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# THE COMPLEXITY OF ELECTRON TRANSPORT: A STUDY OF VARIEGATION THROUGH CHEMICAL INHIBITION OF PLASTOQUINOL TERMINAL OXIDASE (PTOX) IN TOBACCO (NICOTIANA TABACAUM)

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by

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**Abstract**

The photosynthetic electron transport (PET) chain relies on the chlorophyll and carotenoid biosynthetic pathways for pigments that harness light, and protect the photosynthetic apparatus from damage by light. The pigment pathways also connect to the PET chain through electron transfer where the plastoquinol (PQ) pool accepts electrons from phytoene desaturase (PDS) of the carotenoid pathway and the aerobic cyclase (ACS) of the chlorophyll biosynthetic pathway. Genetic studies have shown a plastoquinol terminal oxidase (PTOX) protein functions to regulate the redox state of the PQ pool. Without PTOX to maintain balance between the two redox states of the PQ pool it is at high risk of becoming hyper-reduced. The same studies have shown mutated PTOX causes variegation (abnormal white sectors within the normal green tissue on a plant's leaves). The lack of PTOX due to mutation allows the PQ pool to become hyper-reduced. Hyper-reduction prevents PDS and ACS from using the PQ pool as a source to deposit electrons from their respected pathways. This stops pigment production and variegation becomes apparent on the plants' leaves. The current study explores the hypothesis that in addition to being caused by genetic mutations, variegated plants can also be generated using a direct chemical inhibitor of PTOX. The study also explores variegation under the condition of drought stress. This study is the first to apply chemicals as a means of inhibiting PTOX activity and in a whole plant system of tobacco (*Nicotiana tabacum*). The observations of the treated tobacco leaves showed variegation had occurred and pigment analysis of the abnormal sections showed there were significant changes in the chlorophyll and carotenoid concentrations of tobacco leaves compared to the control (normal leaf). These findings support the literature in that the PTOX interacts with the pigment pathways since the chemical inhibitors altered pigment concentrations. Using whole tobacco plants, we provided findings that further show the complexity and

integration of the carotenoid and chlorophyll biosynthetic pathways with the photosynthetic electron transport chain.

**Acknowledgments**

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## 1. Introduction

### 1.1 Chlorophyll Biosynthesis

Colours are observed wavelengths of light from the visible light spectrum (400 nm – 700 nm), and are perceived based on the transmittance or reflectance of a particular wavelength (Figure 1) (Tilley, 2010). Photosynthetic organisms typically appear green due to the vast amount of chlorophyll molecules reflecting green wavelengths of the spectrum (Tanaka and Tanaka, 2007). Chlorophyll molecules belong to a family of pigments called tetrapyrroles. Most chlorophylls absorb both blue and red light; chlorophyll *a* favours absorption of red light and chlorophyll *b* absorbs primarily blue light (Lichtenthaler and Buschmann, 2001). Their structure consists of a hydro-carbon ring head, with four nitrogen molecules surrounding a magnesium atom, and a long hydro-carbon tail with double bonds (Figure 2) (Reinbothe and Reinbothe, 1996). The difference between chlorophyll *a* and *b* is that *a* has a methyl group on the seventh carbon of the ring, whereas *b* has a formyl group on the same carbon (Cznecki and Grimm, 2012). Typically, chlorophylls are the reaction centre of the light harvesting (LH) complexes attached to photosystem II (PSII) and photosystem I (PSI) of the photosynthetic electron transport chain. The LH complexes are where the energy from photons are harnessed in order to excite the chlorophyll's electrons, and the energy absorbed by these electrons are later transduced into chemical energy (Tanaka and Tanaka, 2007).

Chlorophyll synthesis is a pathway divided into three main stages (Figure 3). The first begins with a molecule of glutamate being modified in three reactions, to form 5-aminolevulinic acid (ALA). The second stage forms a protoporphyrin IX ring from ALA, and the third stage results in chlorophyll formation (Cznecki and Grimm, 2012). The first two steps are oxidation reactions on the nitrogen atoms in the ring. Their electrons are removed to share electrons with

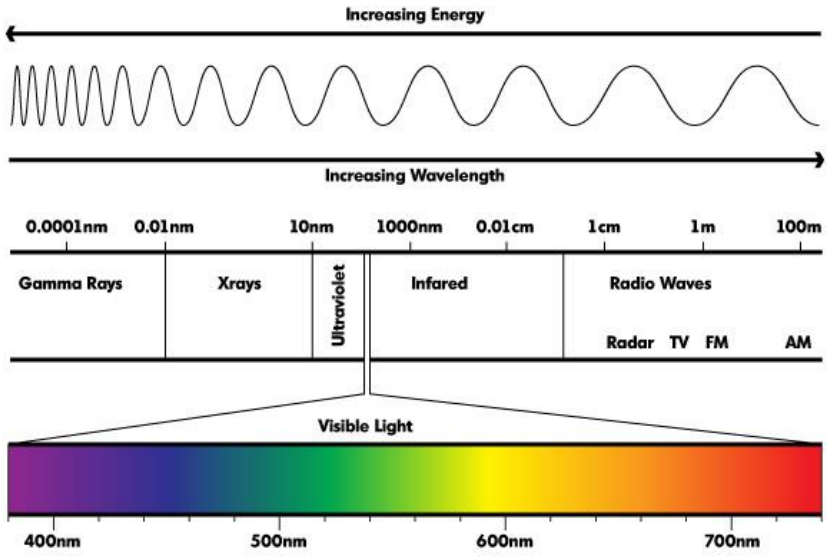


Figure 1. The visible light spectrum. The range of the visible light spectrum shows the wavelengths of visible light relative to the electromagnetic spectrum. The visible light spectrum is also shows the colours of the light within the visible light spectrum ranges. Modified from Cyberphysics.

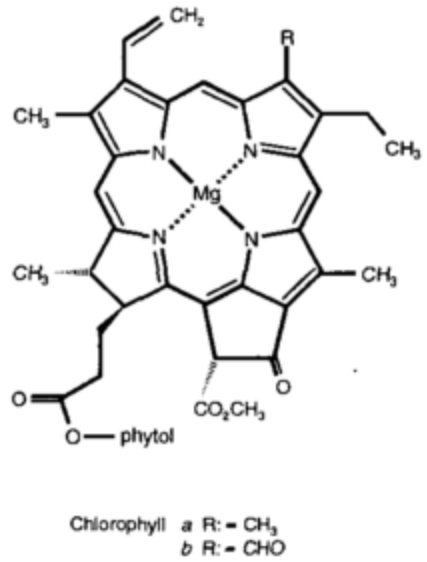


Figure 2. The structure of a chlorophyll molecule. The head of the molecule with a ring structure of four nitrogen atoms supporting the magnesium core is shown. The R group on C-7 is where the methyl or formyl groups are present in chlorophyll *a* or *b*, respectively. The phytol tail is not shown here. From Reinbothe and Reinbothe, 1996.

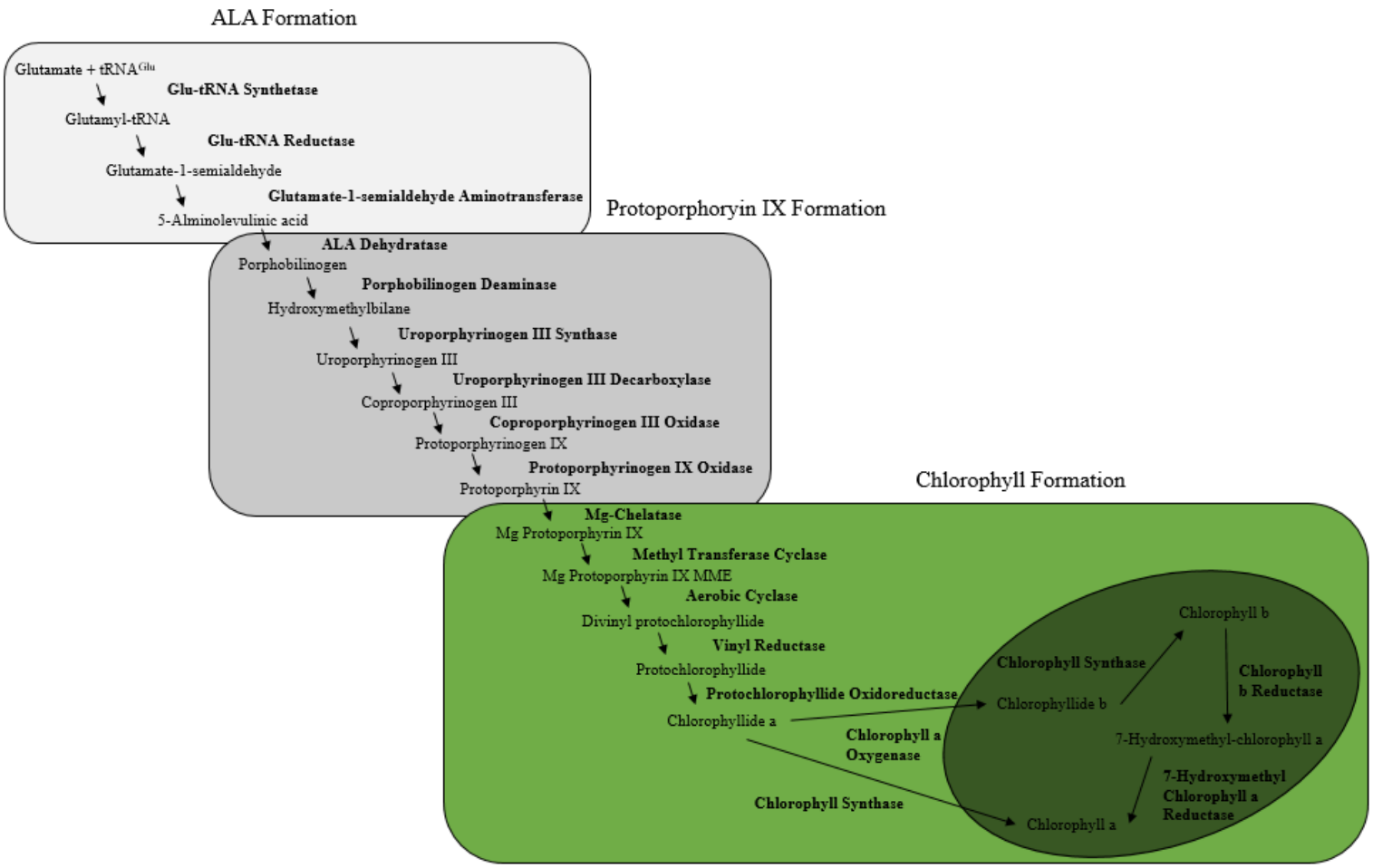


Figure 3. The chlorophyll biosynthetic pathway. Synthesis begins with Aminolevulinic acid (ALA) formation, followed by protoporphyrin IX ring formation, and lastly chlorophyll formation. The ring begins to form from the porphobilinogen molecule to the hydroxymethylbilane molecule. The next few steps are completed to close the ring structure. The protoporphyrin IX molecules is the first molecule that represents the beginning of chlorophyll formation. The magnesium ion is added to the ring by magnesium chelatase. The aerobic cyclase (ACS) that catalyzes the reaction from Mg protoporphyrin IX monomethylester (MME) to divinyl protochlorophyllide takes the electrons and deposits them into the PQ pool. The protochlorophyllide oxidoreductase (POR) enzyme requires light to be activated and catalyze the conversion of a protochlorophyllide molecule to chlorophyllide a. The molecules being to absorb light and turn green. Modified from Cznecki and Grimm, 2012; and, Reinbothe and Reinbothe, 1996.

the magnesium ion. An aerobic cyclase (ACS) enzyme completes this oxidation reaction and removes the electrons from the nitrogen atoms. Research by Steccanella *et al*. (2015) proposed ACS deposits the electrons directly into the plastoquinone (PQ) pool. The next few steps in chlorophyll formation attach the phytol tail to the ring. One step between the monovinyl protochlorophyllide *a* to chlorophyllide *a* catalyzed by the protochlorophyllide oxidoreductase (POR) requires light to be activated (Reinbothe and Reinbothe, 1996). After this point in the pathway the molecules begin to reflect green light, unlike the previous molecules where they are colourless. In total, there are six reactions from the protoprophyrin IX ring step needed to make chlorophyll *a* whereas there are seven needed to make chlorophyll *b* (Cznecki and Grimm, 2012).

The amount of chlorophyll produced by photosynthetic organisms varies due to a variety of factors, including light intensity. Lichtenthaler and Buschmann (2001) created a generalized chlorophyll *a/b* ratio range under different light intensities for plants. Lichtenthaler and Buschmann (2001) looked at fully developed leaves of tree species when they created the chlorophyll *a/b* ratio range, including Beech (*Fagua sylvatica*), Hornbeam (*Carpinus betulus*), and Poplar (*Populus nigra*). In mature leaves under high light, the chlorophyll *a/b* ratio is between 3.0 – 3.8 whereas it is around 2.4 – 2.7 in mature leaves in shade. During the greening process of etiolated leaves (de-etiolation), chlorophyll is synthesized rapidly to harness light (Hopkins and Hüner, 2009). Lichtenthaler and Buschmann (2001) also tested the chlorophyll *a/b* ratio during the greening of etiolated leaves, which was found to be between 4.0 – 10.0.

## 1.2 Carotenoid Biosynthesis

Since chlorophyll molecules are a vital component of photosynthesis, they need to be protected which is the function of another group of pigments called carotenoids. Carotenoids are found in the antennae complexes surrounding the reaction centres and are used to funnel the energy from light to the chlorophyll *a* reaction centre. In doing this, the carotenoid pigments prevent the chlorophyll *a* reaction centre from receiving light directly. If the carotenoid pigments were absent, the chlorophyll *a* reaction centre would receive light directly which would result in photo-oxidation of the chlorophyll *a* molecule. Photo-oxidation can occur in two ways, either electrons are donated to oxygen or due to the exposure of ultraviolet light (Foyer *et al*., 1994). Chlorophyll molecules can become photo-oxidized to form singlet oxygen ($^1O_2$) which is a toxic form of reactive oxygen species (ROS) that causes damage to cells (Demming-Adams and Adams, 1992). When chlorophyll *a* has become photo-oxidized, it cannot harness light which prevents photosynthesis from occurring. In order to protect the photosynthetic electron transport (PET) chain, photo-protective carotenoids are necessary (Bartley and Scolnik, 1995). Like chlorophyll, these pigments transmit and reflect certain wavelengths of the visible light spectrum. They are not usually visible to the human eye when observing a leaf due to the massive amount of chlorophyll pigments reflecting green wavelengths, which masks the reflected light from other less abundant pigments such as carotenoid. For example, carotenoids become visible in leaves of deciduous trees in autumn after chlorophyll degradation occurs (Hörtensteiner, 2006).

The general carotenoid structure consists of a forty-carbon symmetrical compound that contains double bonds used to absorb light (Simkin *et al*., 2008). In different carotenoids, there are certain groups attached to the ends of the chains; for example beta-rings are characteristic of

β-carotene. The carotenoid biosynthetic pathway (Figure 4) begins with two molecules called geranylgeranyl diphosphate (GGDP) that are subjected to several oxidation-reduction reactions (Simkin *et al*., 2008), similarly to the chlorophyll biosynthetic pathway. An enzyme called phytoene synthase (PSY) takes both GGDP's and produces phytoene which is a long colourless carbon chain. The enzyme phytoene desaturase (PDS) oxidizes phytoene to ζ-carotene and like ACS of the chlorophyll pathway, it transfers the electrons directly to the PQ pool (McDonald *et al*., 2011). ζ-carotene is the first coloured carotenoid made in the pathway, found to reflect yellow light (Table 1). Then lycopene, β-carotene and zeaxanthin are produced in the following steps. In the diagram, their reflected colours are shown (Figure 4).

### 1.3 Photosynthetic Electron Transport and the connection to the pigment pathways

Both the chlorophyll and carotenoid biosynthetic pathways have enzymes that function to oxidize molecules to remove the electrons from their respective pathways where the electrons are transferred to the PQ pool. The enzymes ACS from the chlorophyll pathway, and PDS from the carotenoid pathway, use the PQ pool as an electron acceptor (Steccanella *et al*., 2015; McDonald *et al*., 2011). This shows that photosynthesis is not a linear pathway. The transfer of electrons allows the chlorophyll and carotenoid pathways to synthesize the pigments needed to absorb light and protect the photosynthetic apparatus.

