. This is mediated by a few systems. Many transit peptides have binding sites for Hsp70 and together with 14-3-3 proteins, they will translocate the protein to the chloroplast where it can bind with the TOC protein complex. Another pathway involves the binding of Hsp90 to the transit peptides. In this process, the 14-3-3 proteins are not needed. Hsp90 can directly localize the chloroplast protein to TOC64 where it is then directed to TOC34 (Balsera 2009). Many other TOC proteins are involved in the TOC protein complex which facilitates movement of the chloroplast protein through the outer membrane. At this point the chloroplast protein is in the intermembrane space, but in close proximity to the inner membrane and is transferred to the TIC complex which will facilitate movement into the chloroplast.



Pfanner, 2010

Import into the mitochondria is similar but differs in a few ways. First, the transit peptide for mitochondrial localization does have a secondary structure, forming into an amphipathic helix with one positive and one hydrophobic surface. This is essential for proper import and recognition by the TOM complex. However, some mitochondrial proteins have internal targeting sequences and the features of these sequences are not as defined (Balsera et al. 2009).

Some proteins may also be dual targeted. Their transit peptides may have characteristics of those of both chloroplast transit peptides and mitochondrial transit peptides. All of these proteins seem to be important in regulating cell cycle, synthesis of DNA and synthesis of protein and may be a form of communication between the organelles (Balsera et al. 2009). These transit peptides, which confer dual targeting, can be classified as either twin or ambiguous. The twin sequences comprise a chloroplast transit peptide along with a mitochondrial transit peptide, both separate from the other. The twin sequence is achieved by an alternative transcription start site, alternative translation start sites or alternative splicing (Mackenzie 2005). The ambiguous sequence is a chloroplast transit peptide and a mitochondrial transit peptide combined in one sequence. These concepts are important to this thesis in that *Arabidopsis thaliana* DNA Polymerase Gamma I contains an ambiguous sequence for dual targeting while DNA Polymerase Gamma II contains a twin sequence mediated by an alternative start site for transcription. In DNA Polymerase Gamma II, the alternative start site is a CUG codon upstream of the AUG start codon. When the CUG site is used, a different transit peptide is produced, which is located between the CUG and AUG sites (Christensen et al. 2005).

DNA Polymerase Activity

Eukaryotic DNA polymerases share commonalities of function and structure. Some of the similar structures include a thumb domain, finger domain, palm domain and exonuclease domain. The palm domain holds the active site of replication of DNA. The exonuclease domain is used in proofreading in order to excise an incorrectly added base at the end of the DNA chain, at which point the correct base can be added.

DNA polymerases can be categorized into different categories with pol α , pol δ , and pol ϵ belonging to family B. Pol γ , the mitochondrial DNA polymerase belongs to family A along with other polymerases, most of which are usually associated with repair. These groupings are based on homologies between sequences of the different polymerases. *Arabidopsis thaliana* DNA polymerases gamma I and II are referred to as gamma because of their function within the mitochondria. There are six regions which seem to be at least somewhat conserved among DNA polymerases. Regions I and II are most often located within the palm and are part of the active site. Region III is in the finger domain and is also an important part of addition of new nucleotides. Region IV is part of the exonuclease domain and is responsible for the 3' to 5' exonuclease activity of the polymerase. Region V is located within the thumb domain and region VI is in the finger domain (Hübscher et al. 2002).

The palm domain contains the active site of replication, the site where new deoxynucleotides are added to the growing DNA chain. This domain is highly conserved among polymerases. The thumb and finger domains are less conserved among polymerases but perform the same functions with analogous structures. The finger domain is responsible for the addition

of the new dNTP by correctly positioning the template and the next dNTP. The thumb domain is important in processivity (Hübscher et al. 2002).

Thesis Objectives

DNA Polymerases Gamma I and II in plants are not well studied. Initial studies have determined that the two proteins are dual-targeted to mitochondria and chloroplasts, but did not address whether the proteins are equally targeted to these organelles (Christensen et al. 2005). While their nucleotide and protein sequences suggest the proteins may have identical and redundant functions, their promoter sequences suggest there may be differences in expression of the two genes. Expression of the two genes has not previously been analyzed. My research focus was (1) to determine the expression patterns of the two genes in order to determine if they were the same or different and (2) to help build an understanding of the potential roles of these two DNA polymerases in organelle and plant development.

Organellar DNA Polymerases Gamma I and II
in *Arabidopsis thaliana*

Introduction

Chloroplasts and mitochondria arose over a billion years ago through a process of endosymbiosis where one cell engulfed another cell in a symbiotic relationship. Afterwards there was a rapid transfer of genes from the chloroplasts and mitochondria to the nucleus of the cell. The mitochondria and chloroplasts arose from two different types of progenitor cells, both of which were adept at storing energy, a huge plus to the cells in which they were engulfed. Progenitors of mitochondria could take glucose and produce many molecules of ATP. Progenitors of chloroplasts could use the energy of sunlight and convert it into carbohydrates and chemical energy. This provided a huge advantage to eukaryotic cells, the only type of cells to contain mitochondria and chloroplasts. These organelles and the energy and carbohydrates they were able to produce, allowed for the evolution of tissue and multicellular organisms.

Knowing the importance of these organelles, it is easy to understand that maintaining them is of great importance. DNA Polymerases Gamma I and II in *Arabidopsis thaliana* are two proteins which help in the maintenance of the organellar genomes. While most of the genes from the original organellar genomes have long been transferred to the nucleus, including the genes which encode these two polymerases, there are still many important genes encoded in the organellar genomes. These genes must be replicated with high fidelity like other genes. This is the role of DNA Polymerases Gamma I and II.

Both of these polymerases have been shown to be dual targeted to the mitochondria and chloroplasts through GFP tagging experiments. However, these experiments did not show if the localization of these polymerases was equal. While both polymerases target both mitochondria

and chloroplast, they are not both localized equally to both organelles. In fact, initially, experiments with constructs of GFP tagged to the first 100-113 amino acids of the coding sequence showed that Polymerase Gamma I was dual targeted to the mitochondria and chloroplasts while Polymerase Gamma II was targeted to the chloroplasts only. However, by tagging the 5' UTR region of Polymerase Gamma II and translating from an alternative start site of translation upstream of the normal AUG start site, the protein would then localize to the mitochondria as well. This can be accomplished in real time in the cell by an alternative CTG start codon upstream of the normal ATG start codon. These DNA Polymerases play a role in DNA replication within the mitochondria and chloroplasts. Polymerase Gamma I is dual targeted to mitochondria and chloroplasts, while Polymerase Gamma II is preferentially targeted to the chloroplasts but can also localize to the mitochondria (Christensen et al.).

Both of these genes are encoded in the nucleus. They seem to be duplicates of each other, based on their sequence similarities, meaning that originally there was one gene which was duplicated to form two genes. This is based on their sequence homology. Both genes are localized to the organelles and both are dual targeted, albeit by different mechanisms. The importance of these genes in replicating the organellar genomes will be discussed.

Materials and Methods

Insertion Lines

T-DNA insertion lines were obtained for DNA Polymerase Gamma I (At3g20540) and DNA Polymerase Gamma II (At1g50840) from the Arabidopsis Biological Resource Center (ABRC). The insertion line used in DNA Polymerase Gamma I was FLAG_463C09 and is located in the 5th exon of the gene (Figure 1). The insertion line used in DNA Polymerase Gamma I was SALK_022638 and is also located in the 5th exon of the gene (Figure 1). Plants which were heterozygous for an insert in DNA Polymerase Gamma I were crossed with plants which were heterozygous for an insert in DNA Polymerase Gamma II. Progeny were screened as described below in order to obtain plants which were heterozygous for an insert in both genes. These plants were then crossed with each other in order to produce plants with various combinations of inserts.

