DET TEKNISK-NATURVITENSKAPELIGE FAKULTETET

BACHELOROPPGAVE

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Abstract:

Plants undergo photosynthesis, one of the most important process on Earth. This happens through the help of chloroplasts, which uses sunlight, water and carbon

dioxide to create oxygen. Oxygen is used by almost all forms of life in the process of respiration. Sugars are used by both other organisms as a source of energy, and by the plant itself during harsh conditions and periods with little sunlight. But in order for the process of photosynthesis to work, the chloroplast itself needs to be assembled.

This process requires a couple of enzymes to be in place and functional, the most important being protochlorophyllide oxidoreductase (POR) and chlorophyll synthase. These help with the last two steps of the chlorophyll synthesis.

In the present experiment, we investigated if the process of chlorophyll synthesis can be accomplished in conditions deprived of sunlight or any photon emitting sources. POR needs only a single photon to help phototransform protochlorophyllide (Pchlide) into chlorophyllide (Chlide) via NADPH, making it the only light-dependent step in the entire process. Although there are some plants that manage to accomplish this on their own e.g pine cones, usual flowering plants cannot.

The flowering plant used in this paper is barley, as it is easy to grow and cultivate, and does not require constant watering. Thus, this papers focus was trying to synthesize chlorophyll in the dark and in vitro, using barley plastid extracts. Data indicates that the conversion of Pchlide to Chlide via the Pchlide:POR:NADPH conjugate is not possible in the dark. There was a constant accumulation of the Pchlide:POR:NADPH conjugate, with no subsequent conversion and detectable trace of Chlide, both in vivo and in vitro. Although, after a quick 10 second burst of strong, white light to either samples, mass accumulation of Chlide could instantly be detected by the absorbance spectrophotometer. At the same time, biomolecular imaging indicated presence of chlorophyll a and b after exogenous GGPP addition to the phototransformed chlorophyllide.

These results could still have significant future implications when it comes to the amount of electricity used by plant cultivating greenhouses. If 10 seconds of white light was all it took for the light-dependent step to be completed, then plants could be grown in greenhouses. These sudden bursts of light at determined intervals, would decrease electrical cost and carbon footprint size in the long run, worldwide.

Introduction:

Chloroplasts, the reason Earth, is able to host all the complex life that it has today. According to a study led by the University of Bristol, chloroplasts as we know them have existed in aquatic algae for approximately 2.1 billion years (Baracaldo, Raven, Pisani, & Knoll, 2017). There, it was determined that cyanobacteria, underwent a primary symbiotic change in habitats with low salinity concentrations. Cyanobacteria are the precursor and ancestor of the chloroplasts, rhodophytes and glaucophytes. This was done through stochastic mapping analyses of the terrains.

Cyanobacteria are also the oldest living organisms on Earth, appearing in fossils as old as 3.5 billion years ago according to the University of Berkeley (University of California Museum of Paleontology (UCMP), 1995). But more interestingly, in a study recently published in the scientific journal Nature, intact thylakoid membranes were

found in fossils, dating back 1.75 billion years (Demoulin, Lara, Lambion, & Javaux, 2024). In both cases of the cyanobacteria and the thylakoid membranes, the remarkable observation was that both completely resembled their modern counterparts both morphologically and functionally.

The focus of this introduction section is to introduce and explain the background theory behind chloroplasts. A deeper dive into its precursor molecules will be taken, showcasing what their roles are in the function of a chloroplast. This subsequently means, how it helps a plant develop, grow and make energy for itself. The general scope of this thesis will also be explained and an outline of the paper will be given at the end of the section. This is to help organize and categorize, what topics are going to be discussed.

1. Background theory

Chloroplasts are a type of membrane-bound organelle, more commonly known through the scientific nomenclature as etioplasts. These organelles conduct photosynthesis in unicellular organisms like algae or in multicellular organisms like plants. Photosynthesis commences when the photosynthetic pigment chlorophyll (Chl) harvests the energy carried by the sunlight emitted photons. Then, through oxidative photophosphorylation, that energy is converted into molecules like adenosine triphosphate (ATP) and Dihydronicotinamide-adenine dinucleotide phosphate (NADPH). The ATP and NADPH are then utilized by the chloroplast to fix carbon from CO_2 in the air into organic molecules, e.g glucose. This is done through the Calvin cycle, from which the plant can then in turn use for growth or storage.

Plants contain two main types of chlorophyll pigments, a and b. The way that chlorophyll absorbs light in the first place is through photosystems I and II, which have their own respective reaction centers called P700 and P680. The naming of the reaction centers corresponds to the different wavelengths, in nanometres, at which the red-peak absorption maximum occurs. The reason why chlorophyll itself is green is because it absorbs the wavelengths of light corresponding to the colours blue and red, meaning between 450 and 495 nanometres and 620 and 750 nanometres, respectively. Then, the plant ends up reflecting the green wavelengths, and these get picked up by our eyes and registered as green.

Chlorophyll is biosynthesized through the collaboration of two distinct pathways called the MEP and Tetrapyrrole pathways. But although the MEP pathway plays an important role in the last step of chlorophyll synthesis, most of the work is done by the Tetrapyrrole pathway. All the explanations that follow are accredited to the website Microbenotes and the process can be seen in Fig.1. (Sapkota, 2023). There are 10 major steps in total, beginning with the:

I. **Ligation of glutamate to tRNA**: Is the initiatory step of chlorophyll biosynthesis. In this step, glutamate is ligated to tRNA in the presence of glutamyl-tRNA synthetase.

II. **Reduction of the glutamyl-tRNA by glutamyl-tRNA reductase**: Catalyzes the reduction of the activated α-carbonyl group of glutamyl-tRNA. In order for

this reaction to end up taking place, the presence of pyridine nucleotides and NADPH is required, which then ends up releasing glutamate-1-semialdehyde.

III. **Transferring of an amino group**: From the C-2 of the glutamate-1-semialdehyde to the C-5 neighbouring carbon. This happens through the enzyme of glutamate-1-semialdehyde which is an aminomutase and converts it into 5-aminolevulinate.

IV. **Porphobilinogen synthesis**: Condensation of two 5-aminolevulinate molecules in the presence of porphobilinogen synthase to be able to form porphobilinogen.