The photosynthetic electron transport chain can be thought of as a large redox reaction and begins when a photon of light is absorbed by the light harvesting complexes (Hopkins and Hüner, 2009). The photon is converted to chemical energy as it excites two electrons of the chlorophyll molecules in the reaction centre of PSII, which become oxidized (Figure 5). Within PSII, the electrons make their way through the complex and are eventually accepted by

Table 1. Plant pigment characteristics. The table shows the absorption range, wavelength colour observed and the reflected colour for each pigment.

| Pigment | Absorption range | Wavelength colour | Reflects |
|---|---|---|---|
| Chlorophyll *a* | 645 nm – 675 nm<br>400 nm – 440 nm | Red<br>Blue | Green |
| Chlorophyll *b* | 440 nm – 475 nm<br>625 nm – 655 nm | Blue<br>Red | Green |
| ζ-carotene | 400 nm – 500 nm | Blue/Green | Yellow |
| Neurosporene | 400 nm – 500 nm | Blue/Green | Orange |
| Lycopene | 400 nm – 500 nm | Blue/Green | Red |
| β-carotene | 400 nm – 500 nm | Blue/Green | Orange |
| Zeaxanthin | 400 nm – 500 nm | Blue/Green | Yellow |

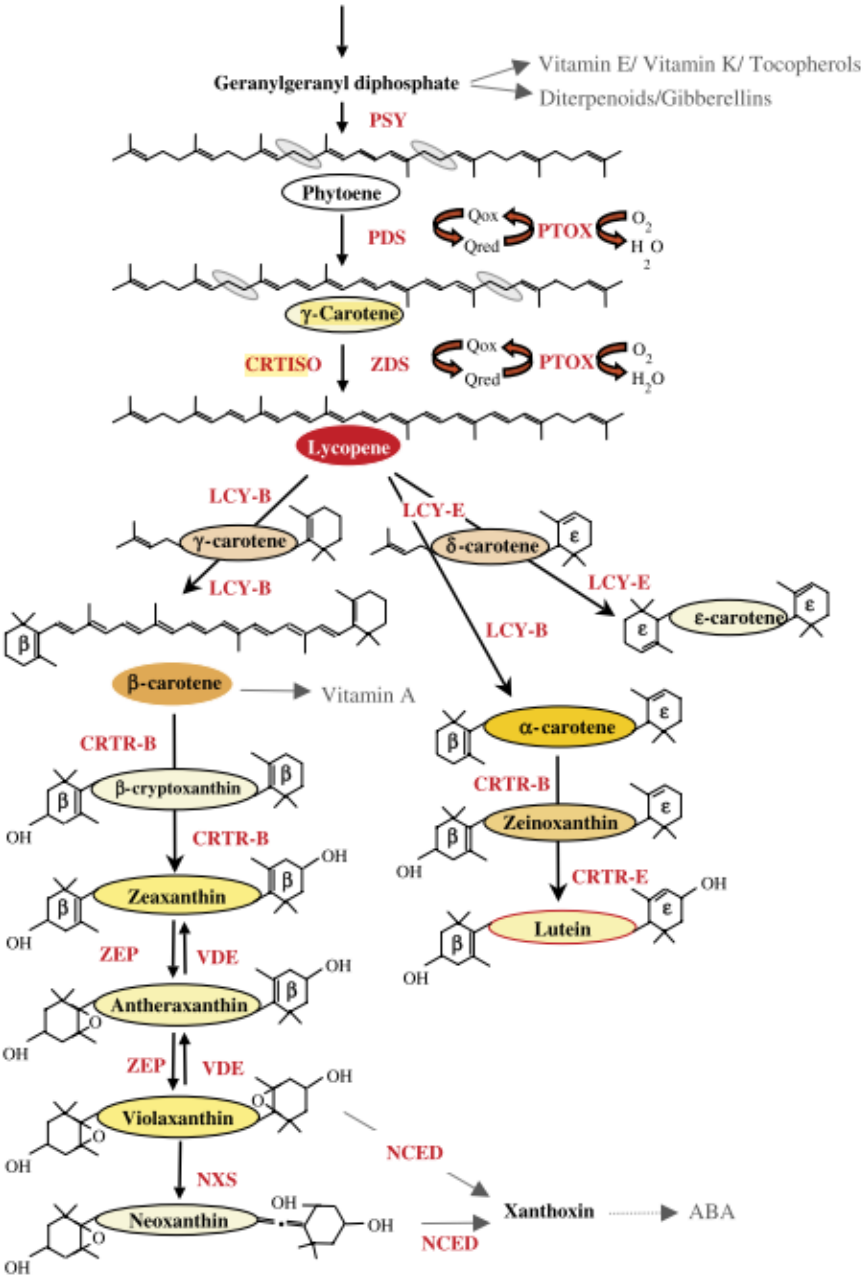


Figure 4. A general overview of the carotenoid biosynthetic pathway. Geranylgeranyl diphosphate is the beginning molecule used to synthesize the carotenoid pigments. PSY, phytoene synthase, creates phytoene from the geranylgeranyl diphosphate. PDS, phytoene desaturase, oxidizes the electrons from phytoene and creates ζ-carotene while depositing the electrons into the PQ pool. ζ-carotene is the first pigment within the pathway to show a light orange colour. ZDS, ζ-carotene desaturase, synthesizes lycopene which reflects red light. Lycopene is transformed into β-carotene by LCY-B, lycopene β-cyclase. Zeaxanthin is formed from CRTR-B, β-carotene hydroxylase. From Simkin *et al*., 2008.

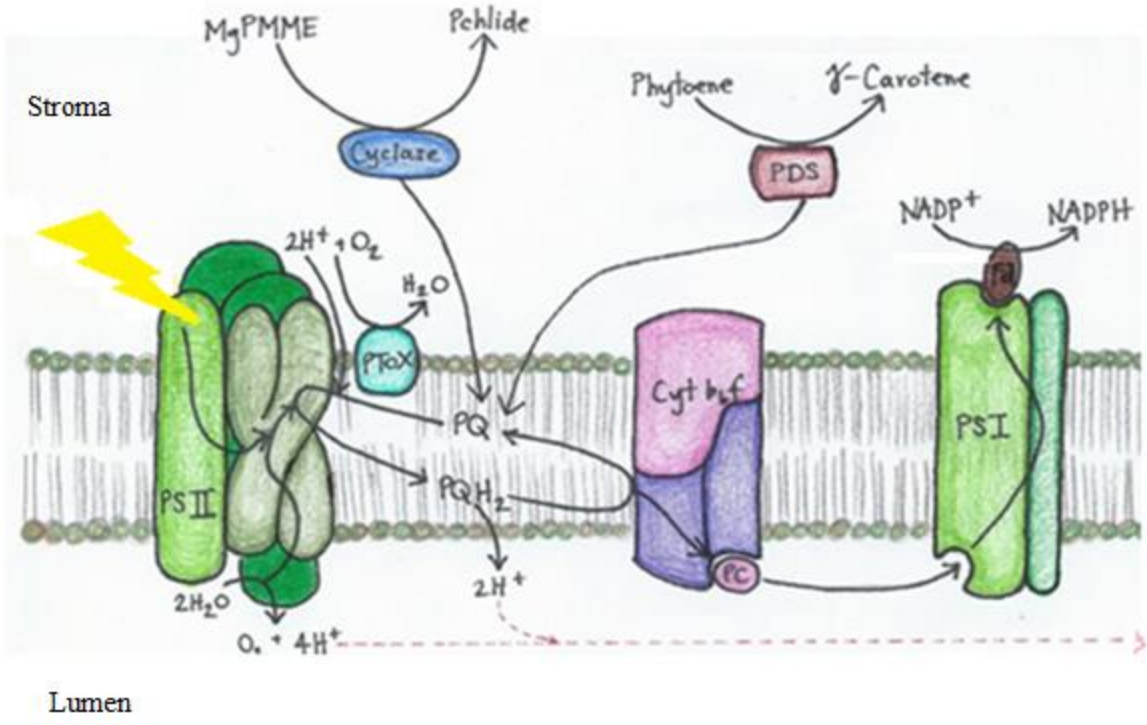


Figure 5. The photosynthetic electron transport chain. The yellow zigzag arrow represents a photon of light being absorbed by a chlorophyll molecule of photosystem II (PSII). The black arrows represent the electrons being transferred between the photosynthetic complexes PQ (plastoquinone), PQH2 (plastoquinol), PTOX (plastoquinol terminal oxidase), Cyt $b_6f$ (cytochrome $b_6f$ complex), Pc (plastocyanin), PSI (photosystem I), and Fd (ferredoxin). The blue circle represents the enzyme aerobic cyclase (ACS) that catalyzes a step in the chlorophyll biosynthetic pathway and is theorized to deposit electrons into the PQ pool. PDS (phytoene desaturase) is an enzyme that catalyzes a step in the carotenoid biosynthetic pathway and it too deposits electrons into the PQ pool Modified from Hopkins and Hüner, 2012.

plastoquinone (PQ) of the PQ pool. As the electrons are transferred from PSII to the PQ pool, the oxidation of water occurs in the lumen of the thylakoid membrane. The protons that are liberated from the oxidation of water remain in the thylakoid lumen where they contribute to the proton gradient that is used to drive ATP production by ATP synthase. The oxygen that is released during the oxidation of water is released into the environment as a by-product, and the two electrons are used to replace those lost from the chlorophyll reaction centre of PSII. When PQ takes the two electrons from PSII, it becomes reduced to plastoquinol ($PQH_2$) and loses affinity for the PSII complex. The reduced $PQH_2$ moves the electrons in the thylakoid membrane to the cytochrome $b_6f$ complex (Cyt $b_6f$). The electrons are moved through the rest of the chain and end up in a NADPH carrier protein that transfers the electrons to the light-independent reactions to produce carbohydrates through carbon fixation (Hopkins and Hüner, 2009).

The PQ pool is usually depicted as two molecules, one in each of the redox states. This is only to simplify the diagram as there are multitudes of PQ being oxidized and reduced as electrons enter and exit the pool (Hopkins and Hüner, 2009). Since the PQ pool can receive electrons from PSII, ACS and PDS, there is a lot of reducing power entering the pool. Balance needs to be maintained otherwise the PQ pool can become hyper-reduced (Eberhard *et al.*, 2008) where many problems can arise and cause problems for electron transport. Hyper-reduction of the PQ pool results in a quiescent state where electrons cannot enter or exit through the pool and the electrons cause damage in the form of photo-oxidation to the PET chain (Eberhard *et al.*, 2008). To overcome hyper-reduction of the PQ pool, plants have a specialized protein called plastoquinone terminal oxidase (PTOX).

### 1.4 Plastoquinol terminal oxidase

All plants contain a plastoquinol terminal oxidase (PTOX) in their chloroplasts. PTOX is integrated in the stromal side of the thylakoid membrane located above the PQ pool. The biochemical function of PTOX is to oxidize plastoquinol ($PQH_2$) to plastoquinone (PQ), likely as a means of regulating the balance between the two states of oxidized PQ and reduced $PQH_2$ (Figure 5). The purpose of PTOX has been debated for years, and there is evidence suggesting PTOX may be pertinent for early chloroplast biogenesis (Aluru *et al*., 2006), that PTOX may be the connection to the chlororespiratory pathway (Peltier and Cournac, 2002), and PTOX may have a role in stress tolerance (Kambakam *et al*., 2016). Ultimately, PTOX prevents hyper-reduction of the PQ pool which allows for continuous electron flow through the PET chain. The structure of PTOX was found to resemble that of the mitochondrial alternative oxidase (AOX) enzyme in that they both contain a di-iron centre (Figure 6) (Berthold, 2000).

### 1.5 Genetic studies of PTOX, the carotenoid biosynthetic pathway and variegation

Previous work has investigated PTOX through its gene *immutans* using *Arabidopsis* plants. When mutated, *immutans* does not produce PTOX (Cournac *et al*., 2000). To determine if PTOX was absent within the mutants, the sections were applied with known chemical inhibitors that PTOX is sensitive to, including n-propyl gallate (nPG) (Siedow and Bickett, 1981) and salicylhydroxamic acid (SHAM) (Goyal and Tolbert, 1990). Without PTOX to regulate electrons and balance the redox state of the PQ pool, it is more likely to become hyper-reduced under environmental stress (Carol and Kuntz, 2001). When hyper-reduction occurs, the carotenoid enzyme PDS cannot deposit the electrons into a reduced PQ pool which means phytoene cannot be oxidized. This leads to an accumulation of phytoene within the plant's leaves which means the

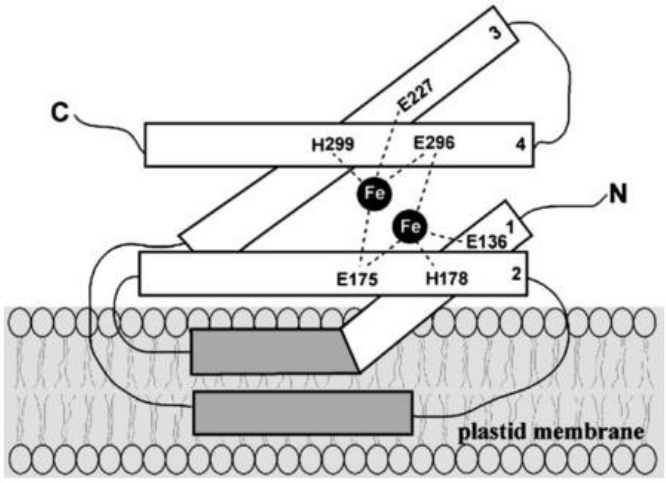


Figure 6. A representation of PTOX showing its predicted structure and topology in the membrane. PTOX is an interfacial membrane protein within the chloroplast, localized to the thylakoid membrane. PTOX contains a di-iron centre similar to AOX. From Aluru, 2006.

photoprotective carotenoids are not being made (Simkin *et al*., 2008). When the dysfunction of PTOX occurs, white-yellow abnormal sectors appear within the green leaves of plants. The reason these abnormal sectors are observed is due to the lack of photoprotective carotenoids (Rodermel, 2002). The accumulation of phytoene occurs in the variegated sections however phytoene is a colourless molecule (Wetzel *et al*.,1994). The abnormal sectors also do not have functional PET chains (Carol and Kuntz, 2001) and chlorophyll biosynthetic pathways (Steccanella *et al*., 2015).

In addition to the effects of PTOX dysfunction on carotenoid biosynthesis, there may be a direct or indirect effect on chlorophylls. Wetzel *et al*. (1994) completed a study on PTOX mutants using high-performance liquid chromatography (HPLC) to compare wild-type (WT) *Arabidopsis* leaves, the green sectors of the *immutans* mutant in *Arabidopsis* leaves, and the variegated white sections of the *immutans* mutant in *Arabidopsis* leaves. The chlorophyll concentration for the WT *Arabidopsis* leaves was 581.22 µg $g^{-1}$ per fresh weight of tissue, the β-carotene was 11.72 µg $g^{-1,}$ and there were no detectable phytoene concentrations within the WT leaves. In the green sectors of a mutated plant, the chlorophyll was 638.93 µg $g^{-1}$, the β-carotene was 9.68 µg $g^{-1,}$ and again, there was no detectable levels of phytoene. This is interesting because there is an increase in the amount of chlorophyll in the green sectors of mutant leaves indicating that photosynthesis is occurring and that the green sectors may be attempting to compensate for the lack of photosynthesis in the white sectors. In the abnormal white sectors, there were no detectable levels of the chlorophyll and β-carotene pigments, however, the phytoene level was 22.19 µg $g^{-1}$.

How variegation arises is a complex process that has provided us with a deeper understanding of the integration of the photosynthetic and pigment biosynthetic pathways,

electron flow and regulatory mechanisms, as well as plant stress tolerance. Genetic studies of PTOX inhibition have only focused on the biological mutation where the PTOX protein is not being made and therefore not functional from the start, for example the pigment concentrations of Wetzel's *et al*. (1994) work. In this study, chemical inhibitors were used to target PTOX and PDS to test whether carotenoid and chlorophyll concentrations are undetectable in pale sectors as was reported by Wetzel *et al*. (1994).

Based on the studies of Siedow and Girvin (1980), Siedow and Bickett (1981), and Cournac *et al*. (2000), that have worked with nPG and SHAM in testing the presence of PTOX, this study used both to cause variegation. Using two PTOX inhibitors will allow for a comparison between the chemicals effectivity to inhibit PTOX. nPG has been found to be a stronger inhibitor than SHAM (Cournac *et al*., 2000). Initially nPG and SHAM were chemical inhibitors primarily used to test if the alternative oxidase (AOX) protein was present in isolated mitochondria in respirometry experiments (Josse *et al*., 2000). In AOX studies, inhibition of AOX does not result in a phenotypic colour change (Fiorani *et al*., 2005). In this study, they will be used differently than the previous work. Instead of an *in vitro* application nPG and SHAM were applied to whole living tobacco leaves to determine their inhibitor properties in a live system. If the inhibitors are successful at inhibiting PTOX we expect that it will results in the development of a variegated phenotype which should be observed on the leaves of the tobacco plants.

In comparison, the herbicide norflurazon is known to inhibit PDS of the carotenoid biosynthetic pathway and cause 'bleached' leaves as reported by Bartels and Watson (1977). The 'bleached' phenotype is an extreme of variegation in that the abnormal sectors are completely

white. Norflurazon is used to control grass and broad-leafed weeds. Due to its known effect on the colour of plants, it was used as my positive control.

### 1.6 Purpose

The purpose of this study is to induce variegation in whole living tobacco leaves using chemical inhibition of PTOX, as well as PDS, to allow real time observations and detect the colour change of normal green sectors to abnormal white sectors in wild-type tobacco leaves (*Nicotiana tabacum*). In addition, we will investigate the chlorophyll and carotenoid pigment concentrations within the variegated sections to quantitate the pigment concentrations in normal green leaves compared to abnormal sectors.

### 1.7 Hypothesis

I hypothesize that the application of chemical inhibitors chosen to target PTOX and PDS will cause variegation, due to changes to the chlorophyll *a* and *b*, and carotenoid pigment concentrations in tobacco leaves.

Since norflurazon is a known herbicide that targets PDS and causes bleached leaves, I predict the leaves will show variegation. Inhibition of PDS prevents the production of carotenoids downstream of phytoene, therefore I predict that the chemical inhibition of PDS will cause a large decrease in the total carotenoid pigment concentration. Since PDS will not be able to oxidize phytoene, there will be a higher concentration of it in the variegated sections. With the lack of photo-protective carotenoids, the chlorophyll pathway will also be affected in directly because of the lack of protection in the LH complexes. I therefore predict a decrease in the chlorophyll concentration in the norflurazon-treated plants as well.