Sequence Alignments

Sequences were obtained from GenBank for nucleotide sequence and GenPept for protein sequences and were aligned with Clustal (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The mRNA accession number for DNA Polymerase Gamma I is NM_112946 and the mRNA accession number for DNA Polymerase Gamma II is NM_103965. The protein accession number for DNA Polymerase Gamma I is NP_188690 and the protein accession number for DNA Polymerase Gamma II is NP_175498.

Isolation of Seeds

Plants were grown until they produced siliques. The siliques were allowed to remain on the plant for 2-3 weeks and were then removed and dried for a period of 3-7 days. The siliques were then crushed over 4 layers of cheesecloth, which was laid on top on a piece of paper. The seeds were filtered through and poured onto new cheesecloth and filtered a second time. The seeds were then placed in a microcentrifuge tube, labeled and placed in the cold, out of the light until they were planted.

Growing Seeds on Soil

Two methods were employed to grow seeds on soil. The first method, cupcakes, was used when a large amount of tissue was desired. Small pots were filled to the rim at which point fertilizer was added. More soil was added until the top was rounded above the rim of the pot, giving it its name of cupcake. A double layer of cheesecloth was then placed over the top of the soil in order to facilitate removal of tissue without bringing the soil with it. The sides of the cheesecloth were sprayed with water on the outer edge of the pot at which point they would stick when they dried, keeping the cheesecloth in place. Seeds were sprinkled on top of the cheesecloth at a rate of 20-50 seeds per pot.

The second method was used when just a few plants were planted per pot, usually for crossing purposes. Soil was filled $\frac{3}{4}$ of the way to the rim of the pot, at which point fertilizer was added. Soil was added to fill the pot to the rim. Seeds were spread onto a piece of paper and tweezers were wetted with sterilized water. By bringing the tweezers close to a seed, the seed would stick to the tweezers and the seed could then be wiped off on the soil. If the tweezers are

not wetted, the static electricity is too great (the seeds are extremely small) and the seeds will continually move away from the tweezers.

Suspension of Seeds for Media

Seeds were washed in a 50% bleach solution with 1 drop Tween 20 for 10 minutes. Seeds were centrifuged at 5000rpm for 10 seconds and the bleach solution was poured off. Seeds were washed in 1ml of autoclaved RNase, DNase free water. Seeds were centrifuged again at 5000rpm for 10 seconds. Washing was repeated 3 more times. 1ml of 0.1% autoclaved agarose solution was then added to the seeds. Seeds were transferred to a 15ml tube and filled with agarose to a final volume of 12-15ml and vortexed to mix.

Plant Media

One packet of Murashige and Skoog media was poured into 2L of double distilled water in an Erlenmeyer flask. The solution was stirred on a stir plate. The pH of the solution was adjusted to approximately 5.7 (should not be less than 5.6 or greater than 5.8). 1L of the solution was then poured into a separate Erlenmeyer flask. 8g of Phytoblend agar was added to each liter of solution (between 7 and 9 grams may be used). Media was autoclaved for 40 minutes. Media is allowed to cool no more than 20 minutes and is then poured immediately into plates in a biosafety hood. Plates will take 3-4 hours to set and then autoclaved filter paper can be added on top of the media.

Planting Seeds on Media

Autoclaved filter paper is added to each plate if not already done. Spread 600-800 μ l of seeds suspended in 0.1% agarose onto the filter paper by first ejecting the solution with a pipette. Seeds were spread with a bacterial loop. Bottoms of the plates were labeled with the date the seeds were spread, the date they should be taken out of the cold, what line of seeds they were and any other necessary information. After all seeds were spread, plates were placed in a 4°C cold room for 5 days. After this period, plates were removed and immediately placed in the light room.

DNA Extraction Buffer 1

DNA Extraction Buffer 1 was made by dissolving 0.8g SDS in 80ml Salts Buffer on low heat while stirring. 160mg phenanthroline was dissolved in 1ml of 100% ethanol and then added to the Salts Buffer/SDS solution. 56 μ l of β -mercaptoethanol was added in a vent hood.

Salts Buffer

Salts Buffer is made by adding 800ml deionized water to a beaker. 12.1g of Trizma base was added while stirring. 29.0g NaCl was then added and then 18.6g EDTA sodium while continuously stirring. The pH of the solution was adjusted by adding 6M HCl until pH reached 8.0. The volume was then brought up to 1000ml with additional deionized water.

DNA Extraction Protocol 1

One or two leaves were isolated from *Arabidopsis thaliana* plants and placed in a 1.5ml microcentrifuge tube. 600 μ l of DNA Extraction Buffer 1 was added. The solution was mixed by

vortexing. Samples were then placed in a 65°C heat block for 10 minutes. 200µl of 5M KOAc (1/3 Volume) was then added and vortexed to mix. Samples were put on ice for 20 minutes. Samples were then centrifuged for 15 minutes at maximum speed. Supernatant was transferred to new, clean, labeled 1.5ml microcentrifuge tubes containing 500µl of cold isopropanol. Samples were centrifuged for 15 minutes at maximum speed. The supernatant was poured off. Samples were washed with 750µl of cold 80% ethanol three times and then placed upside down on a paper towel to air dry for at least 15 minutes. DNA was resuspended with 100µl 0.5X TE Buffer.

Seed DNA Extraction Protocol

10µl of RNase and DNase free water was placed in a 1.5ml microcentrifuge tube. Tweezers were dipped in sterilized water. Tweezers were touched to the seed from which you want to isolate DNA (seed adheres to the tweezers). Tweezers were dipped in the water in the microcentrifuge tube and shaken gently if needed. The seed dislodges and settles into the water in the microcentrifuge tube. 50µl Seed Extraction Buffer (formula not given) was added to the seed in the microcentrifuge tube. Samples were placed in a water bath or heat block at 65°C for 30 minutes. Samples were removed from the heat block and incubated at room temperature for 45-90 minutes (longer time gives better results). 50µl DNA Extraction Buffer 1 was added to each tube. Samples were mixed well by vortexing. Samples were placed in a 65°C water bath or heat block for 10 minutes. Samples were removed from the heat block or water bath and 40µl of 5M KOAc (1/3 volume) was added to each sample. Samples were mixed well by vortexing and placed on ice for 15-20 minutes. Samples were centrifuged at maximum speed for 5 minutes. The supernatant was transferred to new, clean, labeled tubes containing 75µl cold isopropanol. Samples were centrifuged at maximum speed for 10 minutes. The supernatant was discarded.

Samples were washed in 100µl of 80% ethanol three times and placed upside down on a paper towel to air dry for at least 15 minutes. Samples were re-suspended in 30µl of 1X TE Buffer. 1µl of re-suspended DNA was sufficient for PCR. If a low quantity of DNA was extracted, nested PCR was used to first increase the amount of template DNA.

DNA Extraction Protocol 2

Tissue, one leaf or whole plant, was placed in a 1.5ml microcentrifuge tube. 10µl 0.5N NaOH was added. Tissue was ground in the NaOH. 5µl of the solution was then taken and added to a new tube containing 495µl TRIS at a pH of 8.0. 1-3µl of this solution was then used in PCR.