V. **Coproporphyrinogen III formation**: Four molecules of porphobilinogen combine to form hydroxymethylbilane which is a linear tetrapyrrole with the help of porphobilinogen deaminase. In the end, hydroxymethylbilane gets bound by the enzyme uroporphyrinogen decarboxylase in order to produce coproporphyrinogen III.

VI. **Protoporphyrin formation**: Oxidative decarboxylation of propionate side chains on rings A and B on coproporphyrinogen to produce protoporphyrinogen IX. It is then catalysed by protoporphyrinogen oxidase and it results with the oxygen-dependent aromatization of protoporphyrinogen into protoporphyrin.

VII. **Magnesium chelation**: Converts protoporphyrin into Mg-protoporphyrin by inserting Mg^{2+}-ions in the presence of Mg-chelatase. This reaction occurs in two distinct step, both of which are ATP dependent.

VIII. **Protochlorophyllide synthesis**: Protochlorophyllide (Pchlide) is made from Mg-protoporphyrin through catalysis by the enzyme Mg-protoporphyrin cyclase which results in the formation of an isocyclic ring E of the Mg-protoporphyrins. Bacteria usually complete this step anaerobically while algae and higher order plants complete this step aerobically.

IX. **Chlorophyllide conversion**: The only light-requiring step of the tetrapyrrole pathway, the conversion of protochlorophyllide to chlorophyllide (Chlide). It occurs through the enzyme protochlorophyllide reductase which catalyses the reaction and adds two more carbons C17 and C18, on ring D.

X. **Chlorophyll synthesis**: Involves the conversion of chlorophyllide into chlorophyll by the help of the enzyme chlorophyll synthetase and geranylgeranyl pyrophosphate (GGPP) which was made in the MEP pathway as seen in Fig. 1.

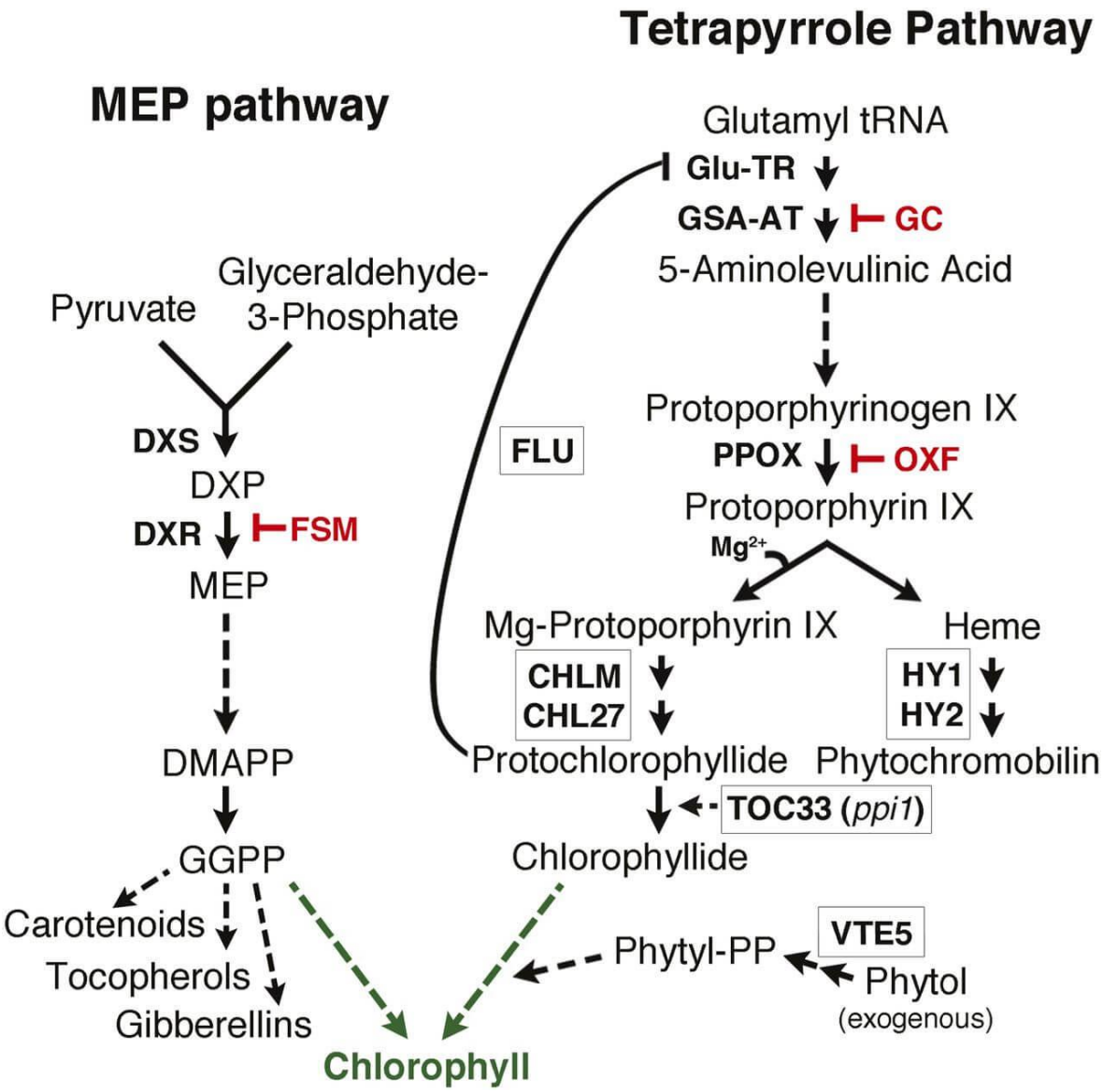

Fig. 1 | The MEP and Tetrapyrrole pathways. This figure shows the two pathways together with all of their different steps and intermediates, most importantly how chlorophyll and GGPP are biosynthesized. Source: Microbenotes (Sapkota, 2023)

The process in which protochlorophyllide gets transformed to chlorophyllide, can be seen in Fig.2. It shows that the only difference between protochlorophyllide and chlorophyllide is the existence of a double bond in protochlorophyllide that gets reduced to a single bond, turning it into a chlorophyllide. This means that the only difference between these two intermediates are 2 hydrogen molecules (Garrone, Archipowa, Zipfel, Hermann, & Dietzek, 2015).