I predict n-propyl gallate (nPG) and salicylhydroxamic acid (SHAM) will also cause variegation. Since these inhibitors prevent PTOX from functioning and impair its ability to regulate the PQ pool, both the carotenoid and chlorophyll biosynthetic pathways that rely on the PQ pool to receive their electrons will be affected. This means phytoene and the chlorophyll precursor molecule, Mg protophorphryin IX monomethylester, will increase. I predict a decrease in the total carotenoid and chlorophyll concentrations in the nPG and SHAM treated plants.

### 1.8 Objectives

This study has two main objectives:

1. Develop a direct method of inhibiting the enzyme PTOX using the chemical inhibitors nPG and SHAM thereby causing variegation.
2. Quantify pigment concentrations (*i.e.* total chlorophyll and carotenoids) within the variegated white and normal green sectors of treated plants.

## 2. Methods

### 2.1 Seed source, cleaning, and storage

*Nicotiana tabacum* WT tobacco seeds were obtained from Dr. G. Vanlerberghe at the University of Toronto, Scarborough Campus. After delivery, the seeds were stored at 4 °C.

The seed surface was cleaned with a 10 % (v/v) bleach solution for 2 minutes and then bleach was removed. 1 mL of filtered MilliQ water was transferred and the tubes were gently inverted for 2 minutes. The water was removed and 1 mL of the 10 % (v/v) bleach solution was added again for 2 minutes. The bleach solution was removed and 1 mL of MilliQ water was used to rinse the seeds of the bleach solution; this cycle was repeated 3 times to ensure the bleach

solution had cleaned the seeds. Finally, the seeds were kept in 1 mL of unsterilized MilliQ water, labeled with the day they were cleaned, sealed with parafilm, and stored at 4 °C until use.

### 2.2 Hoagland's solution

Hoagland's solution was used to water the seedlings and plants as the source of nutrients (Hoagland, 1937). The concentration of Hoagland solution used to water the seedlings and plants was [1x] (Appendix A). Depending on the number of replicates on the needle-less injections experiments, the stock solution changed (Appendix B); *i.e.* 2-3 experiments were watered with [1x] Hoagland solution in 1 L autoclave bottles, whereas, if 4 or more experiments were being conducted, a 20 L carboy of [5x] was made and left in the greenhouse, then the solution was diluted to [1x] for watering. Plants were watered with Hoagland's solution occurred every other day, after the seedlings were 15 days old, however before the seedlings were 15 days old they were only watered with DI water (Dahal *et al*., 2014).

### 2.3 Seed Propagation

For the purpose of seed propagation, plants were grown in 4-inch pots that had been washed with soap and water. The pots were filled to the top with vermiculite (Therm-o-rock, Plant Products) and clean seeds were placed on non-sterile vermiculite and spread throughout the top layer using a scoop. A beaker was filled with DI water and poured over the seeds until the vermiculite was saturated. A drip tray was used under the pots to catch the excess water. The watering schedule consisted of pouring 50 mL of DI water over each pot every other day, as well as filling up the drip tray. The seeds germinated in the vermiculite for 15 days and then were transferred to soil (see below). A [5x] Hoagland solution was prepared and stored in a 20 L

carboy in the greenhouse (Appendix B). The [5x] Hoagland solution was diluted to [1x] when needed to water the plants.

The growth conditions of the greenhouse included a range of temperature from 21 °C in the day to 18 °C at night, the photoperiod was maintained at 16 hours light, and 8 hours of darkness by using supplementary lighting. The light intensity was within the range of 180 LUX to 250 LUX, depending on the weather conditions outside of the greenhouse (*i.e.* a cloudy day vs. a sunny day). The high-pressure sodium light bulbs provided the supplemental lighting to reach the full 16 hours of light during the winter months.

On day 16 after the seeds were planted in the vermiculite, the seeds were transplanted into soil. The composition of the soil consisted of 4 parts soil (Pro-Mix BX, Plant Products) and 1 part vermiculite (Dahal *et al*., 2014). In a large Nalgene bin, 4 litres of soil and 1 litre of vermiculite were mixed together with 1.5 litres of [1x] Hoagland's solution, which was only used to moisten the soil. Usually there were 15 pots per trial and either the 4-inch pots or 7-inch pots were used. If the 7-inch pots were used, then the soil, vermiculite and Hoagland's solution increased to fit the pot size. The moist soil mixture was scooped and packed into each pot then set aside in a drip tray until all pots were filled with the soil-vermiculite mixture. In the middle of the soil, the scoop was used to make a hole which was approximately 5 cm in depth.

To remove the 15-day old seedlings from the 4-inch pots filled with vermiculite, a scoop was used to lift and separate the vermiculite from the bottom of the pot, to make it easier to pull the seedlings out. Sterilized tweezers were used to grab one cotyledon of each seedling while it was carefully removed from the vermiculite. Special care was taken not to break the roots or tear the cotyledons. Once the seedling was free from the vermiculite, the root was threaded into the hole of the soil-vermiculite mix and once in place, the soil was packed around the seedling; there

was only one seedling per pot. Once all the seedlings were transferred the scoop was used again to aerate the soil around the seedling. Lastly, the seedlings were watered with 10 mL of [1x] Hoagland's solution. Irrigation occurred every other day and depending on the age and size of the plant, the volume of solution increased. Appendix C provides the full watering schedule.

After the mature plants produced capsules, several weeks were required for the capsules to turn brown and become dry enough for the seeds to be harvested. In the greenhouse, scissors were used to remove the dry brown capsules off the maternal plant, which were then placed in a Ziplock sandwich bag for transport back to lab. Under the Laminar flow hood in the lab, 2 sets of tweezers were used at a time to pull 1 capsule apart over a medium weigh boat, allowing the seeds to fall into it. The empty capsules were discarded on another weigh boat. The seed removal was repeated with 7 capsules until a 1.5 mL microcentrifuge tube was filled with the seeds and labelled as WT seeds with the date and year. A strip of parafilm was wrapped around the top of the tube to seal it. The seeds were stored at 4 ºC.

In the greenhouse, the mature plants were disposed of by removing and autoclaving the excess soil and discarding the plants.

### 2.4 Inhibitor solutions

The three inhibitors used in the study were: norflurazon, nPG and SHAM. Stock solutions were made for each inhibitor, then depending on the experiment they were diluted (see Appendix D for dilution chart). The stock solution for norflurazon was always 5 mM in 1 L, whereas nPG and SHAM had stock solutions of 15 mM in 1 L. All inhibitor stock solutions were made in the lab and stored at 4 °C until use.

### 2.5 Injection trials

The seedlings for experiments were grown and transplanted into soil as described in the propagation section and watered as described above. All the pots were labelled with the treatment prior to the experiments. The plants were between 35-60 days old when the leaves were injected. The age range of the plants was large due to the uncertainty of the most appropriate time to apply the inhibitors. The seedlings between the age of 20-29 days old were not strong or large enough to handle injections but around day 35, the leaves were large and broad as well as strong enough to allow for injections. There were two ways to inject the inhibitors into the leaves: a) using syringes with needles, and b) using needle-less syringes.

#### 2.5.1 Injections with needles

With gloves on, a small incision was made on the ventral side of the leaf, directly on the main vein. The cutting of the vein did not go through the vein but rather only created a small opening for the needle to fit into the incision. The needle was gently inserted in the incision site and the plant was injected with 1 mL of the inhibitor solution. For the control, the leaf was injected with DI water. At times, there was some solution or DI water that leaked out of the incision site, which meant the injection was too fast, so the syringe was depressed more slowly. Each leaf was injected 5 times with a total of 5 mL of solution. On the same plant, 4 other leaves were injected the same way for a total of 5 leaves injected per plant. There were 3 control plants per experiment. This was repeated for the other experimental groups (*i.e.* norflurazon, nPG and SHAM), and there were 3 plants for each treatment group per experiment. The concentration of the inhibitors for the two experiments completed using injections with needles was 0.1 mM norflurazon, 1 mM nPG, 1 mM SHAM, plus the DI water control. Once all the plants were

injected, the needles and blades were disposed of in the sharps container whereas the syringes were washed with soap and water. If any inhibitor solutions were left over, they were stored at 4 °C for later use. Observations and pictures were made 7 days' post injection. Only two trials used this method.

### 2.5.2 Injections with needle-less syringes

This method of using needle-les syringes was favoured and majority of the experiments were conducted using this technique. Similarly to the injections with needles, this procedure was completed by injecting 1 mL of DI water into the ventral side of the leaves. The needle-less syringe was gently placed onto the leaf surface (between the multiple branching veins) and the solution was pushed into the air spaces of the leaf. When done successfully, the tissue turned a dark green colour and swelled. Similarly to the injections with needles there were times when some solution leaked out and did not enter the leaf. If this happened, the syringe was adjusted on the leaf tissue by changing the angle of the syringe to be flat against the leaf tissue. The injection was repeated 5 times on the same leaf, which meant a total of 5 mL of inhibitor was injected into one leaf. The injections were repeated on 4 other leaves of the same plant, which in total was 20 mL of inhibitor into 1 plant. There were 3 plants for the control and 3 plants per treatment group for each experiment. Different inhibitors were not used on the same plant. The method of needle-less syringe injections remained the same except for the inhibitor concentration. The needle-less injections were replicated 45 times over 2016. Observations were made every time the plants were watered and pictures were taken during the 7-day post-injection period. After each experiment was completed, the injected leaves were either removed and disposed of as hazardous waste, or processed for spectrophotometry analysis.

**2.6 Spectrophotometry analysis and data collection**

Treated leaves from injected plants were cut off at the base of the petiole (where the leaf attaches to the stem), labeled and their appearance documented by taking pictures of them beside a ruler. A Vernier caliper was used to measure a 2 cm diameter circle around the injection site, and scissors were used to cut the sections out. All the veins within the section were removed and only the fresh weight of leaf tissue was weighed. In a fume hood, the leaf tissue was ground up using a pestle and mortar for 1 minute until it was a liquid pulp. A 10 mL volume of 80 % (v/v) acetone was added to the mortar and the pulp was ground again. The pulp was poured into a 50 mL beaker and 3 mL was transferred into a 10 mL beaker. 1 mL of 80 % acetone was added to the 3 mL of pulp and the contents were gently swirled together. Next, 1.5 mL of the diluted homogenate was transferred to a 1.5 mL microcentrifuge tube and centrifuged for 10 minutes at 2500 rpm at 4 °C (Porra *et al*., 1989). 1 mL of the centrifuged supernatant was removed, carefully to avoid disturbing the pellet, by a micropipette and placed into a labeled, clean cuvette. The spectrophotometer (BioMate 3S UV-Visible Spectrophotometer, Thermo Scientific) held five treatment extracts and were processed with a control cuvette filled with 80 % (v/v) acetone. The pre-determined test was selected where the machine scanned the cuvettes between the 260 nm – 720 nm range. The spectrophotometer range was selected to encompass all potential absorbances of the pigments in the variegated tissue. For example, phytoene was found to absorb in the UV-B range at 286 nm by Rabourn and Quackenbush (1953).

The absorbance data was analyzed to calculate the chlorophyll *a, b* and carotenoid concentrations according to Lichtenthaler and Buschmann (2001). See Appendix E for sample calculation. I used the calculations from Lichtenthaler and Buschmann (2001) specifically for the 80 % (v/v) acetone extraction; see Appendix A for an example. Using the absorbances at 470

nm, 646.8 nm, and 663.2 nm, the total chlorophyll and carotenoid concentrations were calculated. The average concentrations of 8 samples per treatment group were calculated (*i.e.* 8 leaves from different plants in the norflurazon treatment group).

### 2.7 Statistical Tests

Once the data were collected, statistical tests were conducted to determine if there were any significant differences between the level of pigments (i.e. chlorophyll *a*, *b* and carotenoids) in the treatment groups as compared to the control. Boxplots were produced to show the data distribution for the pigment concentration between the three treatment groups and the control. An ANOVA was used to determine the variance between the treatment groups based on the pigment concentrations for each group, and the Tukey HSD test was used to determine which treatment groups were significantly different from each other. All statistical data was analyzed through the software R.

### 2.8 Topical spray

The seedlings for treatment by topical spraying were grown the same as in the propagation and injection trials. Similarly to the injection experiments, the plants were between 35 and 60 days old when they were treated in these trials. There were 3 plants for the control and treatment groups, but this experiment of topical spray was only replicated 3 times. The concentrations of the inhibitors used were 0.1 mM norflurazon, 5 mM nPG, and the control treatment was DI water (Figure 11). The inhibitors were diluted in spray bottles and mixed with 0.01 % (v/v) Tween-20. Tween-20 is a viscous, non-ionic detergent that was used in the topical


Figure 7. Results of the topical spray application of inhibitors. The pictures are of leaves sprayed with inhibitors mixed with 0.01 % (v/v) Tween: 0.1 mM norflurazon (A), 5 mM nPG (B), and 0.01 % (v/v) Tween with a DI water control (C), taken seven days after the spray was applied. Scale bars (white lines) are 1 cm.

spray application to ensure the inhibitors would stick to the leaves and be absorbed. When the plants were sprayed, they were moved to a separate area away from the other experiments to avoid cross-contamination. Each plant was completely sprayed (*i.e.* dorsal and ventral sides of all the leaves, and the stem) as was the top of the soil, and the plants were only sprayed once. Observations were made throughout the trial and after 7 days' post-treatment.

### 2.9 Watering trials on seedlings

The 4-inch pots were prepared by cleaning with soap and water before the experiment began. All the pots were labelled and filled to the top with vermiculite. Clean seeds were placed on top of the vermiculite using a sterilized scoop. Once the seeds were on the vermiculite, all pots were watered with 100 mL of DI water. The rest of the trial followed a pre-set watering schedule where the seedlings were watered with inhibitors (Appendix F). The trial targeted the seedlings' first twenty days of development. Observations were made throughout and after the treatment stopped.

### 2.10 MSO media plate trials

First the Murashige and Skoog (MS) vitamin stock solution was made at a concentration of [1000x] in 100 mL of MilliQ purified water. The entire bottle of MS vitamin solution was dissolved to produce a clear, colourless solution. The stock solution was poured into two labelled 50 mL centrifuge tubes and stored at 4 °C.

An ESCO Laminar flow cabinet was used to prevent contamination of the media when the plates were being made. The flow hood was cleaned by wiping the table with 100 % (v/v) EtOH before the equipment was put under it. Typically, there were four inhibitor treatment

groups and three replicates for each, which meant twelve plates were needed for a trial. To make twelve media plates, a 1000 mL beaker was filled with 500 mL of MilliQ water, a large stir bar was added, and it was placed on a stir plate. 2.150 g of Murashige and Skoog Basal Medium and 0.5 mL of MS vitamin stock solution was added to the beaker. 15.0 g of sucrose was added in stages so that it would dissolve effectively. The pH of the solution was tested and was brought to 5.7. Then, 4.0 g of phytoblend agar and was added to a labelled 1000 mL autoclave bottle. Then the 500 mL solution in the beaker was poured into the autoclave bottle which changed the solution a pale cloudy yellow colour. The lid was sealed tightly and autoclave indicator tape was placed on it and it was autoclaved on the liquid cycle for 30 minutes. Petri plates (VWR) were labelled in the Laminar flow hood. After the 500 mL of MSO media had cooled in that the bottle was cool to handle, the inhibitors were added and swirled in the autoclave bottles. Then the media-inhibitor solution was poured in the Petri plates (see Appendix H). The volume of media in each plate was ¾ of the bottom plate. Over several minutes the media was left alone as it set and then clean seeds were placed on the media or the plates were stored at 4 ºC, up to 5 days.

Media plates were generally used within 24 hours of being made. The sterilized seeds were transferred onto the plates using a 200 µL pipette. The pipette was used to suck up a few seeds and they were distributed gently onto the media. Once the designated number of seeds were on the plates (~5 seeds/plate) the lids were put on. The Petri plates were sealed by Micropore tape (3M Health Care, NHRIC) to allow for the seedlings to breathe without bacteria contaminating the media. Finally, they were placed under the light bay in the laboratory to start the experiment and each experiment ran for 30 days. The conditions the seedlings were subjected to included, 21 °C room temperature, photoperiod of 12 hours of light and 12 hours of darkness.

The light intensity ranged from 150 LUX to 250 LUX under the fluorescent bulb lighting. The media plate experiments were replicated 8 times over the year of 2016.

**3. Results**

Experiments were conducted to determine the effects of three different inhibitors (norflurazon, nPG and SHAM) on tobacco plant growth and development. Four methods were used to treat the tobacco plants with inhibitors and included: 1) needle-less injections into the leaves; 2) topical application, where the leaves were sprayed with inhibitor solutions; 3) watering the plants with inhibitor solutions from early development stages; and 4) growing on media with inhibitors mixed in the media for root absorption. The first three applications were completed in the greenhouse while the last was performed in the lab under a light bay. The inhibitor concentrations ranged between 0.1 mM to 25 mM for the different experiment types. The germination and developmental stages were observed in all experiments; however, the growth rate was dependent on several variables including: the inhibitor applied, the inhibitor concentration, the light intensity, and the age of the plant. To see the number of replicates for each experiment, see Appendix G.