PCR Zygoty Screening

DNA was taken from samples as explained above and mixed in a PCR reaction containing 12.5µl of Promega Go Taq solution with 1µl of forward primer and 1µl of reverse primer (30µM) and 1-2µl of DNA (10-50ng/µl). RNase, DNase free water was added to bring the total volume to 25µl. Each sample had four reactions. The first sample used a gene forward primer for DNA Polymerase Gamma I and gene reverse primer for DNA Polymerase Gamma I. The second reaction used a FLAG insert primer in place of the forward primer and a gene reverse primer for DNA Polymerase Gamma I. The third reaction used a gene forward primer for DNA Polymerase Gamma II and a gene reverse primer for DNA Polymerase Gamma II. The fourth reaction used a SALK insert primer and a gene reverse primer for DNA Polymerase Gamma II. PCR was run with an initial denaturing step at 95°C for 5 minutes followed by thirty five cycles with a denaturing step at 94°C for 30 seconds, an annealing step between 51°C and 60°C for 30 seconds (temperature depended on which set of primers was used), and by an

elongation step at 72°C for 80-90 seconds. This was followed by one round of elongation at 72°C for 10 minutes.

QPCR

QPCR was done to look at both mitochondrial and chloroplast DNA copy number. DNA samples were isolated as described above. DNA was then quantified using a nanodrop spectrophotometer to determine concentration. Approximately 9-12ng of DNA was added to each reaction (each reaction containing equal amounts of DNA). Ubiquitin was used as a nuclear control for comparison with both the mitochondria and chloroplast DNA levels. Four regions were analyzed for each organelle; MT26, MT53, MT77 and MT104 for the mitochondria and CT1-1, CT2-1, CT2-2 and pSBA for the chloroplasts (see Table 1 for primer sequences). Copy number was then analyzed by comparing Cp values of wild type and mutant plants. The ubiquitin Cp value was subtracted from the Cp values for the organelle DNA regions above in order to obtain Δ Cp values. Wild type Δ Cp values were subtracted from mutant Δ Cp values in order to obtain $\Delta\Delta$ Cp values. Wild type Δ Cp values being subtracted from themselves lead to $\Delta\Delta$ Cp values for wild type of 0. Fold change was determined by taking $2^{-\Delta\Delta\text{Cp}}$. This led to a fold change for wild type of 1. Mutant fold change was then compared to wild type values of 1.

RNA Slot Blot Analysis

RNA was first isolated with Invitrogen™ Purelink™ RNA Mini Kit as outlined on pgs. 29-33 of the user manual, for isolation from plant tissues. The slot blot was performed using a BioRad slot blot apparatus per BioRad instructions. One piece of Whatman filter paper was dampened with double distilled water and placed in the slot blot apparatus. A 0.45 μ m

MagnaCharge nylon membrane was soaked in 6X SSC and placed on the filter paper. The upper manifold was then placed on top of the membrane and secured with the provided bolts. Equal amounts of total RNA were diluted in 200µl of double distilled water for each sample to be analyzed and heated at 65°C for 10 minutes and then immediately placed on ice for 5 minutes. The vacuum was applied to the slot blot apparatus for approximately 30 seconds. Each 200µl sample was added to the appropriate wells. Once the level liquid approached the bottom of the well, 1ml of 6X SSC was added to each well. The membrane was then removed from the slot blot apparatus and air dried. Once the membrane was dry, it was crosslinked at 1200kJ ultraviolet light.

RNA slot blot probe preparation

Probes were designed by using primers specific to each polymerase (Table 2). Wild type *Arabidopsis thaliana* DNA was used as the template. Probes were created by running PCR reactions as previously described with the probe primers. Probes were labeled with an Invitrogen™ Biotin labeling kit for Nucleic Acids.

Hybridization

RNA slot blot membranes were hybridized with a Sigma Perfect Hyb Plus Hybridization Buffer at 65°C for 20 minutes in a Robbins Scientific Hybridization Incubator. Heat-denatured biotin labeled probes were added and the membrane was incubated in the same buffer at 65°C overnight. The membrane was washed in 2X SSC for 5 minutes and 15minutes in 1X SSC at room temperature. The membranes were processed using the Pierce Chemiluminescent Nucleic

Acid Detection Module protocol. The slot blot was exposed to X-ray film with intensifying screens for 10-20 seconds and developed.

Results

Sequence Alignments

The sequences of DNA Polymerases Gamma I and II were aligned to determine sequence identity. The identity of the mRNA regions of the genes is 72% (Figure 1). The identity of the amino acid sequence is 72% (Figure 2a). These high sequence identities suggest that the two polymerases carry out the same or very similar functions. However, the sequence alignment of the N-terminal regions suggests that their targeting sequences may not be the same. This has been shown in previous experiments (Christensen et al.). The alternative CTG start codon for DNA Polymerase Gamma II, which is upstream of the usual ATG start codon, is needed to confer mitochondrial targeting (Christensen et.al. 2005). Because of the high sequence similarity, differences in plants with inserts in DNA Polymerase Gamma I or DNA Polymerase Gamma II is most likely due to differences in localization to the mitochondria or chloroplasts or due to a difference in expression and/or localization to the different organelles, but not a difference in function of the two proteins.

Promoter Regions

Due to the preliminary data which shows a different expression pattern for DNA Polymerases Gamma I and II, an alignment of the region upstream of and including the start codon of the polymerase genes was performed. This alignment shows a possible explanation for a difference in expression since the two regions have dissimilarities that could account for a different expression pattern because of possible differences in promoter and regulatory regions.

While the genes may have originally been duplicated, it appears their promoter regions have changed to a point where the genes could be regulated to have different expression patterns.

Zygoty Screening

T-DNA insert lines were obtained which contained inserts in DNA Polymerases Gamma I and II. FLAG_463C09 has a T-DNA insert in the 5th exon of DNA Polymerase Gamma I and SALK_022638 has a T-DNA insert in the 5th exon of DNA Polymerase Gamma II. FLAG and SALK refer to the type of insert within the line. These two lines were crossed and seeds were harvested and planted. These F2 plants were then checked for zygoty and plants which were heterozygous for both the SALK and FLAG inserts were crossed in order to obtain double mutants. Zygoty screenings of hundreds of plants never revealed a plant which was homozygous for an insert in both DNA Polymerase Gamma I and DNA Polymerase Gamma II. Plants were identified with various combinations of inserts.

Phenotypes

Plants which were homozygous mutant for DNA Polymerase Gamma showed an extreme phenotype. These plants were extremely dwarfed, growing to only about 1 inch tall and producing very few seeds (Figure 4). Plants which were homozygous mutants for DNA Polymerase Gamma II showed little phenotype other than growing a little slower than wild type plants (Figure 5). These plants took about 3-4 weeks longer to grow to maturity and begin to produce seed than do wild type plants (wild type plants takes 6-8 weeks). Once they reached maturity, they were indistinguishable from wild type plants. Plants which were heterozygous for an insert in DNA Polymerase Gamma I showed only a phenotype of slowed growth (Figure 6).

They took 2-3 weeks longer than wild type plants to grow to maturity. Plants which were heterozygous for an insert in DNA Polymerase Gamma II took about 1-2 weeks longer than wild type plants to grow to maturity than wild type. These heterozygous plants in the two polymerases did show a phenotype among themselves as to how much seed they produced per plant. Plants heterozygous for an insert in DNA Polymerase Gamma I, while growing slower than plants heterozygous for an insert in DNA Polymerase Gamma II, produced more seed than those which were heterozygous for an insert in DNA Polymerase Gamma II (Figure 7). These plants which were heterozygous for an insert in DNA Polymerase Gamma I produced an amount of seed comparable to wild type plants (data not shown) while those which were heterozygous for an insert in DNA Polymerase Gamma II produced almost no seed.