But how does the reduction happen? It was found out that when light particles, or photons, hit the protochlorophyllide clusters, the protochlorophyllide oxidoreductase (POR) enzyme activates. This causes the oxidation of NADPH to $NADP^+$ that reside in between each Pchlide, reducing the double bond to a single bond and yielding Chlide. This can also be seen in Fig.2.

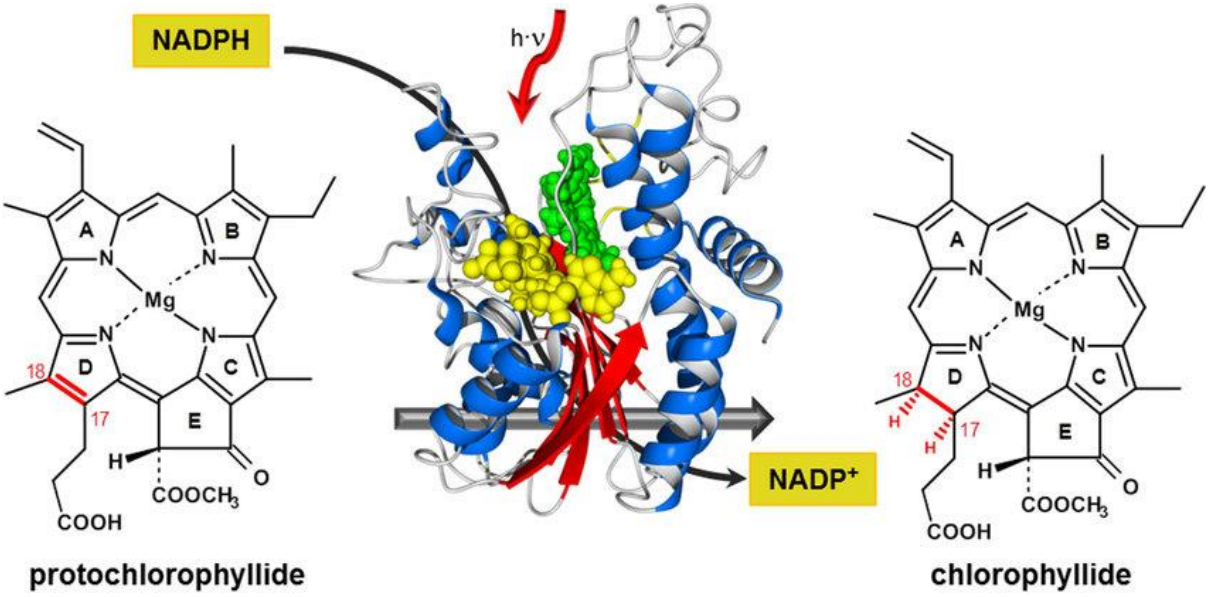

Fig. 2 | The conversion of protochlorophyllide to chlorophyllide. This figure shows protochlorophyllide (on the left) being converted to chlorophyllide (on the right) with the help of the POR enzyme (in the middle). Source: (Garrone, Archipowa, Zipfel, Hermann, & Dietzek, 2015).

2. Scope of thesis (Research Question):

Now that the process by which chlorophyll is produced has been thoroughly explained, it is time to look at why that information was relevant. Step IX is the only light-dependent step in the biosynthesis of chlorophyll. This means that without exposure to light, plants cannot end up producing the pigment, and thus will wither away fast.

But what if we could find a way to produce chlorophyll without the assistance of light? What if we could synthesize chlorophyll in the dark? In this paper, we will try to find a way to yield chlorophyll from its precursors. This will be done with one goal in mind, ensuring that no photons come into contact with the protochlorophyllide.

3. Outline:

Now that the theory has been laid out and the basic knowledge understood, it is time to explain how the thesis will be structured. First of all, the materials will be laid out in a table deducing the different concentrations, molecular weights and volumes. Then the methods used for the thesis are going to be laid out and explained thoroughly in a chronological order, meaning, in the order that they are performed in. This section will not include any calculations or tables.

Then following, each method will get its respective analysis and results presented thoroughly and chronologically. This involves in depth calculations, figures and tables with detailed descriptions of its contents and reasons of inclusion.

The discussion section will focus on the discoveries that were made during this journey. This includes looking at if the set expectations were met, and what implications our discoveries have for the future of this field. Finally, in what ways our methods could be improved in order to ensure that the procedures become more efficient both in terms of time and yield.

At the end, a conclusion will sum up the entirety of the thesis. This, followed by a bibliography where all the sources will be citated. And finally, the appendices, where raw data tables are displayed using a google drive folder, containing all excel sheets necessary to represent the figures.

Materials and solutions:

Table 2 | Table summarizing the materials and solutions along with the molecular weights, grams, and volumes for the chemicals used.

Material/Solution	Molecular Weight (g/mol)	Grams	Volume (mL)	Final Volume (mL)
HEPES Buffer Solution (1M)	-	-	-	500
HEPES	238.30	119.15	-	-
Distilled H_2O	18.01	-	500	500
Potassium Hydroxide (KOH)	56.11	-	-	-
5X Isolation Buffer	-	-	-	100
Sorbitol (0.2 M)	182.17	36.44	-	-
HEPES Solution	-	-	25	-
DTT (1 M)	154.25	-	0.5	-
Distilled H2O	18.01	-	74.5	100
1X Isolation Buffer	-	-	-	1000
Sorbitol (0.4 M)	182.17	72.88	-	-
HEPES Solution	-	-	50	-
DTT (1 M)	154.25	-	1	-
Distilled H2O	-	-	949	1000
40% Percoll Solution	-	-	-	100
100% Percoll	-	-	40	-
Sorbitol (0.4 M)	182.17	7.2872	-	-
HEPES KOH (50 mM)	238.30	-	5	-
DTT (1 mM)	154.25	-	0.1	-
Distilled H2O	-	-	54.9	100
80% Percoll Solution	-	-	-	100
100% Percoll	-	-	80	-