In the greenhouse, observations were recorded and pictures were taken of 5 plants used for seed propagation. In Figure 7, the pictures of the plants were compiled to show the chronological order of development. Germination typically occurred within the first 7 days after imbibition as shown in Figure 7A, where the green tobacco cotyledons had emerged and are visible in the vermiculite. At day 11, 5 seedlings were selected and re-planted into their own 7-inch pot, filled with a soil vermiculite mixture (Figure 7B). The seedlings grew into plants with dark green leaves and displayed flower buds on day 57 (Figure 7C). By day 60, the flowers

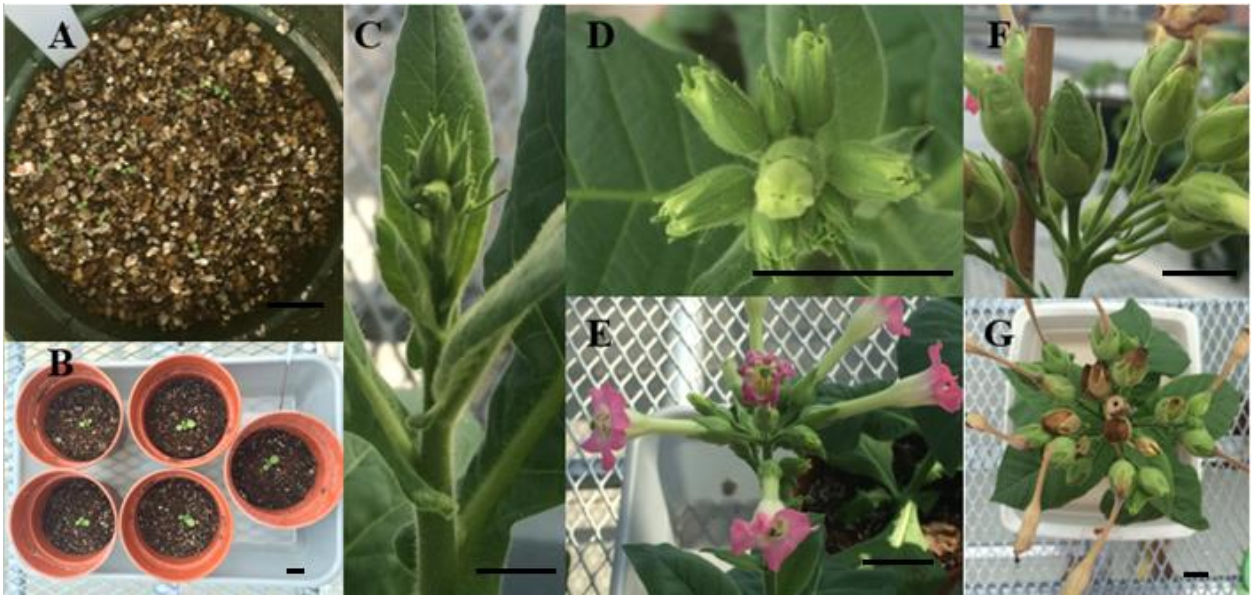


Figure 8. Developmental stages of tobacco plants grown in a greenhouse. A sample of the major life stages of a tobacco plant (*Nicotiana tabacum*) over time are shown. Pictures were taken randomly throughout the lifecycle: A - day 11 after planting; B – day 25 after planting; C – day 57 after planting; D – day 60 after planting; E – day 67 after planting; F – day 76 after planting; and G – day 90 after planting. Scale bars are 1 cm.

emerged and were green (Figure 7D). After a few days, the flowers were pink and had started to fertilize themselves (Figure 7E). The flowers fell off by day 76 and the green ovaries became enlarged (Figure 7F). After a few more days, the ovaries matured and turned brown in colour as seen in the aerial view of Figure 7G. From germination to reproductive maturity, the life cycle of tobacco is about 90 days, or 3 months (Hopkins and Hüner, 2012). Death was not observed naturally since the tobacco plants produced multiple rounds of seeds, however the plants showed signs of decay at 120 days, or 4 months. Plants were grown to obtain fresh seed, as the seeds received from Dr. Vanlerberghe showed poor germination rates, likely due to the effects of long term storage.

### 3.1 Normal tobacco germination and growth rate in the greenhouse

The typical tobacco growth pattern observed in the greenhouse began on day 5 after imbibition and planting in vermiculite, when the radical sprouted out of the seed coat. Observation of the shoot growth was ideal for this project because PTOX is located in the chloroplasts and therefore in the shoot system. The next 3 days allowed for the radical to elongate and produce the cotyledons. After 10 days, the seed coat fell off and revealed 2 small, green cotyledons. Between days 10 and 15, the cotyledons grew rapidly in size and started to spread apart from the apical meristem. The cotyledon growth started to plateau as more investment was directed into the true adult leaf growth. Between days 21 and 24, there was a distinct adult leaf bud emerging between the cotyledons. Over day 25 to 31, the stem elongated and the bud developed into a leaf and became larger than the cotyledons. Next, the plants began to grow another adult leaf bud, which resulted in an alternate leaf growth pattern as the stem elongated. From day 30 to 50, the plants continued to grow and mature. By day 50, the older

Table 2. The chronological steps of the typical development of tobacco plants (Nicotiana tabacum). The timeline begins from seed to reproductive maturity in the Laurier greenhouse.

| Days after planting | Average development for tobacco seedlings |
|---|---|
| 5-7 | Radical germinated out of seed coat |
| 7-10 | Radical elongates, cotyledons start to emerge out of seed coat |
| 10-12 | Both cotyledons are out, small to medium in size |
| 12-15 | Cotyledons are growing, are large, and start to pull away from each other to make room for further leaf growth |
| 16-20 | One tiny bud for the first true adult leaves can be seen between the large cotyledons |
| 21-24 | Adult bud is now distinct and looks more like a smaller cotyledon |
| 25-30 | Adult leaf is the same size as cotyledons, a second adult bud develops |
| 30-50 | The first true adult leaf is larger than the cotyledons and a green, the second true adult leaf is about the same size as the cotyledons; additional adult leaves begin to develop |
| 50-60 | Beginning of the development of flower buds |
| 60-70 | Pink and white flowers emerge from the buds and they take a few days to self-fertilize |
| 70-80 | The ovaries swell and the flowers fall off |
| 85+ | Brown dried up capsules are ready for harvest of seeds |

bottom leaves had a range of size from 15-17 cm in length and 10-12 cm in width, whereas the younger leaves, closer to the top of the plant, ranged from 12-14 cm in length and 5-8 cm in width. These leaves were measured because they were the size and age the plants were treated on. Then the apical meristem began to produce flower buds, indicating reproductive phase around day 60. They produced pink flowers around day 65 which lasted for one week as they completed self-fertilization. Then the dried floral parts fell off the developing ovary. Between 70 and 80 days, the ovaries swelled and turned from green to brown as they also matured. The ovaries were now dead capsules holding the seeds, and during this time were cut off from the maternal plant as propagation ended, typically after 85 days after planting. The average development order and time are summarized in Table 2.

The normal root development appeared within the first few days after planting. The radical emerged before the cotyledons from the seed coat and elongated down into the vermiculite. The observed tobacco roots were white and covered with small hairs to increase the surface area for water and nutrient absorption. The development of root growth is an important observation in this study that will be further discussed in the media plate trials.

### 3.2 Needle-less injection results

Since the inhibitors were applied after the plants were fully established in the greenhouse, the germination rate was normal as they displayed a normal growth rate and pattern as outlined in Table 2 and Figure 6. Most of the treated leaves and plants did not show signs of delayed growth, however the phenotype differed depending on the inhibitors and concentrations applied.

Most of the norflurazon-treated leaves (Figure 8A) were covered with variegated sections. Compared to the other treatment groups and the control, norflurazon was the only


Figure 9. Example of injection results and observations from greenhouse trial 10. Norflurazon (A) was 0.1 mM, nPG (B) and SHAM (C) were 0.3 mM, and EtOH (D) was 5 % (v/v). The leaves were injected at 50 days old, however these pictures are seven days afterwards (i.e. 57 days old). Scale bars are 1 cm.

treatment to result in the formation of large white sections. The leaves did not have normal green tissue mixed with opaque white sections, and it appeared that norflurazon was effective at turning the green leaf tissue white, as previously demonstrated by Bartels and Watson (1977). Bartels and Watson (1977) grew wheat seedlings in petri plates with 10 mL of distilled water or 0.1 mM norflurazon under a light bay. The method conducted by Bartels and Watson (1977) was a different application compared to this study since the tobacco leaves were injected with the inhibitors rather than absorbing the inhibitors from the roots. The injections sites were also visible along with some tearing where the tissue was damaged, see Figure 8.

The leaves in Figure 8 treated with 0.3 mM nPG, 0.3 mM SHAM, 0.1 mM norflurazon and DI water, were all injected 5 times. Leaves treated with 0.3 mM nPG (Figure 8B) were a normal dark green colour, but had variegated sections of small lighter spots mixed in with the normal sections of the leaves. Unlike the norflurazon-treated leaves, the pale spots induced by nPG treatment were contained to areas around the injection sites and near the veins, as if the veins blocked the spots from spreading into adjacent areas. The injection sites were clearly visible by the indentations of where the syringe contacted the leaf. There was also some noticeable necrotic tissue around the injection sites which made the leaves treated with nPG, look stressed. The 0.3 mM SHAM (Figure 8C) treated leaves were similar to the nPG leaves in that the variegation was evident as white spots within the normal green leaves, the variegation was contained to certain sections of the leaf, and the leaves showed some curling around the damage caused by the injection and syringe.

The photos were taken of each needle-less experiment of the treated leaves at 7 and 14 days after the injection. The reason the leaves were observed after 7 days post-injection was chosen to allow for the inhibitors to be absorbed and inhibit PTOX and PDS. The second 14 days

after the injection was chosen to determine if the variegation remained or if the leaves senesced. Figure 8 is an example of one needle-less experiment showing the results of the treated leaves 7 days after the injection, which meant the leaves were 57 days old. At this age, the leaves did not grow any more but over time there was more leaf curling and tears in the leaves. The variegated leaves were found to senesce earlier than untreated leaves. A total of 45 injection trials were completed in the greenhouse, and 4 in the growth chamber, in the fall (2015) and winter (2016). The norflurazon treatments were consistent at causing extensive large white opaque sections at the concentration 0.1 mM, however, the nPG and SHAM treatments were less consistent. In the injection trials, various the nPG and SHAM concentrations were tested to find the range that would cause variegation. The range included 0.1 mM to 25 mM of inhibitor concentration. In Figure 8, the nPG and SHAM concentrations were 0.3 mM which was only effective when injected into plants younger than 55 days old, based on all the experiments conducted in this study. These experiments were performed on whole living plants and testing *in vivo* meant there were multiple variables that could influence the experimental outcomes including seasonality, the age of the plant, and defense mechanisms.

### 3.3 Pigment analysis on variegated injected leaves

A week after the plants were injected with inhibitor, pigments were extracted with 80% (v/v) acetone for analysis using spectrophotometry. Samples from leaves treated with all inhibitors were analyzed and the data were compiled into a graph (Figure 9). The instrument was blanked with 80% acetone. The control sample from a non-treated leaf (light blue line) had the highest absorbance peaks at 340 nm, 430 nm and 660 nm. The control line was used as a baseline for comparing the absorbance and later the pigment concentrations of the inhibitor treated leaves.

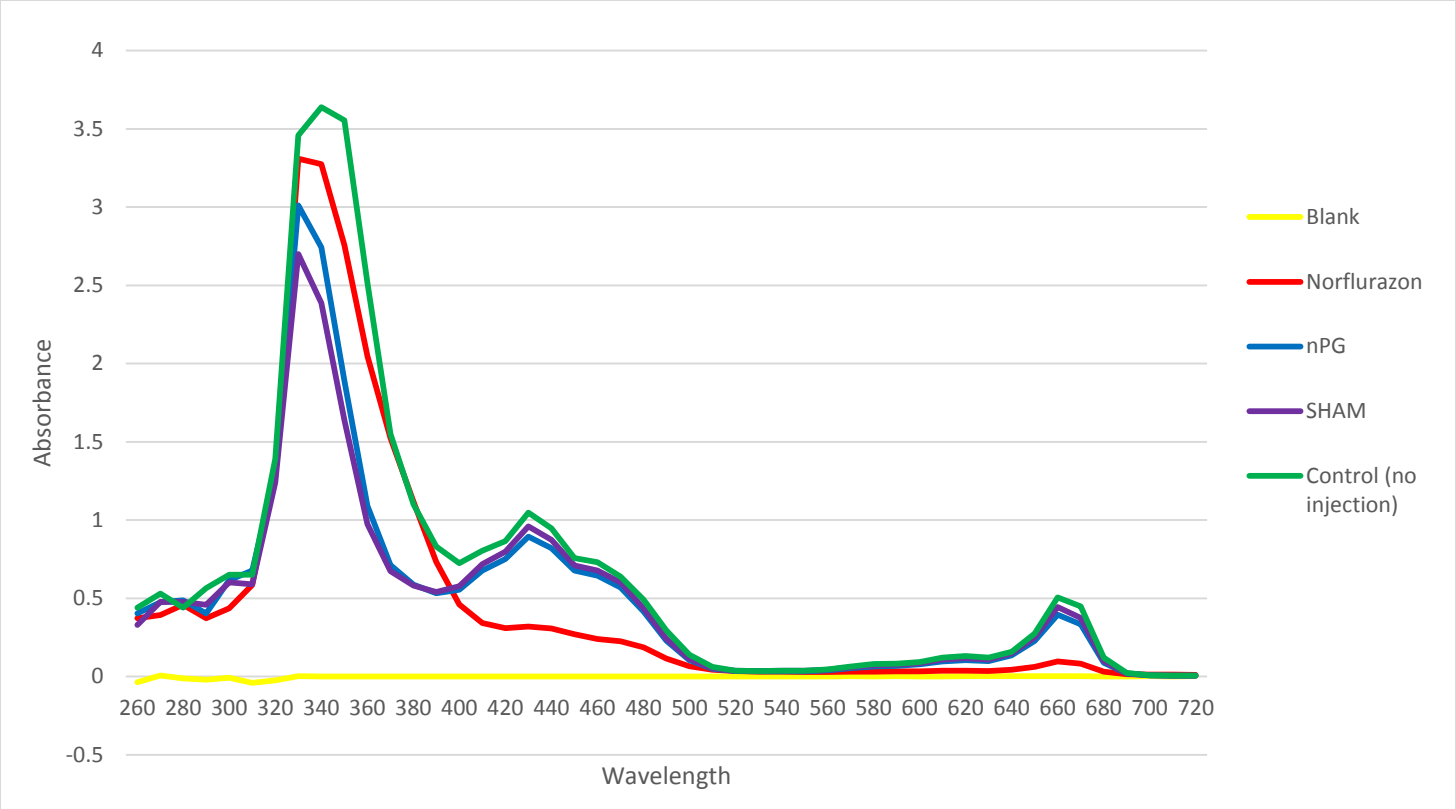


Figure 10. The absorbance spectra of leaf extracts from inhibitor injection experiments. The absorbance spectrum was measured from 260 nm to 720 nm. The blank (dark blue) was 80 % (v/v) acetone and the control (light blue) was a leaf that was not injected. Inhibitors injected into leaves were: 0.1 mM norflurazon (orange), 0.3 mM nPG (grey), and 0.3 mM SHAM (yellow). There are three major peaks within this graph that show that the extracted pigments are primarily absorbing wavelengths within the ranges of 320 nm – 400 nm, 400 nm – 500 nm, and the 640 nm – 680 nm.

In the graph, there are three peaks that represent the range of wavelengths absorbed by the pigments (Figure 9). Chlorophyll *a* is known to absorb strongly in the 640 nm – 680 nm range and chlorophyll *b* absorbs in the 320 nm – 400 nm range, whereas the middle peak between 400 nm – 500 nm is where the carotenoid pigments absorb (Lichtenthaler and Buschmann, 2001). The treatment groups follow a similar pattern to the control line in that the absorbances of nPG and SHAM are similar to the control line at each peak.

### 3.4 Chlorophyll and carotenoid concentrations within variegated sections

Chlorophyll and carotenoid concentrations of only the variegated sections of the tobacco leaves were calculated according to Lichtenthaler and Buschmann (2001). The chlorophyll and carotenoid concentrations were expressed as the amount of pigment per unit fresh weight (µg/g) (Appendix E). The variegated sections were cut out of the leaves and the pigments were extracted from the variegated sections only. The variegated sections that were compared were from different leaves treated with the same inhibitor (*i.e.* 8 different leaf samples for the nPG treatment group). The control group had the highest concentration for all the pigments, as expected. For example, the control had a chlorophyll *a* concentration of 461.64 µg/g, compared to nPG at 377.72 µg/g, SHAM at 372.97 µg/g, and norflurazon at 185.42 µg/g (Table 3).