QPCR

QPCR was performed as described in Materials and Methods on plants which were heterozygous for an insert in DNA Polymerase Gamma I, heterozygous for an insert in DNA Polymerase Gamma II and homozygous for an insert in DNA Polymerase Gamma I to determine genome copy number in mitochondria and chloroplasts. The plant which was heterozygous for an insert in DNA Polymerase Gamma I showed an approximately 20% reduction in DNA copy number in both the mitochondria and chloroplasts. The plants which were heterozygous for an insert in DNA Polymerase Gamma II showed a 15% reduction in DNA copy number in the mitochondria, but a 50% fold change reduction in DNA copy number in the chloroplasts. The plant which were homozygous for an inserts in DNA Polymerase Gamma I showed a 55%

reduction in DNA copy number in the mitochondria and a 50% reduction in DNA copy number in the chloroplasts (see Figures 8 and 9).

Slot Blot

Preliminary expression data obtained via a RNA slot blot suggests that DNA Polymerase Gamma I is expressed early in development of *Arabidopsis thaliana*. Expression of DNA Polymerase Gamma I seems to be at its highest in weeks 3 and 4, tapering off in weeks 6 and 8 with another possible surge of expression in week 10 (Figure 10a). DNA Polymerase Gamma II is expressed later on in development with expression at its highest in weeks 4-8 and tapering off in week 10 (Figure 10b). This experiment was performed twice with the same results each time. This may be the reason why we see such a stunted phenotype in plants deficient in DNA Polymerase Gamma I, but not those deficient in DNA Polymerase Gamma II.

Discussion

DNA Polymerases Gamma I and II are essential to maintain the genomes of the mitochondria and chloroplasts in *Arabidopsis thaliana* and these proteins have also been identified in rice and tobacco (Ono et al.2007). The genes seem to be redundant based on their sequence homologies (see Figures 1 and 2). However, phenotypic differences arise when the two genes are knocked out. A homozygous knockout of DNA Polymerase Gamma I causes a stunted phenotype that never reaches the size of a wild type plant, while a knockout of DNA Polymerase Gamma II causes a slow growth phenotype but eventually reaches the size of a wild type plant. These differences in phenotypes may be due to the expression patterns of the two polymerases. Insertions in DNA Polymerase Gamma I cause a more drastic phenotype (Figures 4-6), most likely due to its high expression early on in development, a period when adequate amounts of this polymerase would be crucial to proper development (Figure 10). Expression of DNA Polymerase Gamma II later in development, possibly regulated by promoter elements different from DNA Polymerase Gamma I, may explain why insertions in this gene cause a reduction in the amount of seeds produced (Figure 7).

Research done on the homologs of DNA Polymerases Gamma I and II in tobacco showed a high sequence similarity among the two tobacco polymerases. These two proteins had a nucleotide sequence identity of 98.3% and an amino acid identity of 97.2%. Due to this sequence similarity, the function of these two proteins is assumed to be the same. The N-terminal regions including the first 93 amino acids were analyzed and found to contain 100% sequence identity. A targeting program, TargetP, predicted their localization to the chloroplasts. However, GFP fusions showed that both proteins were dual targeted (Ono et al. 2007). The *Arabidopsis DNA* Polymerases Gamma I and II show a 72% identity at the mRNA nucleotide level (Figure 1). An

alignment of the N-terminal first 100 amino acids shows only a 51% identity, allowing for differences in localization (Figure 2B).

Based on the QPCR data (Figures 8 and 9) and evidence provided in early experiments by another group (Christensen et al. 2005) it seems that DNA Polymerase Gamma I almost equally localizes to the mitochondria and chloroplasts, while DNA Polymerase Gamma II seems to localize more often to the chloroplasts. This is in harmony with data that suggest that DNA Polymerase Gamma I has an ambiguous targeting sequence, meaning it has a single sequence allowing it to be localized to the mitochondria and chloroplasts, and that DNA Polymerase Gamma II has a twin targeting sequence, meaning it has two targeting sequences, one of which is produced through the usual AUG start site of translation and the other (for the mitochondria) is only produced when an alternate CUG start site of translation is used(Christensen 2005).

Preliminary expression data obtained from a RNA slot blot suggests that DNA Polymerase Gamma I is expressed early in development, while DNA Polymerase Gamma II is not. This may be the reason for the stunted phenotype of those plants deficient in DNA Polymerase Gamma I (see Figure 4). Since these plants are deficient in DNA Polymerase Gamma I and DNA Polymerase Gamma II is not expressed at high levels until week 4 of development, these plants may be stunted due to a lack of sufficient levels of polymerase to replicate their organellar genomes early in development. On the other hand, those plants deficient in DNA Polymerase Gamma II would still have high levels of DNA Polymerase Gamma I early in development and this may account for their ability to mature into plants of the same size as wild type plants (Figure 5). DNA Polymerase Gamma II seems to be expressed until later in development. Once the plant reaches a sufficient size, it may then begin producing larger amounts of chloroplasts as cell size increases. This increase in the number of chloroplasts may

account for the increase in expression of DNA Polymerase Gamma II which seems to localize more often to the chloroplast and may be more responsible for the maintenance of the chloroplast genome at this point when copy number may be increasing.

According to phenotypic data between plants which are heterozygous for an insert in DNA Polymerase Gamma I and plants which are heterozygous for an insert in DNA Polymerase Gamma II, it seems that DNA Polymerase Gamma I may be necessary for proper growth of the plant, while DNA Polymerase Gamma II may be necessary for proper seed production to occur (see Figures 6 and 7). The implications of this are many and must be further studied, but one possibility is that proper maintenance of the mitochondrial genome and its copy number are essential for proper development, while maintenance of the chloroplast genome and its copy number may be more essential for proper seed production.