Sorbitol (0.4 M)	182.17	7.2872	-	-
HEPES KOH (50 mM)	238.30	-	5	-
DTT (1 mM)	154.25	-	0.1	-
Distilled H2O	-	-	14.9	100
80% Acetone Solution	-	-	-	50
Acetone (100%)	58.08	-	40	-
Distilled H2O	18.01	-	10	-
40:60 TLC-Running Buffer	-	-	-	100
Acetone (100%)	58.08	-	40	-
Petroleum Ether (100%)	86.18	-	60	-
NADPH Solution	-	-	1	-
NADPH (Stock 12 mM to 31.68 mM)	833.35	0.0264	-	-
1X Isolation Buffer	-	-	1	-
GGPP Solution	-	-	-	-
GGPP (95% solution, 1 M)	-	-	1	-
Methanol/NH4OH (7:3 ratio)	-	-	-	-
Distilled H2O	-	-	34	-

Notes:

- Molecular weights are provided only for specific chemicals where relevant.
- Volumes are given in milliliters (mL).
- The final volume is the total volume after all components are added and mixed.
- For the GGPP solution, the methanol and NH4OH components' volumes are not specified but are in a 7:3 ratio.

1. HEPES buffer solution (1 M):

119.15 g of HEPES (1 M) was dissolved in 375 mL of distilled H_2O and adjusted to pH 8 with KOH tablets. The rest of the 500 mL bottle was filled with distilled H_2O, autoclaved and then stored at 4°C.

2. 5X isolation buffer:

For a 100 mL solution, 36.44 g of sorbitol (0.2 M) was weighed in and added to a 100 mL storage flask. Then 25 mL of HEPES solution (1 M) and 500 µL of 1 M dithiothreitol (DTT) were measured and added to the flask. The flask was filled to 100 mL with distilled H_2O.

3. 1X isolation buffer:

For this solution, to 72.88 g of sorbitol (0.4 M) was dissolved in 50 mL of the HEPES solution and 1 mL of 1 M, DTT solution and distilled H_2O to a final volume of 1 L.

4. 40% percoll solution:

The 40% percoll solution was measured at 100 mL and contained 40 mL of percoll, 0.4 M of sorbitol which corresponds to 7.2872 g for the 100 mL solution, 50 mM of HEPES KOH which corresponds to 5 mL for the 100 mL solution and 1 mM of DTT which corresponds to 100 µL for the 100 mL solution.

5. 80% percoll solution:

The 80% percoll solution was measured at 100 mL and contained 80 mL of percoll, 0.4 M of sorbitol which corresponds to 7.2872 g for the 100 mL solution, 50 mM of HEPES KOH which corresponds to 5 mL for the 100 mL solution and 1 mM of DTT which corresponds to 100 µL for the 100 mL solution.

6. 80% acetone solution and 40:60 TLC-running buffer:

The 80% acetone solution was created in a 50 mL Falcon tube, by mixing 40 mL of 100% acetone with 10 mL of water. The 40:60 TLC-running buffer was prepared by mixing 40 mL of the 100% acetone with 60 mL of the 100% petroleum ether.

7. NADPH solution for the light dependent reaction:

A 12 mM stock NADPH solution was calculated, resulting in 1 mg of NADPH tetrasodium salt hydrate with a molecular weight of 833.35 g, having to be dissolved into 100 µL of 1X IB. But since it is very difficult to accurately measure 1 mg on a normal scale, a measurable amount was needed. The final concentration of the NADPH solution needed to be around 30 mM, so it was calculated that 26.4 mg of NADPH would be dissolved in 1 mL of 1X IB to arrive at a concentration of 31.68 mM.

8. GGPP solution for the process of chlorophyll synthesis:

The GGPP is a 95% solution with a stock concentration of 1 M in a 7:3 ratio of methanol and NH_4OH respectively. The final concentration of the GGPP solution needed to be 63 µM so 1 µL of GGPP was added to 34 µL of water. This was done to have the GGPP concentration match up with the NADPH and plastid concentrations.

Methods:

1. Percoll gradient plastid isolation:

The 4.75 day old barley leaves were cut approximately 2 cm from the base of the plant. This was done with the help of a single blade scissor and collected into a large beaker. Then, some 1X IB was added to the beaker to drench the cut leaves.

After all the drenched etiolated leaves were cut again using a multi-bladed scissor, they were blended using the Ultra-turrax to cut down the leaves into smaller pieces.

After the leaves were cut down, they were then filtered through both a cheese cloth and a 22 µm pore nylon gauze. This was done to filter all the plant fibers and potential vermiculite out of the sample.

The remaining plant matter was then added to a beaker again and enough 1X IB added to re-dissolve the remaining matter. This was followed by another round of the Ultra-turrax. This maximizes plastid yield and ensures that no large leaf chunks remain uncut. The remaining blend is then filtered again through both a cheese cloth and a 22 µm pore nylon gauze. The solid fibre was then carefully squeezed to remove any excess liquid that remained trapped in it, before discarding it.

The filtrate is then transferred into two 500 mL centrifugation tubes, and balanced until both tubes have around similar amounts of liquid each, followed by a centrifugation at 4650 RPM for 3 minutes using the F10S-6X500Y rotor.

The tubes were then removed from the centrifuge, followed by the discarding of the supernatant, and the addition of 1 mL of 1X IB to one of them. Then the pellet was resuspended and transferred to the other tube. The pellet in the other tube was resuspended using the liquid transferred.

The resuspended pellet was taken and filtered again over a 22 µm nylon gauze to get rid of the nuclei in the plant cells. This helps filter out broken nuclei pieces from the etioplasts, ensuring a higher purity plastid sample.

Then two fresh 30 mL swing-out centrifugation tubes were taken and a 10 mL layer of 40% percoll was pipetted into each one. Then a layer of 10 mL 80% percoll was pipetted under the surface of the 40% percoll solution, at the bottom of the tube. In the end, 1 mL of the sample was pipetted on top of the 40% percoll layer. This was done to filter out mitochondria and broken plastids that were suspended in the sample. The broken plastids and the mitochondria were left floating at the top of the 40% percoll solution, while starch sinks to the bottom of the 80% percoll layer. This left the intact plastids at the bottom of the 40% percoll layer.