The data show that nPG and SHAM have an effect on the concentrations of chlorophyll *a*, *b* and carotenoids, but not to the same extent as norflurazon (Table 5). Boxplots were generated to show the distribution of the data to determine if the pigment concentrations of the nPG, SHAM and norflurazon induced variegated sections were significantly different from each other and the control (Figure 10). The three coloured graphs represent the carotenoids (green boxes), chlorophyll *a* (red boxes) and chlorophyll b (blue boxes) pigments. For all the boxplot

Table 3. The average pigment concentrations (µg pigment/g fresh weight) of the variegated sections 4 days after treatment with inhibitors, as compared to the control. The sample size was eight plants for each treatment.

| | Control (no injection) | nPG | SHAM | Norflurazon |
|---|---|---|---|---|
| Chlorophyll a (µg/g) | 461.64 | 377.72 | 372.97 | 185.42 |
| Chlorophyll b (µg/g) | 136.84 | 108.39 | 107.32 | 68.84 |
| Carotenoid (µg/g) | 183.96 | 141.32 | 132.46 | 75.28 |

Table 4. The ANOVA p-values for all treatment groups for the three pigment types (i.e. carotenoid, chlorophyll a and b) to determine if they are significantly different. (p-value <0.001).

| Pigments | All Treatments |
|---|---|
| Carotenoid | $4.90 \times 10^{-9}$ |
| Chlorophyll *a* | $5.52 \times 10^{-10}$ |
| Chlorophyll *b* | $1.89 \times 10^{-9}$ |

Table 5. The Tukey HSD p-values between all the combinations of treatment groups for the three pigments types to determine between the treatment groups are different or not. (p-value <0.001).

| | Chlorophyll *a* | Chlorophyll *b* | Carotenoids |
|---|---|---|---|
| Norflurazon-Control | <0.0001 | <0.0001 | <0.0001 |
| nPG-Control | 0.0200 | 0.0014 | 0.0043 |
| SHAM-Control | 0.0131 | 0.0009 | 0.0005 |
| nPG-Norflurazon | <0.0001 | <0.0001 | <0.0001 |
| SHAM-Norflurazon | <0.0001 | <0.0001 | 0.0001 |
| SHAM-nPG | 0.9979 | 0.9985 | 0.8630 |

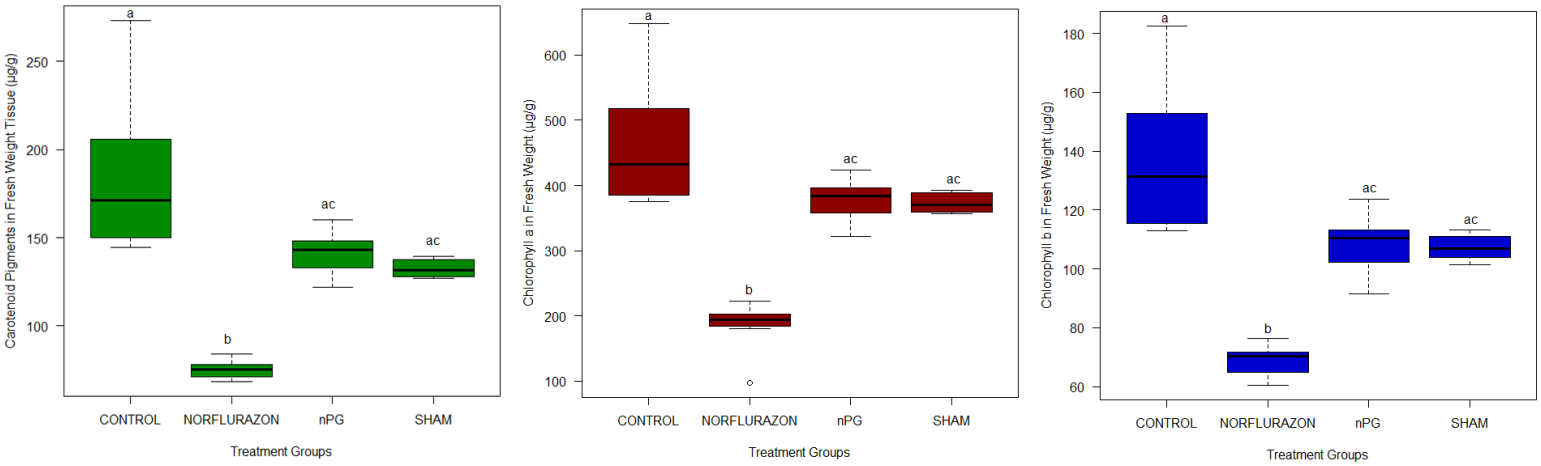


Figure 11. Boxplots showing the distribution of the pigment data for each treatment group. The carotenoid (green boxes), chlorophyll a (red boxes) and chlorophyll b (blue boxes), are showing a similar trend in concentration among all the treatment groups. The distribution shows the control with the highest concentration, nPG and SHAM are in the middle and norflurazon is low, in comparison. Through ANOVA and TukeyHSD tests, the data is significantly different between all treatment groups, except for SHAM-nPG for the three graphs. The letter above the boxes represent those that are different with those that are the same. For example, the control, norflurazon, and nPG are different from each other so they are appointed the different letters, whereas nPG and SHAM receive the letter because they are not different.

graphs, the control group has the highest pigment concentration for all 3 of the pigments that were measured (*i.e.* chlorophyll *a*, chlorophyll *b*, and carotenoids), then nPG and SHAM in the middle whereas norflurazon has the lowest concentration of the 3 pigments. The position of the boxes show a difference between the chlorophyll *a*, *b* and carotenoid pigment concentrations for each treatment group, except for SHAM-nPG. To confirm there is a difference, ANOVA was conducted on the three pigment types and the treatment groups to find the p-values (Table 4). For example, the variance between the chlorophyll *a* pigments among the treatment groups were determined to be different from each other since the ANOVA p-value was <0.001 (Table 4). However, the nPG and SHAM groups are not significantly different from each other and the control (p = >0.001). The Tukey HSD test showed the differences between the treatment groups and it was found that all the treatments were significantly different from each other for the chlorophyll *a* concentration (p-value = <0.0001), except the SHAM-nPG groups (p-value = 0.735) (Table 5). Carotenoid pigment levels were also compared the same way as the chlorophyll *a* and *b* pigment concentration of fresh weight. All treatment groups contained carotenoid pigment amounts that were lower than the control (Figure 9). In comparison to the boxplots, the carotenoid pigment concentrations followed the same trend as the chlorophyll a and b pigments, in that the control was the highest, followed by nPG, then SHAM, and lastly norflurazon with the lowest concentration (Figure 10).

### 3.5 Topical Spray

The effects of inhibitors were also tested by spraying directly onto the surface of the tobacco leaves. To avoid the inhibitor solution from rolling off the leaves and in order to promote adhesion and absorption of the inhibitors, they were mixed with 0.01 % (v/v) Tween.

There were 3 plants per inhibitor treatment and each plant was completely sprayed on day 50. The age of the plants to be sprayed was chosen based on the injection age. Plants younger than 50 days old were not sprayed. Observations were made 3 days later and pictures were taken on the seventh day after the treatment. As shown in Figure 11, the leaves were removed and placed on a black background however there was no overall pigment change observed on the tobacco leaves from the topical application. The topical spray application was not effective at causing variegation. The possible reasons variegation did not occur for the topical spray could have been due to low inhibitor concentrations, the trichomes covering the dorsal and ventral sides of the leaves, or the inhibitors did not get absorbed by the leaf.

### 3.6 Watering Trials

In the watering experiments, the plants were watered with inhibitor solutions a few days after the seeds were planted in the vermiculite. The watering experiments began from germination to the emergence of the first true adult leaf (days 1-20). All treatment groups, including the control, were watered every other day. The treatment groups were watered with the same inhibitor concentration every other day. The concentrations of the inhibitor solutions in Figure 12 include, 0.7 mM nPG, 0.7 mM SHAM, 0.1 mM norflurazon which were all mixed in with 1x Hoagland's solution. There were 2, 4-day periods where the treatment groups and the control were not watered. The seedlings in the nPG and SHAM treatments became variegated after these periods of drought. When compared to other watering trials where there were no periods of drought, the nPG and SHAM treated seedlings did not become variegated.

The nPG treated-seedlings (Figure 12B), were watered with 0.7 mM nPG in DI water from the start of the trial on day 1. Only the first true mature leaf and lateral bud were white on


Figure 12. The results of the watering trials. These tobacco seedlings were watered with inhibitors: 0.1 mM norflurazon (A), 0.7 mM nPG (B), 0.7 mM SHAM (C), and Hoagland's solution for the control (D), every other day. The watering schedule was every other day where the treatments would be watered with the same concentration. Throughout the experiment there were a few periods of drought, where the entire trial was not watered for four days. The results show the variegated white true adult leaves of the seedlings and green cotyledons. Scale bars are 1 cm.

day 20. The cotyledons remained the normal bright green and did not become variegated. There was no variegated effect on the primary or terminal bud either. Similarly, the SHAM treated seedlings (Figure 12C) also showed colour change and only in the adult leaves, however they were only watered with 0.7 mM SHAM for 7 days. The picture, taken on day 20, captured the variegation occurring from the internal portion, closest to the stem, following the veins to the outer tips of the leaves. The variegation remained for the rest of the experiment and the tissue did not revert to green. Both nPG and SHAM were successful in causing variegation, but they appeared spotty compared to the variegation caused by norflurazon.

The norflurazon treated seedlings (Figure 12A) turned completely white and remained small, compared to the control group (Figure 12D). The seedlings were watered with 0.1 mM norflurazon since day 4 of the trial, and after 12 days the seedlings were completely white – the picture was taken on day 16. The seedlings did not grow beyond this stage, and shortly thereafter they shriveled up and died.

### 3.7 Normal tobacco germination and growth rate on media plates

The growth rate of the seedlings on media plates was dependent on several variables including: the inhibitor type, the concentration of the inhibitor, the light intensity, and the age of the plant. Figure 13A-J shows the typical growth pattern of tobacco seedlings on MSO media in Petri plates. The seedlings produced the tiny cotyledons by day seven (Figure 13A), which grew and enlarged by day ten (Figure 13B). On day fourteen, (Figure 13C), the first true adult leaf bud was observed for most seedlings. Two days later, on day sixteen, the second true adult leaf grew, opposite to the first true leaf (Figure 13D). By day eighteen, the true leaves were about the same size and colour as the cotyledons (Figure 13E), although only a few days later the cotyledons

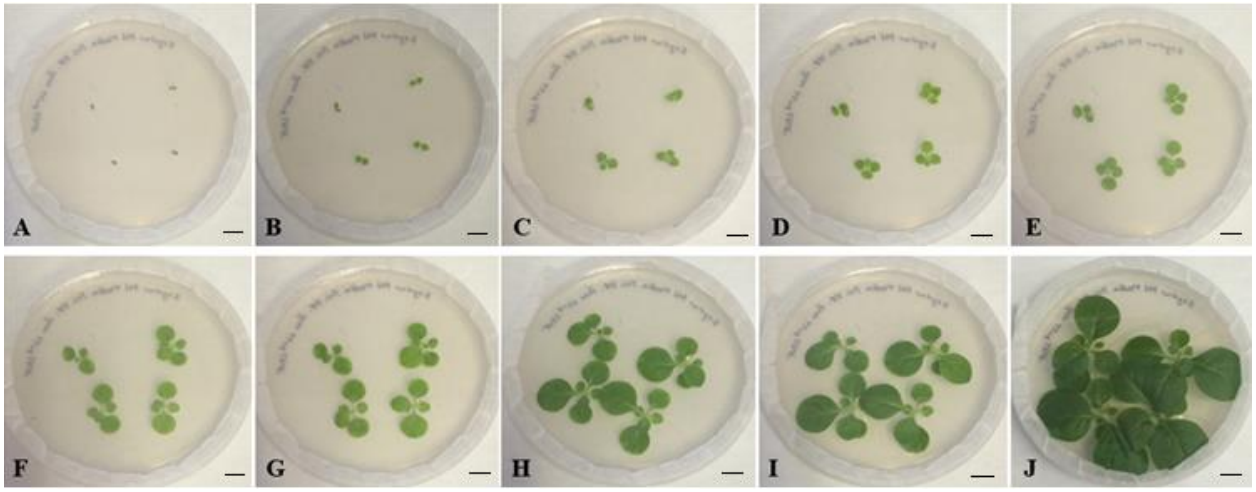


Figure 13. The development of normal tobacco seedlings on regular MSO media from Trial 2. These control plates line up with certain days: A – day 7; B – day 10; C – day 14; D – day 16; E – day 18; F – day 22; G – day 24; H – day 28; I – day 30; and J – day 43. Scale bars are 1 cm.

were distinctly smaller compared to the other leaves (Figure 13F). The growth pattern continued in the alternate leaf emergence pattern observed in the greenhouse seedlings (Figure 13G). There was a change between days twenty-four and twenty-eight (Figure 13G and 13H), in that all the leaves became a darker green and they had spread out to become larger. The vein pattern in the leaves was more noticeable by day 30 (Figure 13I) and by the end of the experiment on day forty-three (Figure 13J), the seedlings had taken up most of the surface area of the plates due to their large size.

### 3.8 Media plate results for inhibitor treatments

Similar to the early development trials, the seedlings grown on media were in contact with the inhibitors from the beginning of the experiment. On day thirty, seedlings in the control plate (Figure 14A) exhibited large true mature leaves and long white roots within the media. When compared to the treated seedlings, the controls were the only ones that demonstrated normal and consistent growth patterns, as mentioned above.

The 0.3 mM SHAM plate (Figure 14B) had an orange colour appearance when the media and inhibitor were mixed together. In the media, the roots of the seedlings were short and brown unlike the control. All the leaves of the SHAM-treated seedlings were small and stunted, which were proportionate to their roots. Adult leaves observed on the SHAM plate were light in colour but the cotyledons displayed the normal green colour. Both the variegated leaves and the media colour did not fade over time.

The 0.3 mM nPG plate also displayed a media colour, like the SHAM plate, however it was grey instead of orange (Figure 14C). Similar to the control, the seedling roots were long, but they were also brown, as seen in the SHAM-treated seedlings. The colour change was also only

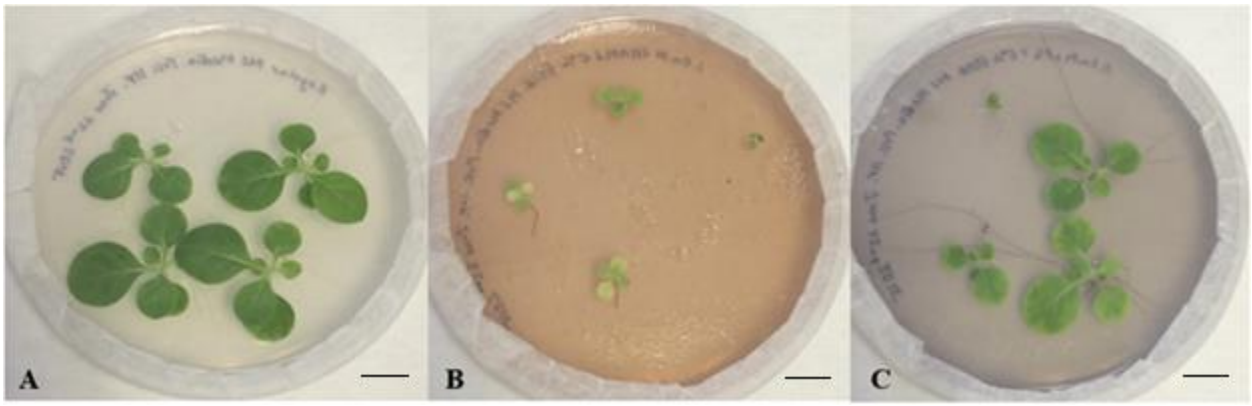


Figure 14. The results from the media plate trials containing inhibitors. The control on the left (A), 0.3 mM SHAM in the top middle (B), and 0.3 mM nPG on the right (C). The seedlings were 35 days old when these pictures were taken. The SHAM seedlings show true adult leaves affected the inhibitor in the media. The nPG seedlings are also affected by the inhibitor however the slight colour change is localized to the margins of the leaves. Seedlings in the inhibitor treatment plates displayed shortened brown roots. Scale bars are 1 cm.

visible in the adult leaves but the pattern was localized on the edges of the leaves, not like the SHAM seedlings which turned the whole leaf white. Some nPG seedlings retained similar growth to the controls since they displayed large true adult leaves, however there were two seedlings which were delayed in development. The small seedlings were not observed in each replicate of the media plate experiment. Across the replicates of the media plate experiments, there were seedlings that displayed a negative chemotaxis reaction to the media and the roots of the seedlings were touching the lid of the Petri plate.

The 0.1 mM norflurazon plate displayed clear media like the control plate (Figure not shown). Although the plate had bacterial growth throughout the media, the seedlings were hard to see because they turned completely white, similar to the plants that were treated with norflurazon by watering. The seedlings only produced cotyledons which were white and the seedlings stopped development. The reason the seedlings did not grow past the cotyledon stage could have been due to the lack of photosynthetic activity occurring in the leaves.

## 4. Discussion

### 4.1 How did we get here? The electron transport that causes variegation

There have been many studies showing that genetically mutating PTOX causes variegation. In 1967, Rédei used the gene *immutans* in *Arabidopsis thaliana* to determine the relationship between RNA metabolism and chloroplast formation. When *immutans* was mutated it caused the formation of both normal green and white plastids in distinct sectors, which were observed under microscopic analysis. From there, research showed that *immutans* encoded a plastoquinol terminal oxidase (PTOX) and it was suggested to be a carotenoid biosynthetic co-factor (Carol *et al*., 1999). Wetzel *et al*. (1994) made this connection because the carotenoid

precursor phytoene was found within the white variegated sections in Arabidopsis leaves. However, Wu *et al*. (1999) suggested the function of PTOX is to maintain redox balance of the PQ pool, essentially acting as a safety valve to oxidize plastoquinol and remove electrons from the pool, as suggested by Peltier and Cournac (2002). Eliminating PTOX as the regulator of the PQ pool leads it to be susceptible to hyper-reduction under stressful conditions (Peltier and Cournac, 2002). If the PQ pool is hyper-reduced, this means there are no available oxidized plastoquinone carriers capable of accepting electrons which creates a blockage within photosynthesis, the carotenoid pathway (Wetzel *et al*., 1994), and the chlorophyll pathway (Rodermel, 2002). This leads to variegation (Rédei, 1967) which means an altered pigment concentration, but our results show that the details of when and what enzyme, can cause a difference in pigment concentrations (Table 3).

Based on our *in vivo* results of the injection trials (Figure 8), watering experiments (Figure 12), and media plates (Figure 14), variegation can be caused by chemical inhibition of both PDS and PTOX, presumably through the same mode of action as hypothesized by the genetic work (*i.e.* the lack of functional PTOX causes the PQ pool to become hyper-reduced). Until recently, the lower chlorophyll concentrations within the variegated sections were thought to be due to the lack of protective carotenoid pigments which would lead to increased chlorophyll molecule degradation due to photo-damage (Rodermel, 2002). However, Steccanella *et al*. (2015) proposed the chlorophyll biosynthetic pathway is directly connected to the PQ pool via the ACS enzyme. Although chlorophyll is dependent on the carotenoid pigments for protection of the light harvesting complexes, the oxidation/reduction status of the PQ pool may be another way the chlorophyll and pigment concentrations are affected during variegation.