In conclusion, it is apparent that these polymerases are vital to the proper development and function of *Arabidopsis* plants. Both are conserved in other plants, indicating they may both play specific roles in development. Further research is needed in order to fully determine the expression of these two genes, what the specific effects of the polymerases are on organelle and plant development and whether one is responsible for growth and one for seed production.

```

PolGammaI -----ATAGAGCAATGGC 13
PolGammaII AAGCGATGGCCACAATCTTCTTCTTCTGCTTAAGCTTTCCTTCTCAGGCAGACCCACTTG 60
                                         *** * *

PolGammaI TACCAGCTCAGCTTTGTGATAAAAACGACAAAATTGGACAACGATAAACCCACTTTTA-T 72
PolGammaII CTTGAAACTAGCTCGGAACGATAACGACAAAATTGCCCACC-ATAAACCCATTTTTTAAT 119
          *      **** *      * ***** * * * ***** * * * *

PolGammaI CTCTGGAAGACTCAAAATT---CCTTCAAGCTGGGGAGATTAGCTTCACTTACTACGGCC 129
PolGammaII CTCCGACTCTGTCTGCTACTTCTCCTTCAAGCTGGGTAGATTAAGTTCGCTTA---TGGCC 176
*** *      ** * * ***** ***** * * * * *

PolGammaI ATGGGGTTCCTCTT-----CGTCACTTGTCTCCTTCTCT 165
PolGammaII ATGGGGTTCCTCTTACTTCCCACAATAACCCTTCTTCTCGCCACTTATCTCCTTCTCT 236
***** ***** * * * * *

PolGammaI TTCTGGGTTTCGCGT-CGTCCTCGTGT-----TTCTTCTCAATTCTTTCTTTCTTGT 219
PolGammaII TCCTGGGTTTCCTCGTTCCCTCCTCTCGCTCTCTTCTTCTCCACTCCCTTCTTCTCTT 296
* ***** * * * * * ***** * * * * *

PolGammaI CCTCGCCGTCGAATCCTTTGCAACCAGGAAAGTAGCAATCATAAAGGGAAATGCGGGATAT 279
PolGammaII CCTTGCCGTCGAACCCCTTCTCAAAGGAACTAGCTTCCACTGATGGAATGTGCGTTAT 356
*** ***** * * * ***** * * * * *

PolGammaI TCGACTGCTACTGATTGTGGTGGTTCTCATGGGTTTCATCATTAGGTTCATCAAAGGTCA 339
PolGammaII TGTAATACTACAGTTTGT-----CAGGGATTTTCAGCATTTCGGTTCATCAACGGTCG 407
* * * * * * * * * * * * * * * * * * * * *

PolGammaI TCCTCTGTTGAGTTTAGTGGAGAGTGGAACTTAATTTGGGATCTAAGACGGCGAGAATG 399
PolGammaII TCTTCTGTTGTTTAAACGGAGAGTGGAACTTCGATCGGAATCTAACAAGGTAAGGATG 467
** ***** * * * * * * * * * * * * * * * * * * * *

PolGammaI GTTCCGCCAACTGT-----GAAACAAG-----CT 423
PolGammaII GTTCCTAAGATTATCAAGGTTGGAATCAGACGGAGGTAGCCGAACTCATCAAGTTCCT 527
***** * * * ***** * * * * *

PolGammaI GGAGCTGTAAGTGCTTGGAGGGAGGAAGTCAATAATAAGTTAAGAGGAAGAAATAGAGAA 483
PolGammaII GGAAGTGTAAAGTGCTTGGAGGGAAGAAGC---TAATAAGCTTAGAGAAAGAAATGGTCAA 584
*** ***** * * * * * * * * * * * * * * * * * *

PolGammaI TATGCTAACAACCAGGATGATGCTTTTGGTAATGGCAGTTATATTTAAAGGCTTTGT 543
PolGammaII ATTGCTAGAAACC-----TTGATGACAATGGTTAT---TTCATGGCTCTGT 629
***** * * * * * * * * * * * * * * * * * * * *

PolGammaI CCCAAAATAGATGATGTCCATTCTTATGAAACGGCCAGAACTTTGATTACAATCTGAAG 603
PolGammaII CCAATTATATCTAGTGCTCCGTCTTATGAAACCTCTCAGAAGATTGATTATGAGTTCAAG 689
** * * * * * * * * * * * * * * * * * * * *

PolGammaI CCTGGAA-----CTGATATTACCACACTTGGTAGAGAATTGAATGGCTTTATG 651
PolGammaII CCCAGAGGGACCCTAGGTCCACTACTGTACTCAATAAGGAATTGATTGGCATTACC 749
** * * * * * * * * * * * * * * * * * * * *

PolGammaI CAAACGAA---TAGTATAAGAGGATCGGTGCTAGCATTACCAAGCAAGGATATTGAGGTT 708
PolGammaII CAATCTGAACCTGTTGTGTCATTGCCAAGGAAGGTTTAGATGTTGGGGATAATATGGAT 809
*** * * * * * * * * * * * * * * * * * * * *

PolGammaI GGGGAAAC---AACAGACGTGACTCTGAAACCTCTTAACAGTGATACGACTCTTGACAAT 765
PolGammaII GTAAATCCTAAAGGAGAGGAATACAGAGACCTCTTATAAGCGATAAGTCTTCTGGTACC 869
* * * * * * * * * * * * * * * * * * * * *

PolGammaI GCCAGCT---ATAAGAAAACAGCAACCATTCCAAAGTGAAAAATGCACCAACCTATCG 822
PolGammaII GCAAATGGAACAAGAATACTGTTGCCATTCCAAAGTGAGAGAAGCACTGAACCTTCG 929
*** * * * * * * * * * * * * * * * * * * * *

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PolGammaI	GTGCTCCTTGAATGGCATCCACAACCTGGACAAGAGAAGCCGCCAGTGCCATTGTTGAAG	2682
PolGammaII	GTGATCCTTGAATGGCATCCAGAACCTGGGAAGACAAGCCACCTGTGCCGTTGTTAAAG	2771
	*** *****	
PolGammaI	GATGCCTTTGCTTCTGAGAGAAGAAAAGCAAAGATGCTTAACTTCTCGATTGCGTATGGG	2742
PolGammaII	GATGCCTTTGTTTCAGAGAGAAGAAAAGCCAAGATGCTTAACTTTTCAATTGCGTATGGG	2831

PolGammaI	AAGACTGCTATTGGGCTGTCTAGAGATTGGAAGGTATCAAGAGAAGAAGCTCAAGATAACA	2802
PolGammaII	AAAACCGCGGTCGGGCTTTCTAGAGATTGGAAGGTCTCCACTAAAGAAGCTCAGGAAACA	2891
	** * * * *	
PolGammaI	GTTAATCTCTGGTATAATGACAGACAAGAAGTCCGAAATGGCAAGAAGTACGCAAGAAA	2862
PolGammaII	GTCGATCTCTGGTACAATGACAGGCAAGAAGTAAGGAAATGGCAAGAAGTACGTAAGAAA	2951
	** *****	
PolGammaI	GAAGCTATACAAAAGGGTATGTACTCACTTTGTTGGGAAGGGCTCGTAAATTCCTGAA	2922
PolGammaII	GAAGCTATAGAAGATGGGTATGTACTCACTTTACTAGGAAGGTCTCGTAGATTTCCGGCG	3011

PolGammaI	TACCGTTCACGTGCCCAAAGAACCATATCGAGCGAGCAGCAATCAACACTCCTGTTCAG	2982
PolGammaII	TCAAAGTCACGTGCTCAGAGAAATCATATCCAAAGAGCAGCAATCAACACTCCAGTCCAG	3071
	* *****	
PolGammaI	GGAAGTGCAGCTGATGTTGCTATGTGCGTATGCTGGAGATATCAAATAATCAACGGCTA	3042
PolGammaII	GGAAGTGCAGCTGATGTTGCCATGTGTCAATGTTGAAATATCGATAAATCAACAGTTG	3131

PolGammaI	AAAGAGCTTGGTTGGAAATTGCTTCTACAAGTTCATGATGAAGTAATCTTGAAGGACCA	3102
PolGammaII	AAGAAGCTTGGTTGGAGATTGCTTCTACAGATTCATGATGAAGTAATCTTGAAGGACCA	3191
	** *****	
PolGammaI	AGTGAGTCAGCGGAGAACGCTAAAGACATAGTTGTGAAGTGCATGTCTGAACCCTTCAAC	3162
PolGammaII	ATTGAATCAGCGGAGATCGCAAAGGACATAGTTGTGGACTGTATGTCTAAGCCCTTTAAC	3251
	* *** *****	
PolGammaI	GGCAAGAATATTCTCTCAGTCGACTTATCTGTTGATGCTAAGTGTGCTCAGAACTGGTAT	3222
PolGammaII	GGAAGGAATATTCTCTCAGTCGATTTATCTGTTGATGCAAAATGTGCTCAAACCTGGTAT	3311
	** * *****	
PolGammaI	GCTGGCAAATAAGAGGAAAATAGAAAAAGATCTT-GCTAGTGAAAGCTAACAATGAGGCA	3281
PolGammaII	GCTGCCAAATA-GAGAAGCTTAGAGACCGCCGTAGTTGAAGGAAGCGATTTTTTGGGTA	3370

PolGammaI	GTGCTTGAGGTTTTTTTTTTTTTTGTCCTATG-GCTCTGAGAAAATCTGTTTCATATGTG	3340
PolGammaII	TA--TTAAGGTTTGGGTTTTGTAAATGTTAGAAAGCCTTGAGAA----CGTTGAGCTGTG	3424
	** *****	
PolGammaI	AGAAAGGGTCATTTTGTATTATGCTAATGAAAGCTAATTGTTGTCATCTTCTTTA-TGTTA	3399
PolGammaII	TAAATTGTTTCTCAAACCTAATCTTCTAAGTACGATGTTTGAATGCAAAAAGTGGTT	3484
	** * * *	
PolGammaI	AAAGATACATTGCTGTGTAAAT--TAGTAGGCTTGAAGAGT-CGGTGTTTTAAATGAGAA	3456
PolGammaII	AAAGTAACCTGAGAACAATCATGATATGAATATTGTGGATTGCTTTGTGTAATAATGAGAA	3544

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PolGammaI AACAGAGTAATCTTATCA----- 3474
PolGammaII TTTGAATAGATTATATAAACAAAAT 3569
          *   **   ***  *
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Figure 1: DNA sequence alignment for the mRNAs for DNA Polymerases Gamma I (PolGammaI) and II (PolGammaII). 72% sequence identity. FLAG_463C09 and SALK_022638 insertion locations are marked.