The 2 tubes were then centrifuged using the swing-out centrifuge module at 4230 RPM for 4 minutes using the HB-6 rotor. This was done to separate the different components across the percoll gradients.

To extract the layer of intact plastids from the 40% percoll layer, a 200 µL pipette was taken with a pipette tip attached, and the tip of the pipette tip was cut off, allowing for an easier extraction. The plastids were then transferred into a 2 mL centrifugation tube together with some 1X IB to resuspend the sample. This tube was then centrifuged for 5 minutes at 4000 G’s.

The supernatant was then removed from the tube, and 200 µL of 1X IB was transferred into the pellet sample and resuspended one final time. The tube was stored either on ice for immediate use, or in the -80°C freezer for longer time storage.

2. Crude plastid isolation:

The 4.75 day old barley leaves were cut approximately 2 cm from the base of the plant. This was done with the help of a single blade scissor, and collected into a large beaker. Then, some 1X IB was added to the beaker to drench the cut leaves.

After all the drenched etiolated leaves were cut again using a multi-bladed scissor, they were blended using the Ultra-turrax to cut down the leaves into smaller pieces. After the leaves were cut down, they were then filtered through both a cheese cloth and a 22 µm pore nylon gauze. This was done to filter all the plant fibers and potential vermiculite out of the sample.

The remaining plant matter was then added to a beaker again and enough 1X IB added to re-dissolve the remaining matter. This was followed by another round of the Ultra-turrax. This maximizes plastid yield and ensures that no large leaf chunks remain uncut. The remaining blend is then filtered again through both a cheese cloth and a 22 µm pore nylon gauze. The solid fibre was then carefully squeezed to remove any excess liquid that remained trapped in it, before discarding it.

The remaining liquid was then evenly distributed and transferred into 4 different 500 mL centrifugation tubes and balanced until all of them had around the same amount of liquid each. This was followed by a centrifugation at 4650 RPM for 3 minutes using F10S-6X500Y rotor.

The tubes were then removed from the centrifuge followed by the discarding of the supernatant. 5 mL of 1X IB was then added to each individual centrifugation tube to resuspend the different pellets.

The total amount of liquid from the 4 tubes were transferred into a 30 mL swing-out centrifugation tube. The opposite tube had an equal amount of water to balance the centrifuge. The centrifugation was run at 4230 RPM using the HB-6 rotor for 4 minutes.

After the centrifugation process, the supernatant was discarded and 1 mL of 1X IB was added to the pellet to resuspend it. This was then transferred to a 2 mL Eppendorf cup and stored in a black box, at -80°C.

3. Plastid dilution and count:

198 µL of 1X isolation buffer was pipetted into two different 2 mL Eppendorf tubes.

Also, two other 2 mL Eppendorf tubes were filled with 80 µL of 1X isolation buffer each.

2 µL of the stored plastid sample was then pipetted into the 198 µL 1X isolation buffer containing tubes.

Then 20 µL was taken from these tubes and added to the 2 mL Eppendorf tubes containing 80 µL of 1X IB. This now makes the etioplast sample diluted 1:500.

Then the etioplasts were counted with the help of a microscope by using the Thoma counting grid. It is 1 × 1 mm^2 with a 0.1 mm counting chamber size as seen in figure 3 below. The goal of this step was to end up determining how many etioplasts there were in the whole sample. The process of calculation will be described in the Analysis and results section.

10 µL of water was inserted into each of the two chamber wells. Afterwards, a glass plate was added on top of the chamber to help disperse the volume across the entire well.

Finally, 10 µL of the 1:500 dilution was carefully pipetted in between the glass plate and the counting chamber, in order to load the sample on the grid. Now count the total amount of plastids in the top four cells.

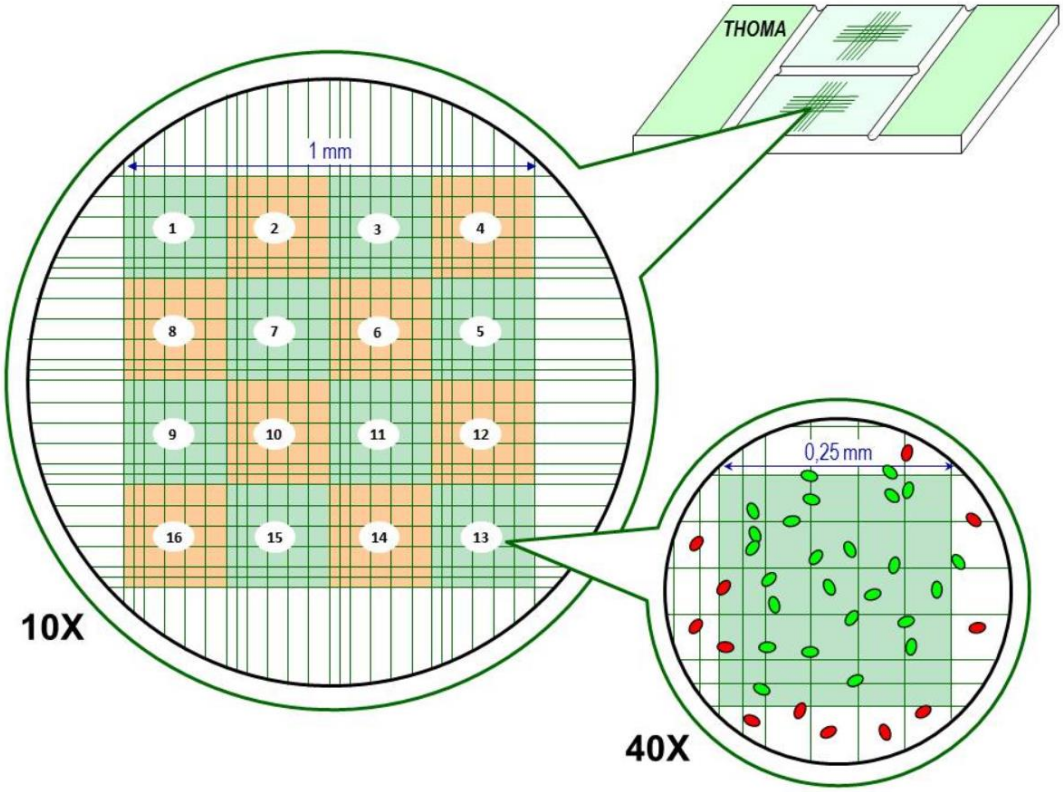

Fig. 3 | Thoma counting chamber. This figure represents and shows how the Thoma counting chamber looks like under the microscope, having a total of 16 individual cells, and those cells in total having an area of 1 mm^2. Source: (University of the Basque Country, n.d.)