Due to the observation of variegation induced by nPG, SHAM and norflurazon, the results suggest that PDS and PTOX enzymes were present in tobacco plants during most developmental stages. The norflurazon inhibitor has long been used as an effective herbicide sprayed to control grass and broad-leafed weeds. Bartels and Watson (1977) found it to inhibit PDS and cause phytoene to accumulate in wheat seedlings. At the same time, it decreased photo-protective carotenoids levels that in turn led to decreased chlorophyll concentrations, ultimately resulting in 'bleached' leaves (Bartels and Watson, 1977). Sandmann *et al.*, (1989) proposed norflurazon inhibits PDS through an allosteric site and is an irreversible inhibitor. Sandmann *et al.*, (1989) used cyanobacteria (*Anacystis nidulans R2*) in "binding studies" where isolated membranes of *Anacystis* were mixed with 3.24 mM [$^{14}$C] norflurazon. The researchers washed the [$^{14}$C] norflurazon off the membranes and then applied unlabeled norflurazon. They found the unlabeled norflurazon was not removed from the membranes after washing. The variegation caused by norflurazon in this study appeared as large opaque white-yellow sections on tobacco and we propose that it is most likely due to the high abundance of PDS throughout the plant tissue. Since carotenoids are used as accessory pigments to assist chlorophyll in harnessing light energy, and they also protect photosystems against photooxidation (Bartley and Scolnik, 1995). Due to the importance of carotenoid pigments, carotenoids are constantly turned over (Gabriele Beisel *et al*., 2010). The study by Gabriele Beisel *et al*. (2010) used $^{14}CO_2$ pulse-chase labeling on mature leaves of *Arabidopsis* to determine carotenoid and chlorophyll turnover rates. A comparison between controlled light and high light, the results indicated in normal leaves there was not a difference in turnover rates between carotenoid and chlorophyll pigments. When comparing the results in *Arabidopsis* mutants (*lut5*), there was indication that carotenoids had a higher turnover rate (Gabriele Beisel *et al*., 2010). When norflurazon is applied, it binds and

inhibits the vast majority of PDS enzymes available. The white-yellow sectors were showing where norflurazon stopped the carotenoid pathway at the level of phytoene production.

Our data indicate that both the chlorophyll and carotenoid pigments within the variegated sections are lower than those seen in untreated tobacco leaves (Table 3). However, depending on where the inhibitors target within the pathway (*i.e.* PDS or PTOX) the intensity of the variegation that occurred was different. Although nPG and SHAM are used in AOX studies (Josse *et al*., 2000), these inhibitors were also found to inhibit PTOX (Cournac *et al*., 2000). AOX knock-out plants do not exhibit an obvious phenotype such as colour change (Fiorani *et al*., 2005) which indicates that it is PTOX inhibition that specifically causes variegation. However, based on these inhibitors, we cannot be completely sure that the inhibitors are only targeting PTOX. Our observations upon chemical inhibitor applications show that variegation is taking place which means that nPG and SHAM are targeting PTOX, although it cannot be completely ruled out that they are not also targeting AOX. The variegation that occurs when nPG and SHAM are applied is different than that seen in the norflurazon-treated leaves (Figure 8). Perhaps this is due to the abundance of PTOX within plant tissue as compared to PDS. Lennon *et al*., (2003) isolated and extracted intact chloroplasts from spinach (*Spinacia oleracea*) leaves. The chloroplasts were lysed and a freeze-thaw treatment was applied 3 times to break the thylakoid membranes. The supernatant and pellets were analyzed via a SDS-PAGE and immunoblotting by being compared to the antibody of IMMUTANS. The results suggested an estimate of the PTOX enzyme is 1 % of the total protein in leaf tissue. We propose the variegation is different between the nPG and SHAM inhibitors compared to the norflurazon inhibitor (i.e. spotty vs. opaque sections), since the PTOX concentration is likely to be much less than PDS.

### 4.2 Pigment concentrations decrease because the inhibitors stop the biosynthetic pathways

Plant pigments absorb wavelengths within the visible light spectrum. Each pigment readily absorbs particular wavelengths, for example, chlorophyll *a* absorbs in the blue (428 nm) and red (661 nm) regions of the spectrum. Chlorophyll does not absorb green wavelengths which are reflected and this is why plants are perceived as green. According to Gitelson and Merzlyak (1997), the total amount of chlorophyll in tobacco leaves naturally varies between 8.37 $\mu g/cm^2$ – 41.1 $\mu g/cm^2$, whereas the total carotenoids have a lower range between 3.2 $\mu g/cm^2$ – 8.25 $\mu g/cm^2$. This confirms that chlorophyll is usually more abundant than carotenoid pigments in tobacco leaf tissue. The fact that chlorophyll pigment concentration is higher than carotenoid pigment concentration is not surprising, however the fact that during variegation chlorophyll concentrations decrease rapidly that the plant loses its green colour in the abnormal section is interesting.

The relative pigment concentrations in leaves will change based on the light intensity the leaves are subjected to. For example, the plant will encounter direct sunlight on sunny days or indirect sunlight, such as shade, on cloudy days. Lichtenthaler and Buschmann (2001) reported high and low chlorophyll *a/b* ratios within sun leaves (3.0 – 3.8) and shade leaves (2.4 – 2.7) respectively, which indicates the functional pigment in the photosystems. In Table 3, the chlorophyll and carotenoid pigment concentration in the control group (461.64 µg/g and 183.96 µg/g) is the highest among the nPG- (377.72 µg/g and 141.32 µg/g) and SHAM- (372.97 µg/g and 132.46 µg/g) treated plants, which have lower pigment concentrations. Although all the plants experienced sun plant conditions (as evidenced by the control group values) plants treated with nPG and SHAM inhibitors show decreased chlorophyll and carotenoid concentrations when compared to the control values. The decrease was expected to be larger than the results in Table

3 based on a report by Steccanella *et al*. (2015) suggesting the ACS enzyme of the chlorophyll biosynthesis pathway, deposits electrons into the PQ pool. Due to this connection, when PTOX is dysfunctional and the PQ pool is hyper-reduced, the chlorophyll concentration is expected to decrease substantially like the norflurazon chlorophyll concentration (185.42 µg/g), because ACS is blocked from transferring electrons into the PQ pool. A potential reason the chlorophyll pathway was not severely affected could be because it is highly regulated (von Wettstein *et al*., 1995; Reinbothe and Reinbothe, 1996). Although ACS is one of the last enzymes to be characterized and fully understood, as reported by Stenbaek and Jensen (2010), and Steccanella *et al*. (2015), it may be possible that ACS functions differently when the PQ pool is hyper-reduced. The reason the chlorophyll concentration decreased within the nPG and SHAM treatments could be due to the decreased carotenoid pigments that could not protect the chlorophyll as suggested by Rodermel (2002), or ACS was blocked from depositing the electrons and caused a decrease in chlorophyll as hypothesized by Steccanella *et al*. (2015). This should be investigated further to determine the possible route the electrons are moving. To test this, it is possible to use a chlorophyll inhibitor, such as Gabaculine (May *et al*., 1987; Cznecki and Grimm, 2012). Using a chlorophyll inhibitor would be interesting to determine the phenotype colour when chlorophyll is not synthesized and measure the chlorophyll and carotenoid pigments present under these conditions.

The chlorophyll and carotenoid pigment concentration per fresh weight in the norflurazon-treated plants was much lower (185.42 µg/g and 75.28 µg/g) as compared to those of the control, and the nPG and SHAM treatments (Table 3). The herbicide targets PDS of the carotenoid pathway, as determined by Bartley and Scolnik (1995) who experimented on wheat seedlings and found the colourless phytoene molecule was present in the white sections. There

was no indication of chlorophyll concentration in Bartley's study because the norflurazon is an effective herbicide targeting PDS that turned the wheat seedlings completely white, which was observed for the watering experiments (Figure 12). Not all herbicides inhibit chlorophyll synthesis but norflurazon seems to have an indirect effect on the chlorophyll concentration through the inhibition of the carotenoid biosynthetic pathway. The only connection to the chlorophyll pathway to cause such a decrease is through the lack of protection from the carotenoids (Rodermel, 2002).

Wetzel *et al*. (1994) compared chlorophyll and carotenoid concentrations between the white sectors of leaves from *immutans* mutants to green wild type leaves using HPLC analysis. The pigments were extracted from WT *Arabidopsis* leaves, the green sectors of mutated leaves and the abnormal variegated sectors of the leaves using 100 % acetone. The extracted pigments were centrifuged then passed through a filter before being tested before HPLC analysis. In the white sectors, there was 22.19 μg $g^{-1}$ of phytoene whereas the chlorophyll and β-carotene levels were not detectable. In Table 3, the pigment concentrations determined through spectrophotometry analysis, showed decreased chlorophyll (norflurazon was 185.42 μg/g, the control was 461.64 μg/g) and carotenoid (norflurazon was 75.28 μg/g, the control was 183.96 μg/g) concentrations in the inhibitor-treated plants. Since phytoene absorbs in the UV light spectrum at 286 nm (Rabourn and Quackenbush, 1953) we included this range in the spectrophotometry test, in attempt to find the phytoene absorbance, however we did not calculate the phytoene concentration.

Aside from the difference in methodology, (*i.e.* HPLC vs. spectrophotometry), and the organism used, (*i.e. Arabidopsis* vs. tobacco), there is also a difference in when variegation occurred. The genetic mutation causes problems with photosynthesis and pigment synthesis as

soon as the seedling began to grow (Wetzel *et al*., 1994), whereas the plants in this work were grown normally for 50 days before they were injected with the inhibitors. In comparing this factor, there would be a normal pigment concentration within the leaves prior to injection and once the inhibitors were in the plant, it took time for the pigments to decrease. It could be that when the leaves were sampled for analysis, the pigment concentration was decreasing but if exposed to the inhibitors for a longer period or at higher concentrations, it is possible that our pigment concentrations would be comparable to those seen in the genetic mutants.

Plants reflect other colours based on their structure (*i.e.* floral parts are colourful and can be white). Even plants that are thought to not have chlorophyll (achlorophyllous), such as the Indian pipe plant (*Monotropa uniflora*), have minimal but detectable chlorophyll *a* and carotenoid pigments, yet they appear completely white to the human eye (Cummings and Welschmeyer, 1996). The white colour of the Indian pipe plant suggests a plant's colour depends on the relative concentrations of the pigments present because of the wavelengths being reflected. Low chlorophyll and carotenoid pigment levels within a plant shows a white phenotype. Although there has been higher phytoene concentrations found within the variegated sections, phytoene may play a role in the process of variegation. A potential future study could be to test and compare the white and green sectors of both genetic mutants and inhibitor injected leaf tissue. Using both HPLC and spectrophotometry methods would be beneficial in determining the chlorophyll and carotenoid pigments in the genetically white sectors.

Prior work has shown that the *immutans* chloroplasts were found to be structurally different to the WT chloroplasts (Rédei, 1967). Reports from Wetzel *et al*. (1994) and others used electron micrographs to find the mutated white chloroplasts to contain multiple vacuoles and lack organized thylakoid membranes. Without intact thylakoid membranes the normal

biosynthetic pathways of chlorophyll and carotenoid pigments, as well as photosynthesis will not occur (Wetzel *et al*., 1994). When Bartels and Watson (1977) tested norflurazon and fluridone on wheat seedlings, the thylakoid membrane displayed an atypical circular pattern. Not only does this mean the inhibitors influence the molecular pathways, but it also means it has an influence on the internal structure of the chloroplast.

The nPG inhibitor displayed similar trends to the norflurazon in that there was an overall decrease in the amounts of chlorophyll and carotenoids (Table 3), however the observed variegation was not the same. The nPG-treated leaves resembled the variegated phenotype displayed by the leaky PTOX knock-out mutant called spotty (Rosso *et al*., 2009). This mutation does not completely knock-out the PTOX gene meaning that some PTOX protein is able to function in chloroplasts (Rosso *et al*., 2009). The phenotype resulted in the leaves having spots. Similar to the spotty variegated mutant (Figure 15), the nPG results (for injection and media plates) showed transparent white sections, throughout the leaf tissue. The nPG treatment resulted in decreases of both chlorophyll and carotenoid content (Table 3).

The SHAM injection was the closest to the control based on the chlorophyll and carotenoid pigments, it also displayed a similar phenotype to nPG and the spotty mutant.

### 4.3 PTOX's role in plant development

There is evidence to suggest that PTOX plays a role during early development and in chloroplast biogenesis (Rosso *et al*., 2009; Putarjunan *et al*., 2013). The theory is that when photosynthesis is beginning, such as in cotyledons or new leaves, PTOX functions to oxidize the PQ pool until the cytochrome $b_6f$ complex is operational and ready to take on the electrons (Josse

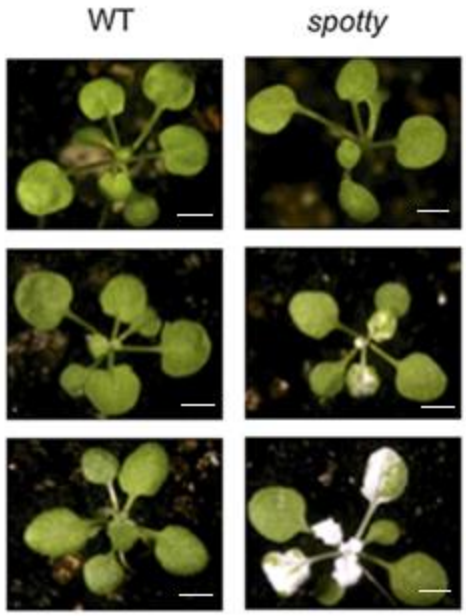


Figure 15. The difference between the WT Arabidopsis plants and spotty mutant grown under 50 (top row), 150 (middle row) and 450 (bottom) µmol $m^{-2}s^{-1}$. The spotty mutant shows sporadic white sections throughout the normal green tissue. Scale bars (white lines) are 1 cm. Modified from Rosso *et al*. (2009).

*et al*., 2000; Carol and Kuntz, 2001; Aluru and Rodermel, 2001). Wetzel *et al*. (1994) showed that the mutated PTOX gene *immutans* resulted in disorganized thylakoid membranes. The structure of thylakoid membranes is necessary for photosynthesis to occur. This indicates regulation of the PQ pool oxidation/reduction state is important to the normal development of young plants. Bartels and Watson (1977) experimented on wheat seedlings with the herbicides norflurazon and fluridone to determine their effects on the chlorophyll and carotenoid pathways. The seedlings were placed in 10 mL 0.1 mM norflurazon or fluridone then subjected to various light regiments. They found a high concentration of phytoene within the norflurazon sections via spectrophotometry, in addition to abnormal thylakoid membrane structure. They found norflurazon to be an effective herbicide because of the damage it causes to the thylakoid membranes, as well as the inhibition of carotenoid production.

Another future experiment that would be interesting to investigate should include the nPG and SHAM chemically-induced variegation to determine if the thylakoid membranes also resulted in abnormal structure. The next experiment should be similar to the work completed here, in that nPG, SHAM and norflurazon are injected into tobacco leaves and compared to PTOX-mutated tobacco leaves. The genetically and chemically altered PTOX leaves should be analyzed through HPLC to determine the pigment concentrations, as well as electron microscopy (Wetzel *et al*., 1994) for chloroplast imaging. This could provide part of a possible explanation why there is a lack of pigments within the variegated sections. The possible process could be the lack of chlorophyll pigments that cause the transformation and destruction of the thylakoid membranes which further prevents the production of pigments and ultimately, the metabolic pathways.

An explanation for the phytoene concentration and a lack of functional pigments observed in Wetzel's (*et al*., 1994) data could be due to when variegation occurred in the plants development (Figure 16). Wetzel and her team were not able to detect chlorophyll or carotenoid pigments because the seedlings were mutated and therefore, they did not produce any pigments from the beginning of their lives. In my work, the plants were allowed to grow under normal conditions which meant functional chlorophyll and carotenoid pigments were produced. Then the plants were injected at fifty days old which lead to the inhibition of the biosynthetic pathways. The data in Table 3 were collected seven days after the injection occurred although it is unknown when PTOX was inhibited. The data are showing the decrease in chlorophyll and carotenoid pigment concentration from normal levels. The difference between the literature and this work leads us to question, if the plants injected with nPG and SHAM are given more time (i.e. 15, 20, 25 days) to decrease the pigments in the leaves, what is the half-life of the pigments? Would the chlorophyll and carotenoid concentrations be depleted or just lower like in Table 3? The potential problem with this could be the timing of senescence. Allowing too much time would allow the pigment levels to decrease and the leaf would die due to the natural aging process. To overcome this problem, more tests regarding the inhibitor concentration range to induce variegation should be completed. Then, if variegation can be produced faster at a higher inhibitor concentration, the problem of timing senescence would not matter.