```

PolGammaI      --MGVS-----LRHLSFSSFWVSRRP-RVSSS-ILSFLVPRRRILCTRKVAIIKUNA 48
PolGammaII     MAMGVSLTSHNNPLLRHLSFSSSWVSRSSRLSSSPLPSFLFPCRRTLLQRKLASTDGNV 60
                ****                ***** ** . *:*** : **.* ** * **:*. **.

PolGammaI      GYSTATDCGGSHGFHSHGHQRSSSVEFSGEWKLNLSKSTARMVPPTVKQ----- 97
PolGammaII     GYCTTTVCQG---FQHSVHQRSSSVFNGEWELRSESNKVRMVKI IKVGNQTEVAETHQ 117
                **.*: * * * *:** ***** *.***:*. *:..***** :*

PolGammaI      -AGAVSAWREEVNNKLRGRNREYANNQDDAFNGSYILKGFVPKIDDVHSYGNGQNFYDYN 156
PolGammaII     VPGTVSAWREEAN-KLRERNGQIARNLDD---NG--YFNGSVPIISSAPSYETSQKIDYE 171
                .*:*****.* ** ** * : *. * ** ** *: * ** *... ** ..*:**.*

PolGammaI      LKPG----TDITTLGRELNGFMQTNISIRGSSVVALPSKDIEVGETTDVTLK-----PLNS 206
PolGammaII     FKPRGTRSTTATLNKELIGITQSE----PVVSLPRKGLDVGDNMDVNPKEGEGIQRPLIS 227
                **:          : :**.:** *: **:          .**:* *.:**:. ** . * ** *

PolGammaI      DTTLDNAS-YKKTATISKVEKCTNLSQVRANLKKIYNRVRVVDNVSSAKETVALLMNQYR 265
PolGammaII     DKSSGTANGNKNTVAISKVERSTEPSNVRENLGKIYDKVLIVDNVQAAKDTVAKLVNQFR 287
                *.: ..* . *:*:*****.:*: *:* ** ***:*: * :*****.:**:* ***:**.*

PolGammaI      NLVHACDTEVSRIDVKTETPVDHGEMICFSIYCGSEADFGDGKSCIWVDVLGENGRDILA 325
PolGammaII     NHVHSCDTEVSGIEVKEETPVDHGELICFSIYCGPEADFGNGKSCIWVDVLGENGREVLA 347
                * **:****** *:** *****:*****.*****:*****:*****:***

PolGammaI      EFKPFEDSSIKKVWHNYSFDNHIIRNYGIKLSGFHGDTHMMARLWDSRRISGGYSLEA 385
PolGammaII     EFKPYFEDSFIRKVWHNYSFDSHIIIRNHGIEISGFHADTHMMARLWDSARRIKGGYSLEA 407
                ****:* ** * :*****.*****:*. :***. *****:***.*****

PolGammaI      LTSDPKVLGGTETKEEAELFGKISMKKIFGKGLKKGSEGKLVIIIPVKELQMEDREAW 445
PolGammaII     LTSDPKVLGGTQTKEEAELFGKISMKTI FGKRKLLKKGSEGKIVVIPPVEELQREDREAW 467
                *****:*****.:*****.*** *****:*****.:**:* ** *****

PolGammaI      ISYSALDISITLKLYESMKQLQAKKWFLDGKLISKKNMDFYQYEWQPFGELLAKMESE 505
PolGammaII     ISYSALDAISTLKLYESMTKKLQMDWHLDGKPVLRGRTMLDFYHEFWRPFGELLVKMEAE 527
                *****:*****.:** .*.*** : :*.***:*.***:*****.***.*

PolGammaI      GMLVDRDYLAQIEIVAKAEQEIIVSRFRNWASKHCPDAKHMNVGSDTQLRQLFFGGISNS 565
PolGammaII     GILVDREYLAEIEKVAKAEQQVAGSRFRNWASKYCPDAKYMNIGSDTQLRQLFFGGISN- 586
                *:***:***:*** *****:.* *****:*****:***:*****:*****

PolGammaI      CNDEDLPYEKLFKVPNVDKVIEEGKKRATKFRNIKLHRI SDRPLPTEKFTASGWPSVSGD 625
PolGammaII     SHDEVLPEKLFKVPNIDKVIEEGKKTPTKFRNIKLHRISDSPLSTENFTASGWPSVGGD 646
                .:* ** *****:*****.***** **.*:*****. **

PolGammaI      TLKALAGKVS AEYDYM EGVLDTCLEENIGDDDCISLPDEVVETQHVNTSVESDTSAYGTA 685
PolGammaII     VLKELAGKVS AEYDFMDDVSDISLEEVEDDD-----VETSETQKSKTDETDTSAYGTA 701
                .* *****:*. * * .*** : ** * .*** : *. * :*****

PolGammaI      FDAFGGGESGKEACHAIAALCEVCSIDSLISNFILPLQGSNVSGKDRVHCSLNINTETG 745
PolGammaII     YVAFGGGERGKEACHAIASLCEVCSIDSLISNFILPLQGSNVSGKDRVHCSLNINTETG 761
                : ***** *****:*****

PolGammaI      RLSARRPNLQNPFALEKDRYKIRQAFIASPGNSLIVADYGQLELRILAHLASCESMKEAF 805
PolGammaII     RLSARRPNLQNPFALEKDRYKIRKAFVSPGNTLVVADYGQLELRILAHLTGCKSMMEAF 821
                *****:***:***:***:*****:***:***:***

PolGammaI      IAGGDFHSRTAMNMPHIREAVENGEVLEWHPPQPGQEKPPVPLLKDAFASERRKAKMLN 865
PolGammaII     KAGGDFHSRTAMNMPHVREAVENGQVILEWHPEPGEKPPVPLLKDAFASERRKAKMLN 881
                *****:*****:*.***:***:*****:***:*****:*****