4. Absorbance spectroscopy and TLC-analysis:

The cuvettes were washed with 100% acetone to get rid of different residuals.

Then the 80% acetone solution was made, and 2 centrifugation tubes were filled, one with 280 µL of 100% acetone and 70 µL of the plastid sample, and one with 350 µL of 80% acetone for balance. The tubes were centrifuged at 20800 G's for 10 minutes.

After the centrifugation was done, 50 µL of the sample supernatant was taken and would later be used to load the TLC-plates.

Then another 250 µL of the supernatant was taken out and added to 250 µL of 80% acetone in another tube, to be added to the cuvettes. The UV-2041PC spectrophotometer was used to run these upcoming graphs.

A blank sample consisting of 500 µL 80% acetone solution was ran on the spectrophotometer to calibrate it.

After the calibration, the cuvette containing the mix of 250 µL sample and 250 µL of 80% acetone was ran through a sample measurement scan and a graph was made. This is shown in the Analysis and results section.

After the graphs were made, the TLC-running buffer was prepared as described in the Solutions section. Following that, the TLC-chamber was filled about 3 mm from the middle bottom of the chamber. The chamber was left for around 30 minutes, to equalize itself with the surrounding atmosphere.

The TLC-plate was stained with the 50 µL of the sample supernatant, one small spot containing 10 µL and one big spot containing 40 µL. When the running buffer reached the spots, the samples were taken out and dried. This step was repeated until 2 clear yellow lines could be seen on the TLC-plate. The plate was then reinserted into the chamber and let to soak up the running buffer. The plate was a normal phase TLC sheet.

After the TLC process is completed, let the plate dry, and then insert it into the Typhoon biomolecular scanner to print an image of it. Configure the machines' settings, as shown below, and then let the printer run until its finished. The different channel photos were examined and enhanced to show how the different pigments moved up the TLC-plate.

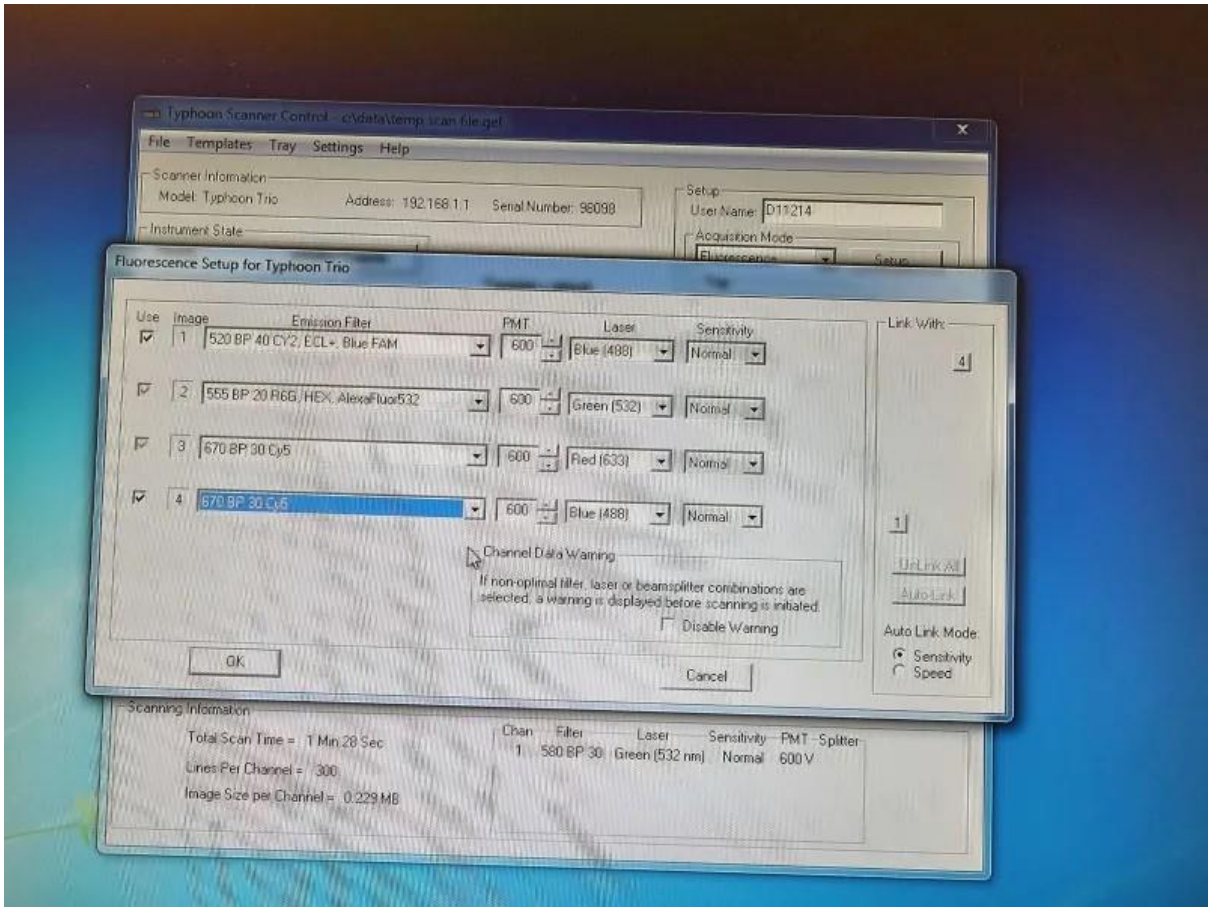

Fig. 4 | Setup for the Typhoon Trio fluorescence scan. This figure shows the different emission filters and laser wavelengths used to complete 4 different scans on the Typhoon Trio machine.