There is evidence that the age of a plant plays a role in the development of variegation. The injected inhibitors did not influence the tobacco leaves if they were applied after the plants were 55 days old. In Figure 8, the tobacco leaves were injected at 50 days old and were affected by the inhibitors since variegation occurred. The reason the plants were injected at this age was because the leaves were broad and had enough space between the veins to allow a needle-less

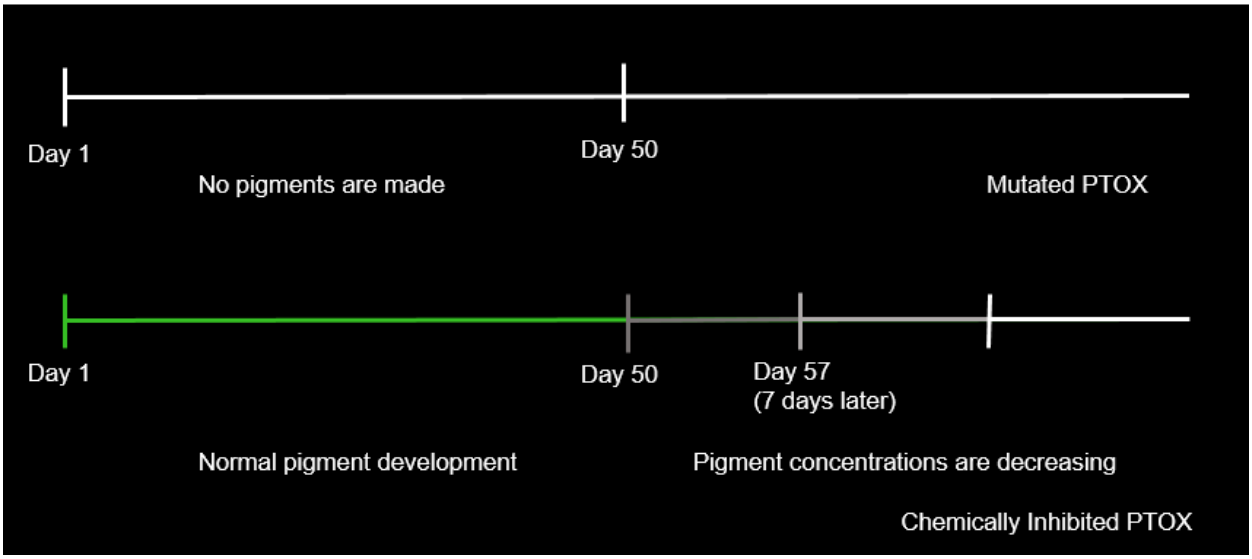


Figure 166. The timeline of chlorophyll synthesis in the mutated PTOX plants compared to the chemically injected PTOX plants. The results of dysfunctional photosynthesis, chlorophyll and carotenoid synthesis pathways are assumed to be affected the same between the two plants. However, the pigments concentrations are different, probably due to when the variegation occurred. Represented as the white line, the mutated PTOX plants were not making chlorophyll or carotenoid pigments from Day 1 because of the unorganized thylakoid membranes. The chemically injected plants were grown under normal conditions so the pigments were produced normally, then the pathways were inhibited at Day 45. The pigment concentrations could be lower if the plants were tested after longer periods.

syringe to fit between them for injection. The age chosen is in the upper limit for the definition of 'early development'. At this point, the plants were either successfully established, and therefore the inhibitors were less effective and had negligible impact on metabolism, or perhaps PTOX was inactive and could not serve as a target for the inhibitors. A future directions study should include the determination of the PTOX transcript levels and protein during this time, for example, by performing quantitative RT-PCR and Western blots. A study on the carotenoid biosynthetic pathway of two coffee plant species by Simkin *et al*. (2008) used quantitative RT-PCR on PTOX and several other proteins. Normal and stress conditions were applied and the results showed an increase in PTOX transcript levels under stress. To our knowledge, there is a lack of measured amounts of PTOX during each stage of a plants development. We suggest another experiment should target this knowledge gap by establishing PTOX levels at time (daily, weekly, etc.) and age (Day 1, Day 10, Day 25, etc.) intervals as well as new leaf development under normal conditions, under stress to compare the changes. This would provide a baseline for the PTOX transcript levels within tobacco plants.

When the topical spray was applied, the leaves remained unaffected. All plants have a variety of constitutive defence mechanisms and tobacco plants have many trichomes covering the ventral and dorsal side of their leaves in addition to the waxy cuticle (Wagner *et al*., 2004). We suspect that with this mode of application that the inhibitors were not effective at infiltrating the leaves. Another study could re-examine this application but use seedlings or young plants (*i.e.* less than 35 days old) to determine if the topical application could be a possible method.

The watering experiments and media plate results show variegation occurring in young seedlings but only in the real adult leaves, not the cotyledons (Figures 10 and 12). Wetzel *et al*. (1994) and Wu *et al*. (1999), showed *immutans* produces variegation on cotyledons of

*Arabidopsis thaliana* when the gene was knocked out (Rodermel, 2002). This means PTOX is present in the cotyledons however the cotyledons do not become variegated. Cotyledons are infant leaves predetermined in the seeds that sprout after the radical germination has ended (Hopkins and Hüner, 2009). A reason the cotyledons were not variegated may be that the cotyledons do not use environmental resources when they are growing and the cotyledons only use the reserves from endosperm in the seed coat (Hopkins and Hüner, 2009). Since PTOX is in the cotyledons but there was no indication of variegation occurring in the cotyledons during chemical inhibitor treatments, this suggests that the cotyledons of tobacco do not rely on the use of nutrients from the surrounding environment for development and growth.

### 4.4 Environmental stress – a critical factor in causing variegation

To simplify variegation, it can be broken down into two steps; the first being the dysfunction of PTOX, either through mutation, or using chemical inhibition as in this study. Removing PTOX's ability to regulate the PQ pool will cause its reduction state to rise above Rosso's *et al.* (2009) theorized threshold and the PQ pool becomes hyper-reduced. Our results show that the tobacco leaves treated with the PTOX-inhibiting chemicals nPG and SHAM produced variegation (Figure 8). Since variegation was observed in the leaves, it is reasonable to suggest the PQ pool was hyper-reduced as a result of PTOX inhibition, as this connection between the PQ pool and PTOX was proposed by Rodermel (2002). We cannot be sure that nPG and SHAM are only targeting PTOX within the tobacco leaves.

The second component needed to cause variegation is some form of environmental stress (Escoubas *et al*., 1995). In the presence of non-functional PTOX, stress may cause an increase in excitation pressure and the influx of electrons, or a decrease in the efflux of electrons that alters

the balance of the now-susceptible PQ pool in that it becomes hyper-reduced. Many researchers, such as Wetzel *et al*. (1994), have used high light (above 100 µmol $m^{-2}$ $s^{-1}$) intensity as a stress to increase electron input. Experimenting with high light mimics a condition that most plants encounter throughout their lives. Quiles (2006) subjected oat plants (*Avena sativa*) to high light and high temperatures to find that there was an increase in PTOX activity under these conditions. Conversely, Ivanov *et al*., (2012) conducted an experiment on Arabidopsis where low temperatures and high light were used. The results indicated PTOX was used within the plant system to adjust to the low temperatures as well as the high light intensity. This suggests PTOX is active during acclimation to a change in temperature. Feilke *et al*., (2014) altered the pH in the lumen and stroma of the thylakoid membranes to slightly acidic (6.5) and slightly basic (8.0) within spinach leaves. The goal of this experiment was to disturb PSII activity to determine if PTOX function was coupled with PSII or PSI activity. Under the acidic experiment, the results showed PSII and PTOX activity decreased together, suggesting they were closely linked. There are many studies testing the purpose of PTOX however it can be determined that PTOX plays are role in variegation and stress.

The results of our chemical inhibition experiments showed the environmental stress of drought during early development was successful at causing variegation (Figure 11). The definition of drought, within this experiment, is simply a lack of watering for four days to allow the vermiculite medium the seedlings were growing in to dry out. This differed from the normal watering schedule which consisted of watering the plants every other day. Simkin *et al*. (2008) found PTOX transcript levels increased 3-fold in mature coffee leaves after three weeks without water. In this study, there is possibility that the seedlings were increasing PTOX levels in attempt to balance the stress of drought, although it is unknown what the PTOX levels were in the

seedling leaves during the experiment. This may explain when the adult leaves of the cotyledons were completely white and looked similar to the norflurazon injection observations (Figure 8). Based on the stress of drought, the plant may have increase PTOX production in that more PTOX enzyme available in the seedlings. With a higher concentration of PTOX in the leaves, this would have given more enzyme for nPG and SHAM to inhibit and thus the variegation that occurred was opaque rather than spotty.

There were other stresses introduced during our work which have not been investigated in genetic studies, including physical damage caused by the needle-less syringes used in our experiments. We observed damage around the injection sites (*i.e.* localized cell death) that looked like a hypersensitive response (HR) (Figure 8). The HR is activated when plants are trying to decrease the spread of a pathogen (Tanaka and Tanaka, 2007) in that the cells will die once they are infected. This type of stress did not appear to cause variegation as it only occurred in the injection experiments and negative controls did not display variegation.

The last environmental stress that was noticeable was the change of colour for the media plate trials. For both the SHAM and nPG inhibitors the media turned either orange or grey, respectively, although there is no knowledge of this occurring in the literature. We attempted the experiment several times and the colour of the media was consistently orange or grey. If the concentration of either inhibitor increased, the colour became darker. We think this may be an oxidation reaction between the inhibitors and the nutrients in media. To our knowledge, any media plate studies for PTOX have not involved mixing of nPG and SHAM with MSO media but there have been no reports of MSO media showing a colour change. With this information, we cannot be certain the plants are absorbing the same amount of inhibitor that was mixed into the MSO media due to the reaction however the concentration that the plants are absorbing does

cause variegation to occur in both the nPG and SHAM plates. This will need to be considered moving forward regarding this work.

Research has shown there is a relationship between PTOX activity and environmental stress, however the studies mentioned were all conducted on different plant species. Since there is an inadequate amount of data on these environmental stress factors with in the same species, and a future study may need to include the different environment stresses (*i.e*. high and low light, high and low temperature, drought, salinity, metals, and pH) to test on one plant species, for example tobacco (*Nicotiana tabacum*).

**5. Summary and the integrative nature of the study**

The goal of this study was to determine if the chemical inhibitors, nPG and SHAM, would cause variegation in tobacco leaves. Since this is the first project to incorporate chemical inhibition of PTOX in a whole plant system, an integrative approach was used. Several methods of injections, topical spray, watering with inhibitor solutions, and media with inhibitors, were conducted to establish an effective method of incorporating the chemicals into the plants, which was the first objective of this study. Success was found as variegation occurred through chemical inhibition of PTOX in most of the application methods that were compared.

The second objective was to quantify the pigment concentrations in the variegated sections to determine if the white sections were caused from a lack of chlorophyll and carotenoid pigments. The second objective was also to find if the chemically-induced variegated sections would exhibit similar pigment concentrations as found by Wetzel *et al*. (1994). This part of the project was focused on the pigment concentrations in the variegated sections rather than the application of the inhibitors. The pigments were extracted from the variegated sections, analyzed

using a spectrophotometer and the data was used to determine that the chlorophyll and carotenoid pigments decreased for all treatment groups. The ANOVA and Tukey HSD tests supported this trend in the data and showed the decrease in pigment concentrations of the treated plants, were significantly lower than the control (p-value = 0.0001). This suggested that the timing of when variegation occurred determined the concentration of the pigments with the genetic or chemical variegated sections. In addition to the two objectives, we also found evidence PTOX plays a role in environmental stress during the watering trials which support the literature findings.

The importance of this knowledge is that now variegation experiments can use chemicals to inhibit PTOX in living plants. Using chemical inhibition may lead to new knowledge for this field of research. Now that a direct mode of inhibition has been determined this knowledge can be used in crop stress tolerance and ornamental design of the variegation pattern.

There is growing concern regarding our crop and food industry in the means of production, competition of land and the quality of the product (Godfray *et al*., 2010). This has led to alteration of food, the increasing in the amount of food produced, in addition to environmental factors of water and soil, that causes stress on plants. A paper by Johnson and Stepien (2016) suggested the idea that PTOX may be modified to increase stress tolerance in crop plants. Based on the current PTOX data, they concluded that there was more information needed to understand the purpose of PTOX in determining if it can be modified in crop plants. This study provided information regarding the stress of drought on tobacco seedlings. Using different approaches, such as in this study, could provide a better understanding in this field of research.

In 2007, Canadians spent approximately $6.3 billion on ornamental plants according to a review by the Canadian Ornamental Horticulture Alliance (Andrews, 2009). Studies show that the activities of gardening and cultivating plants have a high correlation with the retired population of the Western world. According to Andrews (2009) gardening is proposed to increase as the baby-boomer generation retires over the next five years. Variegated plants are valuable in the Canadian horticultural industry as ornamental garden plants. The current production of variegated plants is produced through time consuming breeding methods. The information found in this study can be applied to produce variegated plants in a quick and efficient process. Applying the knowledge of chemically-induced variegated plants to the horticulture industry, there may be an additional sector of custom design of variegated plants.

Overall, this thesis has contributed to this field of research and this work has brought new questions and ideas for future experiments for PTOX inhibition. Variegation is a complex internal process and continuation of using whole plants with chemical inhibition should be considered for the future experiments.

**References**

Aluru MR, Bae H, Wu D, Rodermel S. 2001. The Arabidopsis *immutans* mutation affects plastid differentiation and the morphogenesis of white and green sectors in variegated plants. Plant Physiology. 127: 67-77.

Aluru MR, Yu F, Fu A, Rodermel S. 2006. Arabidopsis variegation mutants: New insights into chloroplast biogenesis. Journal of Experimental Biology. 57: 1871-1881.

Andrews C, Smyth S. 2009. The impact of ornamental horticulture on Canada's economy. Canadian Ornamental Horticulture Alliance. 1-138.

Bartley GE, Scolnik PA. 1995. Plant carotenoids: Pigments for photoprotection, visual attraction, and human health. The Plant Cell. 7: 1027-1038.

Bartels PG, Watson CW. 1977. Inhibition of carotenoid synthesis by fluridone and norflurazon. Weed Science Society of America. 26: 198-203.

Beisel KG, Jahnke S, Hofmann D, Köppchen S, Schurr U, Matsubara S. 2010. Continuous turnover of carotenes and chlorophyll *a* in mature leaves of *Arabidopsis* revealed by $^{14}CO_2$ pulse-check labeling. Plant Physiology. 152: 2188-2199.

Berthold BA, Andersson MA, Nordlund P. 2000. New insight into the structure and function of the alternative oxidase. Biochimica et biophysica Acta. 1460: 241-254.

Carol P, Stevenson D, Bisanz C, Breitenbach J, Sandmann G, Mache R, Coupland G, Kuntz M. 1999. Mutations in the Arabidopsis gene *IMMUTANS* cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. The Plant Cell. 11: 57-68.

Carol P, Kuntz M. 2001. A plastid terminal oxidase comes to light: Implications for carotenoid biosynthesis and chlororespiration. Trends in Plant Science. 6: 31-36.

Cournac L, Josse EM, Joët T, Rumeau D, Redding K, Kuntz M, Peltier G. 2000. Flexibility in photosynthetic electron transport: Newly identified chloroplast oxidase involved in chlororespiration. The Royal Society of London Biological Sciences. 355: 1447-1454.

Cummings M, Welschmeyer NA. 1996. Pigment composition of putatively achlorophyllous angiosperms. Plant Systematics and Evolution. 210: 105-111.

Cznecki O, Grimm B. 2012, Post-translational control of tetrapyrrole biosynthesis in plants, algae, and cyanobacteria. Journal of Experimental Biology. 63: 1675-1687.

Dahal K, Wang J, Martyn G D, Rahimy F, Vanlerberghe G C. 2014. Mitochondrial alternative oxidase maintains respiration and preserves photosynthetic capacity during moderate drought in *Nicotiana tabacum*. Plant Physiology. 166: 1560-1574.

Demmig-Adams B, Adams III WW. 1992. Photoprotection and other responses of plants to high light stress. Annual Review of Plant Physiology and Molecular Biology. 43: 599-626.

Eberhard S, Finazzi G, Wollman F-A. 2008. The dynamics of photosynthesis. Annual Review of Genetics. 42: 463-515.

Escoubas JM, Moas M, LaRoche J, Falkowski PG. 1995. Light intensity regulation of *cab* gene transcription is signaled by the redox state of the plastoquinone pool. Proceedings of the National Academy of Sciences of the United States of America. 92: 10237-10241.

Feilke K, Yu Q, Beyer P, Sétif P, Krieger-Liszkay A. 2014. *In vitro* analysis of the plastid terminal oxidase in photosynthetic electron transport. Biochimica et biophysica Acta. 1837: 1684-1690.

Fiorani F, Umbach AL, Siedow JN. 2005. The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis *AOX1* transgenic plants. Plant Physiology. 139: 1795-1805.

Foyer CH, Lelandais M, Kunert KJ. 1994. Photooxidatice stress in plants. Physiologia Plantarum. 92: 696-717.

Gitelson AA, Merzlyak MN. 1997. Remote estimation of chlorophyll content in higher plant leaves. International Journal of Remote Sensing. 18: 2691-2697.

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson, Thomas SM, Toulmin C. 2010. Food Security: The Challenge of Feeding 9 Billion People. Science. 327: 812-818.

Goyal A and Tolbert NE. Salicylhydroxamic acid (SHAM) inhibition of the dissolved inorganic carbon concentrating process in unicellular green algae. Plant Physiology. 92: 630-636.

Hoagland D R. 1937. Some aspects of the salt nutrition of higher plants. The Botanical Review. 1: 307-334.

Hopkins WG, Hüner NPA. 2009. Introduction to Plant Physiology, Fourth Edition. John Wiley & Sons.

Hörtensteiner S. 2006. Chlorophyll degradation during senescence. Annual Review of Plant Biology. 57: 55-77.

Ivanov AG, Rosso D, Savitch LV, Stachula P, Rosembert M, Oquist G, Hurry V, Hüner NPA. 2012. Implications of alternative electron sinks in increased resistance of PSII and PSI photochemistry to high light stress in cold-acclimated *Arabidopsis thaliana*. 113: 191-206.