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PolGammaI      FSIAYGKTAIGLSRDWKSREEAQDVTNLWYNDRQEVKRWQELRKKEAIQKGYVLTLLGR 925
PolGammaII     FSIAYGKTAVGLSRDWKVSSTKEAQETVDLWYNDRQEVKRWQEMRKKEAIEDGYVLTLLGR 941
                *****:***** :*:**:*:*****:*****:*****

PolGammaI      ARKFPEYRSRAQKNHIERAAINTPVQGSAADVAMCAMEISNNQRLKELGWKLLQVHDE 985
PolGammaII     SRRFPASKSRAQRNHIQRAAINTPVQGSAADVAMCAMEISINQQLKKGWRLLLQIHDE 1001
                *:** :****:***:*****:***** **:*:***:****:***

PolGammaI      VILEGPSESAENAKDIVVNCMSEPFNGKNILSVDLSDAKCAQNWYAGK 1034
PolGammaII     VILEGPESAEIAKDIVVDCMSKPFNGRNILSVDLSDAKCAQNWYAAK 1050
                ***** ** * *****:***:*****:*****:*****.*

```

Figure 2A: Amino acid sequence alignment of DNA Polymerases Gamma I (PolGammaI) and II (PolGammaII). An asterisk indicates an identical amino acid while a double or single dot indicates similar amino acids. The two proteins show a 72% sequence identity. The N-terminal regions shows the highest level of dissimilarity.

```

PolGammaI      --MGVS-----LRHLSPPSSFWVSRRP-RVSSS-ILSFLVPRRRLCTRKVAIIKUNA 48
PolGammaII     MAMGVSLTSHNNPLLRHLSPPSSWVSRSSRLSSSPLPSFLFPCRRTLLQRKLASTDGNV 60
                ****          ***** ** * .*:*** : **.* ** * **:*.**

PolGammaI      GYSTATDCGGSHGFHSHGHRSSSVFESGEWKLNLGSKTARMVPPTVKQAGA 100
PolGammaII     GYCTTTVC---QGFQHSVHQRSSSVVFNGEWELRSESNKVRMV----- 100
                **.*:* * :***:** ***** *.***:*. *:.**

```

Figure 2B: Amino acid sequence alignment of the first 100 amino acids in the N-terminal region showing a 51% identity between the two polymerases and allowing for differences in localization.

```

PolGammaI -----ACATTT 6
PolGammaII GAATCAGTTTTTGCTAAGAAAGGTGCAATCTTTGTTGAAGATGAGCAGAGAAAAATGTAC 60
* *

PolGammaI TAAAGGATTTGCTGAGAATT-TTGTATCTGGGTTTCAAAAAGTTTC-----AGACTTGA 59
PolGammaII TCAACATTTTGCTTTTTTATCATTTTATGGCAAACAAAACAAGTTCCTTCATTAATCTAA 120
* * * * * * * * * * * * * * * * * * * * * *

PolGammaI CTCATCGCTGAACAA-AAATTA-CAGAATTA AAAATCC---CAACTTTATCGGAA----- 109
PolGammaII ATGATGACTGTACAATAAATTAACGCAACATACCATGTGTATAATTTTTTCTAAATTTAG 180
* * * * * * * * * * * * * * * * * * * * * *

PolGammaI AAATGTCTTTTTTTTTTGG-----TTTATGTATTTT--TTTTTAAAAGTGAAATGATT 160
PolGammaII GGATGTACACAATTGACGAAACATCATTTTCATAATTTGATTTTTTAGGGGAAAATCATT 240
* * * * * * * * * * * * * * * * * * * * * *

PolGammaI AATTAAGAAGATAAAAAGGGGAAAAGGGTTTAAGGGTTTGTGCATAGAGCAATGGCTACCA 220
PolGammaII AATACATACATACACAAACCAAGAAAATTTTATTGTCATGAAGAGATTCATGGTCACAT 300
* * * * * * * * * * * * * * * * * * * * * *

PolGammaI GCTCAGCTTTGTGATAAAACGACAAAATTGACAACGATAAACCCCTACTTTT---ATCT 276
PolGammaII CATGAACTAAAAATCACAGAGACGGAGAAACCAACCATGTTTCTTGGTCATCAGGATCT 360
* * * * * * * * * * * * * * * * * * * * * *

PolGammaI CT-----GGAAGACTCAAAATTCCTTCAAGCTGGGGAG--ATTAGCT-----TCACTTA 323
PolGammaII CTCTGAAAGAAAACCTCTGATATGCAACACACCAAGAAGCTATTAGTTAGCTCATAATTTA 420
* * * * * * * * * * * * * * * * * * * * * *