5. Pigment determination:

Following the Typhoon scannings, the different lines that appeared on the TLC plate, were numbered like in the diagram below. The lines scraped off with a sharp razor and put into five 2 mL Eppendorf tubes.

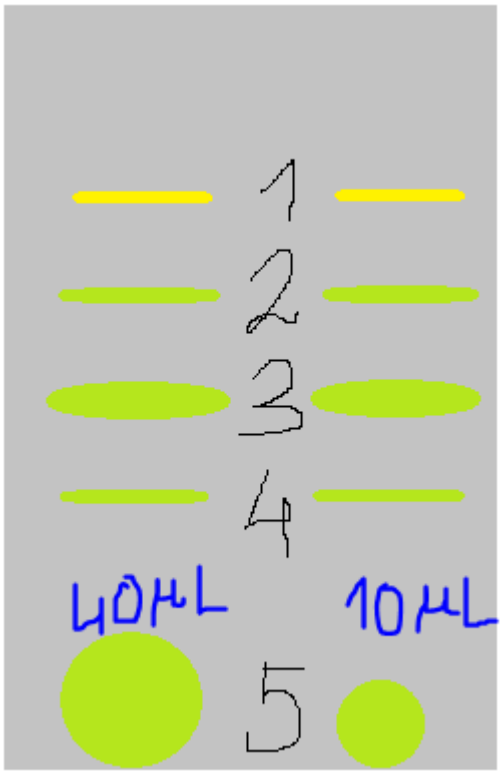

Fig. 5 | TLC-plate after the TLC was ran. This figure shows how the TLC-plate looked like after the process was complete. Two different volumes of the plastid sample supernatant were loaded on the plate, creating two distinctly sized circles. There were 5 distinct spots where pigments were accumulated, and there were numbered 1-5.

The pigments in the scrapes were re-extracted with 550 µL of 80% acetone solution and centrifuged in order to get rid of any residuals left by the TLC plate.

The spectrophotometer was turned on and a baseline was ran using 450 µL of 80% acetone. Afterwards, the different pigment tubes were then analysed and graphed using the UV-2401PC.

6. Protein determination and spectra analysis:

The number of plastids per µL was calculated from the weeks' sample of isolated plastids, and then the sample amount needed to make a 50 µL dilution of sample and 1X IB. In this case, 28.572 µL of sample and 21.428 µL of 1X IB were transferred to an Eppendorf tube. This tube was used for the protein determination.

The spectrophotometer was calibrated, to be prepared for the protein determination. 500 µL of Bradford solution (1X Dye Reagent, BioRad) was added to a cuvette together with 12.5 µL of 1X IB. The baseline was recorded.

The protein determination process was started by taking 5 µL of the isolated plastids and diluting it down to a concentration of 5×10^5 plastids/µL. In this case, 5 µL of

plastid sample needs to be diluted with 30 µL of 1X IB to reach a concentration of 5×10^5 plastids/µL.

From this dilution, 6 new solutions were made, each with different concentrations of plastids: 2.5×10^5, 5×10^5, 7.5×10^5, 1×10^6, 2.5×10^6, and 5×10^6. Since 1 µL of the new plastid sample dilution has a concentration of 5×10^5 plastids/µL; 0.5 µL, 1 µL, 1.5 µL, 2 µL, 5 µL and 10 µL need to be added respectively. Similarly, different amounts of 1X IB need to be added to match the total volume of 12.5 µL: 12 µL, 11.5 µL, 11 µL, 10.5 µL, 7.5 µL and 2.5 µL respectively. 500 µL of Bradford solution was added to each measurement to measure the protein concentration. This was followed by a graphing of each dilution showcasing the absorbance of different concentrations.

7. Excitation and Emission graphs (fluorescence checking):

The plastid sample was re-extracted using 28.572 µL of the sample, together with 21.428 µL of 100% acetone and 450 µL of 80% acetone.

Then, the sample was run and graphed through a fluorolog. The resulting graph was an emission graph for the ranges 465 nm to 840 nm, with an excitation check at 430 nm.

Then, an excitation graph was run for the ranges of 350 nm to 650 nm with an emission check at 675 nm.

8. Protochlorophyllide concentration determination

A 2 mL Eppendorf tube was taken and 800 µL of 100% acetone added to it together with 200 µL of water in order to create an 80% acetone solution.

Another Eppendorf tube was filled with 800 µL of 100% acetone, 10 µL of isolated plastid sample and 190 µL of water. This makes the sample diluted 1:100.

Repeat this step with 20 µL and 30 µL of sample, meaning subsequently 180 µL and 170 µL of water respectively. This makes a dilution ratio of 1:50 and 1:33.3 respectively.

The tubes were mixed and put on ice to cool down to 4°C for 15 minutes. The tubes needed to be chilled for 10 minutes. In addition, it takes around 5 minutes for them to cool down to 4°C.

After cooling down the samples, the tubes were centrifuged for 10 minutes at 16800 G's. Three other, 2 mL Eppendorf tube were prepared with 800 µL of 100% acetone and 200 µL of water. These work as balances for the centrifugation process.

While the centrifugation was running, the spectrophotometer was calibrated using the first tube made. The 80% acetone solution was used to set a baseline for the measurements and analyses of the sample.

After the baseline was set and the centrifugation done, the 1:100 diluted sample was run from 850 nm to 340 nm, followed by the 1:50 and then 1:33.33 samples. The reason 340 nm was chosen as the endpoint was because after 340 nm, the acetone started interacting and interrupting the spectrophotometer, creating a disturbance in the spectra.

The protochlorophyllide concentration was then calculated using the Lambert-Beers' law: $A = \varepsilon \times l \times c$. A is the absorption reading on the y-axis on the graph, ε is the molar extinction coefficient, l is the cuvette light pathway, and c is the concentration of protochlorophyllide that must be found. So, the equation would look like this: $\frac{A}{\varepsilon \times l} = c$.

9. In vivo photoconversion of protochlorophyllide to chlorophyllide followed by chlorophyll synthesis:

The spectrophotometer was turned on and prepared for analysis. Following that, some seedlings were taken out and mounted on the scanning plate. The mounting was done in the dark and through the following procedure.