Johnson GN, Stepien P. 2016. Plastid terminal oxidase as a route to improving plant stress tolerance: Known knowns and known unknowns. Plant and Cell Physiology. 1-10.

Josse EM, Simkin AJ, Gaffé J, Labouré AM, Kuntz M, Carol P. 2000. A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. Plant Physiology. 123: 1427-1436.

Kambakam S, Bhattacharjee U, Petrich J, Rodermel S. 2016. PTOX mediates novel pathways of electron transport in etioplasts of *Arabidopsis*. Molecular Plant. 9: 1240-1259.

Lennon AM, Prommeenate P, Nixon PJ. 2003. Location, expression and orientation of the putative chlororespiratory enzymes, Ndh and IMMUTANS, in higher-plant plastids. Planta. 218: 254-260.

Lichtenthaler HK, Buschmann C. 2001. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS Spectroscopy. Current Protocols in Food Analytical Chemistry. F4.3.1 – F4.3.8.

May TB, Guikema JA, Henry RL, Schluer MK, Wong PP. 1987. Gabaculine inhibition of chlorophyll biosynthesis and nodulation in *Phaseolus lunatus* L. Plant Physiology. 84: 1309-1313.

McDonald AE, Ivanov AG, Bode R, Maxwell DP, Rodermel S, Hüner NPA. 2011. Flexibility in photosynthetic electron transport: The physiological role of plastoquinol terminal oxidase (PTOX). Biochimica et Biphysica Acta. 1807: 954-967.

Peltier G, and Cournac L. 2002. Chlororespiration. Annul Review of Plant Biology. 53: 523-550.

Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et biophysica Acta. 975: 384-394.

Putarjunan A, Liu X, Nolan T. 2013. Understanding chloroplast biogenesis using second-site suppressors of *immutans* and *var2*. Photosynthesis Research. 116: 437-453.

Quiles MJ. 2006. Stimulation of chlororespiration by heat and high light intensity in oat plants. Plant, Cell and Environment. 29: 1463-1470.

Rabourn WJ, Quackenbush FW. 1953. The occurrence of phytoene in various plant materials. Archives of Biochemistry and Biophysics. 44: 159-164.

Rédei GP. 1967. Biochemical aspects of genetically determined variegation in Arabidopsis. Genetics. 56: 431-443.

Reinbothe S, Reinbothe C.1996. Regulation of chlorophyll biosynthesis in angiosperms. Plant Physiology. 11: 1-7.

Rodermel S. 2002. Arabidopsis variegation mutants. American Society of Plant Biologists. 30: 1-28.

Rosso D, Bode R, Li W, Krol M, Saccon D, Wang S, Schillaci LA, Rodermel S, Maxwell DP, Hüner NPA. 2009. Photosynthetic redox imbalance governs leaf sectoring in the *Arabidopsis thaliana* variegation mutants *immutans, spotty, var1*, and *var2*. The Plant Cell. 21: 3473-3492.

Sandmann G, Linden H, Böger P. 1989. Enzyme-kinetic studies on the interaction of norflurazon with phytoene desaturase. Zeitschrift für Naturforschung B. 44: 787-790.

Siedow JN, Girvin ME. 1980. Alternative respiratory pathway: Its role in seed respiration and its inhibition by propyl gallate. *Plant Physiol*. 65: 669-674

Siedow JN, Bickett DM. 1981. Structural features required for inhibition of cyanide-insensitive electron transfer by propyl gallate. Archives of Biochemistry and Biophysics. 207: 32-39.

Simkin AJ, Moreau H, Kuntz M, Pagny G, Lin C, Tanksley S, McCarthy J. 2008. An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea Arabica*. Journal of Plant Physiology. 165: 1087-1106.

Steccanella V, Hansson M, Jensen PE. 2015. Linking chlorophyll biosynthesis to a dynamic plastoquinone pool. Plant Physiology and Biochemistry. 97: 207-216.

Stenbaek A, Jensen PE. 2010. Redox regulation of chlorophyll biosynthesis. Phytochemistry. 71: 853-859.

Tanaka R, Tanaka A. 2007. Tetrapyrrole biosynthesis in higher plants. Annual Review of Plant Biology. 58: 321-346.

Tilley RJD. 2010. Colour and optical properties of materials: An exploration of the relationship between light the optical properties of materials and colour, Second Edition. John Wiley & Sons.

Von Wettstein D, Gough S, Kannangara CD. 1995. Chlorophyll biosynthesis. The Plant Cell. 7: 1039-1057.

Wagner GJ, Wang E, Shepherd RW. 2004. New approaches for studying and exploiting and old protuberance, the plant trichome. Annals of Botany. 93: 3-11.

Wetzel CM, Jiang CZ, Meehan LAJ, Voytas DF, Rodermel SR. 1994. Nuclear-organelle interactions: The immutans variegation mutant of Arabidopsis is plastid autonomous and impaired in carotenoid biosynthesis. The Plant Journal. 5:161-175.

Wu D, Wright D, Wetzel C, Voytas DF, Rodermel S. 1999. The IMMUTANS variegation locus of Arabidopsis defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. The Plant Cell. 11: 43-55.

[Internet Source]: Cyberphysics. The Electromagnetic Spectrum: The family of Light. (Retrieved from http://www.cyberphysics.co.uk/topics/light/emspect.htm). Last accessed: April 5th 2017.

## Appendix

### 1. Appendix A

Table 1: The volume of MilliQ water and the amount of reagents to make the stock solutions of Hoagland's solution.

| Volume of MilliQ water | Amount of Reagent |
|---|---|
| 50 mL | 12.3 g $MgSO_4$ |
| 50 mL | 6.8 g $KH_2PO_4$ |
| 50 mL | 5.055 g $KNO_3$ |
| 50 mL | 11.81 g $Ca(NO_3)_2$ |
| 100 mL | 0.335 g EDTA<br>0.25 g $FeSO_4$*7H2O |
| 500 mL | 1.43 g $H_3BO_3$<br>0.905 g $MnCl_2$*4H2O<br>0.055 g $ZnCl_2$<br>0.037 g $CuSO_4$*$5H_2O$<br>0.0145 g $Na_2MoO_4$*$2H_2O$ |

To make a 1 M stock solution for 50 mL of $MgSO_4$, the example includes:

$$g = Molecular\ Weight\ x\ Molarity\ x\ Volume$$

$$g = 246.47 g/mol\ x\ 1 mol/L\ x\ 0.050 L$$

$$g = 12.3 g$$

### 2. Appendix B

Table 2: The dilution of stock solutions to make the appropriate concentration of Hoagland's solution in the correct volume of MilliQ water.

| [10x] in 1 L | [1x] in 1 L | [5x] in 20 L |
|---|---|---|
| 2 mL 1 M $MgSO_4$<br>4 mL 1 M $KH_2PO_4$<br>10 mL 1 M $KNO_3$<br>10 mL 1 M $Ca(NO_3)_2$<br>2 mL Fe-EDTA<br>2 mL Micronutrient solution<br><br>Add 970 mL of MilliQ $H_2O$ | 100 mL of [10x] Hoagland solution<br><br>Add 900 mL of MilliQ $H_2O$ | 20 mL 1 M $MgSO_4$<br>40 mL 1 M $KH_2PO_4$<br>100 mL 1 M $KNO_3$<br>100 mL 1 M $Ca(NO_3)_2$<br>20 mL Fe-EDTA<br>20 mL Micronutrient solution<br><br>In the greenhouse, add 19.7 L of DI $H_2O$ to carboy |

A written version of diluting Hoagland's solution based on Table 2 includes:

A 150 mL beaker was filled with 50 mL of filtered MilliQ water and the first stock solution of 1 M $MgSO_4$ (Sigma-Aldrich, 1 KG solid, Lot No.: M1880) was made by adding 12.3 g. The next solutions of $KH_2PO_4$ (Bioshop, Biotechnology grade – 500 g, Lot No.: 2F25361), $KNO_3$ (Bioshop, Reagent grade – 500 g, Lot No.: 0K18309), and $Ca(NO_3)_2$ (Sigma, Lot No.: MKBK6089V), were made the same way in 50 mL of filtered MilliQ water. Then they were poured and stored in labelled 50 mL centrifuge tubes. Fe-EDTA was made by adding 0.335 g EDTA (Bioshop, Reagent grade – 500 g, Lot No.: 1A19438) and 0.25 g $FeSO_4$*7H2O (Sigma-Aldrich, Reagent grade – 250 g, Lot No.: SLBC4547V) in 100 mL of MilliQ $H_2O$. This solution was stored in a 100 mL autoclave bottle. The Micronutrient solution was made by adding 1.43 g of $H_3BO_3$ (Bioshop, Biotechnology grade – 500 g, Lot No.: 1D20792), 0.905 g of $MnCl_2$*4H2O (Sigma-Aldrich, Reagent grade – 100 g, Lot No.: SLBD1661V), 0.055 g of $ZnCl_2$ (Sigma-Aldrich, Reagent grade – 100g, Lot No.: MKBR5953V), 0.037 g of $CuSO_4$*5$H_2O$ (Sigma, Bioreagent grade – 500 g, Lot No.: SLBD1241V), and 0.0145 g $Na_2MoO_4$*2$H_2O$ (Sigma, 100g, Lot No.: 109K1464V) into 500 mL of MilliQ $H_2O$. And stored in a 500 mL autoclave bottle.

## 3. Appendix C

Table 3: Chart of watering schedule for propagation and injection trials

| Day range | Amount of water/Hoagland's solution |
|---|---|
| Day 0-15 | 50-75mL of DI water |
| Day 15-19 | 15mL of Hoagland's solution |
| Day 20-29 | 25mL of Hoagland's solution |
| Day 30-39 | 50mL of Hoagland's solution |
| Day 40-59 | 100mL of Hoagland's solution |
| Day 60-79 | 125mL of Hoagland's solution |
| Day 80-99 | 150mL of Hoagland's solution |
| Day 100-119 | 200mL of Hoagland's solution |
| Day 120-150 | 250mL of Hoagland's solution |

## 4. Appendix D

The calculation of a 15 mM stock solution of n-propyl gallate (nPG) in 500 mL was:

$$g = Molecular\ Weight\ x\ Molarity\ x\ Volume$$
$$g = 212.2 g/mol\ x\ 0.015 mol/L\ x\ 0.500 L$$
$$g = 1.59 g$$

Therefore 1.59 grams of nPG was mixed into 500 mL of MilliQ water and stored in a labelled autoclave bottle in the fridge.

To make a 0.5 mM nPG solution in 500 mL of MilliQ water from the 15 mM stock solution, the calculation below was used:

$$C1\ x\ V1 = C2\ x\ V2$$
$$0.0005 M\ x\ 0.500 L = 0.015 M\ x\ V2$$
$$V2 = \frac{0.0005 M\ x\ 0.500 L}{0.015 M}$$
$$V2 = 0.016667 L$$

Therefore, 16.67 mL of the 15 mM nPG stock solution was needed. To get the total 500 mL of the solution, only 483.33 mL of MilliQ water was used.

## 5. Appendix E

Sample calculation of chlorophyll *a* concentration:

Chlorophyll *a* concentration:

$$ca\ (\mu g/mL) = 12.25\ A663.2 - 2.70\ A646.8$$
$$ca\ (\mu g/mL) = 12.25\ (0.103) - 2.70(0.052)$$
$$ca\ (\mu g/mL) = 1.26175 - 0.1404$$
$$ca\ (\mu g/mL) = 1.12$$

Chlorophyll *b* concentration:

$$cb\ (\mu g/mL) = 21.50\ A646.8 - 5.10\ A663.2$$
$$cb\ (\mu g/mL) = 21.50\ (0.052) - 5.10(0.103)$$
$$cb\ (\mu g/mL) = 1.118 - 0.2163$$
$$cb\ (\mu g/mL) = 0.90$$

Carotenoid concentration:

$$c(x+c)\ (\mu g/mL) = (1000\ A470 - 1.82\ ca - 85.02\ cb)/\ 198$$
$$c(x+c)\ (\mu g/mL) = (1000(0.226) - 1.82(1.12) - (0.90))/198$$
$$c(x+c)\ (\mu g/mL) = (226 - 2.04 - 75.52)/198$$
$$c(x+c)\ (\mu g/mL) = (148.44)/198$$
$$c(x+c)\ (\mu g/mL) = 0.75$$

Converting µg/mL to fresh weight (µg/g):

$$FW\ (\mu g/g) = \left[\text{Chl a}\ \left(\mu \frac{g}{mL}\right)\right]\ \text{x Dilution factor / weight of tissue used}$$

## 6. Appendix F

Table 4: Example of the treatment schedule for the watering trials, where the date corresponded to the age of the seedlings and the volume of the solution the seedlings were watered with (not the total solution made).

| Day | Date | Scheduled Watering |
|---|---|---|
| 1 | W. June 29th | 100 mL DI $H_2O$ |
| 2 | R. June 30th | 50 mL DI $H_2O$ |
| 3 | F. July 1st | No watering |
| 4 | Sat. July 2nd | 50 mL DI $H_2O$ |
| 5 | Sun. July 3rd | No watering |
| 6 | M. July 4th | 50 mL inhibitor solution |
| 7 | T. July 5th | No watering |
| 8 | W. July 6th | 50 mL inhibitor solution |
| 9 | R. July 7th | No watering |
| 10 | F. July 8th | 50 mL inhibitor solution |
| 11 | Sat. July 9th | No watering |
| 12 | Sun. July 10th | 50 mL inhibitor solution |
| 13 | M. July 11th | No watering |
| 14 | T. July 12th | 50 mL inhibitor solution |

| | | |
|---|---|---|
| 15 | W. July 13th | No watering |
| 16 | R. July 14th | 50 mL inhibitor solution |
| 17 | F. July 15th | No watering |
| 18 | Sat. July 16th | 50 mL inhibitor solution |
| 19 | Sun. July 17th | No watering |
| 20 | M. July 18th | 50 mL inhibitor solution |

## 7. Appendix G

Table 5: The main trial types in conjunction to the total number of experiments conducted and when they occurred in 2016.

| Trial Type | Number of experiments completed | Semester experiments were conducted |
|---|---|---|
| Injection | 45 | Winter 2016 |
| Topical | 3 | Winter 2016 |
| Watering | 8 | Summer 2016 |
| Media plates | 8 | Winter/Summer 2016 |

## 8. Appendix H

MSO Media has been the choice of media for growing tobacco plant species as it has the necessities for these plants need to germinate and grow.

**Equipment and Materials**

- 600mL beaker
- 500mL of MilliQ water
- Stir bar and hotplate
- 2.150g of MS salts
- 0.5mL of MS vitamin stock solution
- 15.0g sucrose
- 4.0g phytoagar
- Permanent marker
- One 1,000mL autoclave jar
- Autoclave tape
- pH metre and pH coloured buffer tester
- 1M HCl and 5M NaOH
- Inhibitors at the desired concentration

**Methodology**

1. In a 600mL beaker, fill 350mL of MilliQ water. Add the appropriate sized stir bar to the water in the beaker and place it on the hot plate.
2. Turn on the stir bar and keep the dial around 4. The mixing should be moving but not splashing over the sides of the beaker. Make sure the temperature dial is not on.
3. Retrieve the MS salt container from the fridge. Weigh 2.150g of Sigma-Aldrich MS salts in a paper weighing cup then add it to the 600mL beaker.

4. Add 0.5mL of prepared MS vitamin stock solution to the beaker.
5. Weigh out 15.0g of sucrose and add it to the beaker.
   *Note: The blue-based hot plate will not dissolve this large amount of sugar fast enough and the stir bar might get stuck. Use the yellow-based hot plate. The spin dial can be turned up to a higher number (~5-7) to mix all the sucrose well; again not too fast so the solution doesn't spill over.
6. While the sucrose is being mixed in, turn on the pH metre to let it adjust to its solution.
7. Weigh out 4.0g of phytoagar and place it into a labelled 1000mL autoclave jar.
8. Once the sucrose is dissolved keep the stir dial on but turn it down (~2)
9. Remove the pH metre from the standard pH solution and gently rinse the tip with MilliQ water. Then put the metre into the 4.0 pH standard buffer test solution and allow for it to adjust. This may take a few minutes. Only if it reads the right pH units, rinse the tip again with MilliQ water and place it into the 600mL beaker media solution.
10. The solution should be fairly acidic (~3.6-3.8). Use 5.0M KOH and 1.0M HCl to balance the pH level to 5.7 (~1-2 drops of KOH and 2-3 drops of HCl).
11. Add 150mL of MilliQ water and retest the pH; this is to make sure the pH hasn't changed. If it has, then add more of the acid or base to bring it back to the 5.7 level.
12. Remove the pH metre, wash with MilliQ water and put it back in the standard pH solution.
13. Turn off the stir bar and pour the 500mL solution into the labelled 1000mL autoclave jar with the 4.0g of phytoagar. Together it should be a cloudy pale yellow colour.
    *Note: Be careful the stir bar doesn't fall into the autoclave jar! Otherwise use the magnetic stick to retrieve it.
14. Seal the jar tightly via the lid. Place the autoclave indicator tape on the jar.
15. Repeat the steps for however many jars are desired. If 250mL are desired, the steps are the same however cut all the reagents in half; the only exception to this is the pH which stays the same.
16. When the media has been autoclaved, bring back to lab to cool to room temperature. As the media is cooling, gently swirl the solution so it doesn't fall out of the solution.
17. When the media has cooled enough (~20 minutes), pour the desired diluted inhibitor concentrations in each separate autoclave bottle (the inhibitors should NOT mix).
18. Again, swirl the solution of media and inhibitor together and gently pour into the Petri plates.