PolGammaI CTACGGCCATG----- 334
PolGammaII GTACGTTCAAAAATACAAATTA AACACTTAAAACCAACCAGTCCTTAAAAAGATACTA 480
* * * * * *

PolGammaI -----
PolGammaII AAAGCATAAACATCACCCAGCTTATATTGATCTTAGATTACTCTACTTGCATTTGACTAA 540

PolGammaI -----
PolGammaII CTCAAAACAAGATTTAACATGCCTAAATCCAACGAGCTGCTGCTAAAATTTCTCTCTATTA 600

PolGammaI -----
PolGammaII CGGCTCTCATTTTTTTTCAACGTCCATGCCACATTTTTCTGTGTTTCCATAATATACTCCT 660

PolGammaI -----
PolGammaII TTAGCGATAATGCAATAACCTTGAGTTCTTGAACTTTTTTCGTAGGAGTCATTAGCACTT 720

PolGammaI -----
PolGammaII TAGCAGCATCAGCTTCACCAAAATCATTAAGATATTACAGTTAATAAGAAATGATGCAAT 780

PolGammaI -----
PolGammaII GATGATACTTTTGAGAGTGGCTAATAACTCGCTATCAATCATGTCAATATAAGTAACAA 840

PolGammaI -----
PolGammaII GACTTTGTGACATAAAATGATAAAAAAATATGTGCCAATATAGCATTTTAGTAGAATTG 900

PolGammaI -----
PolGammaII TTGGGTCAAGTAGAATAAGGTGTTTCAGAAAAAAAAAAGTTAAAGTAAAGTAGTTTATTTA 960

```

```

PolGammaI -----
PolGammaII TTTTTTAATAAAAATTGGCAAAAAGAAAGGGCTATATTTGTAAATAGTCCTTTTTAGAAAT 1020

PolGammaI -----
PolGammaII TAATAATCCGAAGCTTCTTTTTTGACAGAGCAATGCAATCTTTATCATCTTAGGTTTTAC 1080

PolGammaI -----
PolGammaII AGTGTGATTAGTATAATTAACACTAAAGATTTAATAATAATAGAAGCAAGGTTTAAGGC 1140

PolGammaI -----
PolGammaII TAATACACACAAAAGAGCGTCTTCAAGCGATGGCCACAATCTTCTTCTTCTGCTTAAGCT 1200

PolGammaI -----
PolGammaII TTCCTTCTCAGGCAGACCCACTTGCTTGAACTAGCTCGGAACGATAACGACAAAATTTCG 1260

PolGammaI -----
PolGammaII CCACCATAAACCTATTTTTAATCTCCGACTCTGTCGTCACTTCTCCTTCAAGCTGGGTA 1320

PolGammaI -----
PolGammaII GATTAAGTTCGCTTATG 1337

```

Figure 3: Sequence alignment of the regions upstream of the start codon for DNA Polymerases Gamma I and II showing possible promoter alignment and differences.



1

2

3

4

Figure 4: Starting from the left, plants 1 and 2 are homozygous for an insert in DNA Polymerase Gamma I and heterozygous for an insert in DNA Polymerase Gamma II. Plant 3 is homozygous for an insert in DNA Polymerase Gamma I, but has no inserts in DNA Polymerase Gamma II. This shows that the insert in DNA Polymerase Gamma II had little effect on these first two plants. Plant 4 is heterozygous for an insert in both DNA Polymerase Gamma I and DNA Polymerase Gamma II. All plants were grown at the same time and under identical



1

2

Figure 5: Plant 1 is heterozygous for an insert in DNA Polymerase Gamma II and Plant 2 is homozygous for an insert in DNA Polymerase Gamma II. Both plants were grown at the same time under identical conditions.



Figure 6A: Plants on the left are heterozygous for an insert in DNA Polymerase Gamma II and plants on the right are heterozygous for an insert in DNA Polymerase Gamma I. All plants were 6 week old plants, grown at the same time under identical conditions.



Figure 6B: Plants heterozygous for an insert in DNA Polymerase Gamma I.



Figure 6C: Plants heterozygous for an insert in DNA Polymerase Gamma II.



Figure 7: The seeds on the left came from 45 plants heterozygous for an insert in DNA Polymerase Gamma I and the seeds on the right came from 45 plants heterozygous for an insert in DNA Polymerase Gamma II. These seeds came from the plants in Figure 6 above along with other identical plants grown alongside those plants.

Chloroplast DNA in COWT vs. Polymerase Gamma I (1 insert), Polymerase Gamma II (1 insert) and Polymerase Gamma I (2 inserts) plants

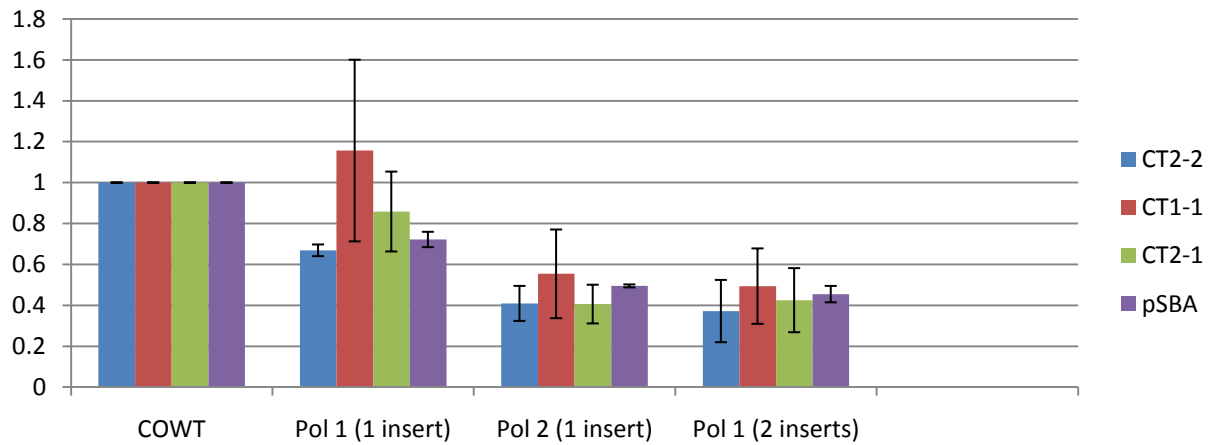


Figure 8: QPCR results for chloroplast DNA copy number.

Mitochondria DNA in COWT vs. Polymerase Gamma I (1 insert), Polymerase Gamma II (1 insert) and Polymerase Gamma I (2 inserts) plants

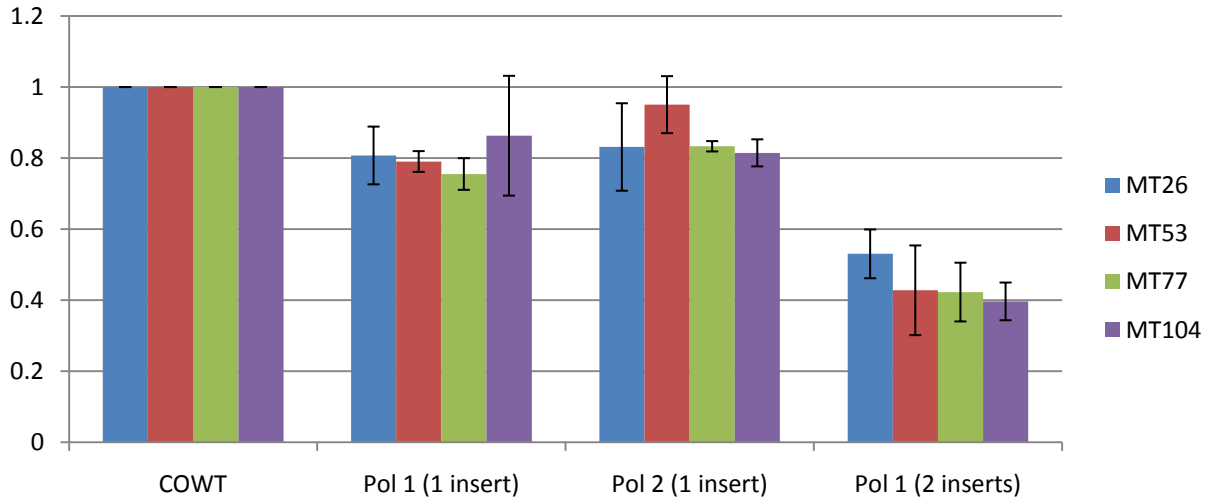


Figure 9: QPCR results for mitochondria DNA copy number.



Figure 10A: Slot Blot Analysis of DNA Polymerase Gamma I expression at weeks 3,4,5,6,8,10



Figure 10B: Slot Blot Analysis of DNA Polymerase Gamma II expression at weeks 3,4,5,6,8,10

Region	Forward Primer	Reverse Primer	T _m
UBC	gattcgtgttgcttcagttc	attgaacctctcacatcac	51.9°C, 52.2°C
MT26	ttgacagttcggctctgatcc	tcaatcggaagctcaagaag	54.4°C, 52.4°C
MT53	atgccacaacgactctatcg	tctcggatcactcatggaac	54.7°C, 53.9°C
MT77	tgactcggatgtaagcgatg	tgactcggatgtaagcgatg	54.4°C, 54.9°C
MT104	tgactcggatgtaagcgatg	ttgtcaatccggttgctt	52.9°C, 51.4°C
CT1-1	cacgacceatataacaagctacacc	gctgcatccggtgatgaatggcta	57.3°C, 59.8°C
CT2-1	tgactgtactctgggacggaagg	agcggtaaggcaacgggttttgggt	59.9°C, 63.5°C
CT2-2	aatgactgtactctgggacg	tagccaagcggtaaggcaa	53.9°C, 56.9°C
pSBA	aactaagtcccactcacga	catccggtgatgaatggcta	53.2°C, 52.6°C

Table 1: QPCR primers used in this study; forward and reverse sequences with melting temperatures. Primer sequences are given 5' to 3'.

Gene	Forward Primer	Reverse Primer	Tm
DNA Polymerase Gamma I	aggccttcatagcatcacctg	tacgagcccttccaacaag	60.2°C, 60.3°C
DNA Polymerase Gamma II	aagccacctgtgccgttgta	actatgctcttgcgatctcc	60.2°C, 60.1°C

Table 2: RNA slot blot probe primers. Primer sequences are given 5' to 3'.

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