Firstly, the scanning plate was prepared, and a piece of black tape was cut. Thereafter, the black tape was folded in on itself and attached to both ends of the scanning plate.

Second, the seedlings were placed evenly spaced on the black tape with the tips pointing towards the bottom of the scanning plate. This is done to keep the seedlings stationary, allowing for an easier and more precise scanning over a larger area. This also ensures that the photons sent out by the spectrophotometer reach and analyse the barley leaves.

Third, for the roots to not dry out, they were placed in between two soft tissues that have been wetted and wrapped in plastic foil. This was performed to keep everything moist and in place. This setup can be seen in the attached figure under.

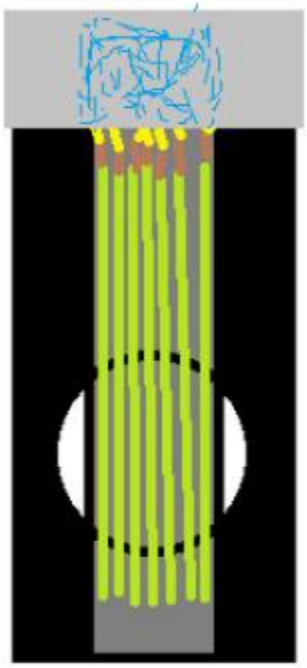

Fig. 6 | Setup for the in vivo analysis process. This figure shows the scanning plate (represented in black) containing the $BaSO_4$ (represented in white), together with the black tape (represented in dark gray) holding the etiolated seedlings (leaves represented in green, seeds represented in brown, and roots represented in yellow), all being held together by the wet paper and plastic foil (represented in light gray + blue lines).

After the setup is finished, the scanning plate was mounted in the spectrophotometer. A dark spectrum was ran in order to check if the leaves were truly etiolated.

Following the dark spectrum, the leaves were briefly illuminated for 10 seconds using a white light lamp at maximum intensity. Now that the leaves were deetiolated, continuous spectra were ran for a duration of 6 hours in order to show the full transformation of protochlorophyllide to chlorophyllide. The next day, 18 hours after the start of the first measurement, another measurement was taken. This was done so that the eventual changes that took place in the 12 hour spacing could be categorized and analysed.

10. In vitro addition of NADPH and GGPP to isolated etioplasts & subsequent photoconversion of protochlorophyllide to chlorophyllide and chlorophyll:

The spectrophotometer was turned on and set up for analysis. The isolated plastids and the NADPH and GGPP solutions were prepared and kept on ice in a black box.

A baseline was ran using 1X isolation buffer. When analysing the plastids, the 1X isolation buffer would make up most of the sample volume due to it being the solute. 450 µL of 1X isolation buffer was added to both the reference and sample cuvettes.

The room was made dark by turning off the lights and making sure that any unwanted source of light was turned off. The spectrophotometer was opened, and the cuvette taken out. 360 µL of isolated plastids were added to the cuvette. A dark spectrum was then ran, showcasing the absorption of protochlorophyllide.

After the dark spectra was ran, the spectrophotometer was opened again and 25 µL of the 31.68 mM NADPH solution was added to the cuvette. The liquid in the cuvette was then mixed to allow for the NADPH to bind to the protochlorophyllide. 20 repeats were ran in order to showcase the changes happening over a longer period of time.

After the 20 repeats, the spectrophotometer was opened again and the white light lamp was turned on. Full power was used and shined on the sample cuvette for 10 seconds before turning off the lamp and closing the spectrophotometer. 5 more repeats were ran in order to get a better idea of what the nature of the changes were. These spectra showcase the transition of protochlorophyllide to chlorophyllide.

After the 5 repeats, the spectrophotometer was opened one last time, and 25 µL of the 63 µM GGPP solution was added to the cuvette. Approximately 100 µL of extra plastid sample was added to the cuvette following the GGPP due to the liquid starting to evaporate. This is a consequence of the high temperature forming inside of the spectrophotometer. The liquid in the cuvette was then mixed again. Lastly 2 spectra were ran in order to show the change of chlorophyllide to chlorophyll through the chlorophyll synthase enzyme.

The same exact process was repeated in a 50 µL mini cuvette, but reducing the amount of repeats to 5 for the protochlorophyllide:NADPH spectra. Then 2 measurements for the chlorophyllide spectra and 1 measurement for the transition of chlorophyllide to chlorophyll. The mini cuvette setup is depicted in figure 7.

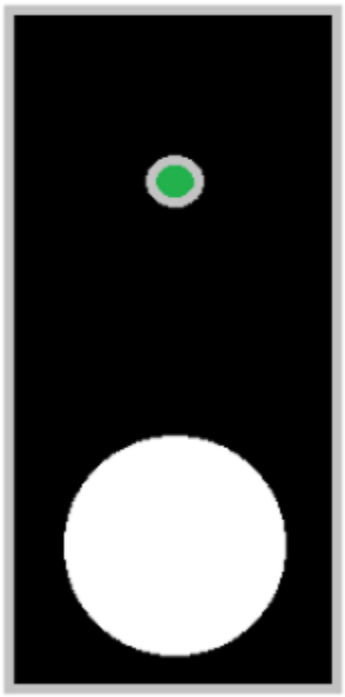

Fig. 7 | Setup for the in vitro analysis process. This figure shows the scanning plate (represented in black) containing the $BaSO_4$ (represented in white), together with the 50 µL cuvette compartment, (represented as the grey circle), and the analysed plastid sample (represented in green).

Since chlorophyllide and chlorophyll readings could not be discerned as easily through spectrophotometry, a TLC was ran using the sample from the normal cuvette. This was done after the graphing was done, to see if it resembles other recorded chlorophyll TLC data.

Analysis and results:

1. Plastid dilution and count:

Count the top 4 cells of the Thoma chamber. Multiply the number gotten by four so that you get an approximate amount for how many plastids there are in the total 16 chambers.

Take this number and multiply that by 10. This is done because the value from the first step denotes the amount of plastids per 0.1 µL.

Multiply this value by the dilution factor. In this case, the dilution was a 1:500 dilution, so the dilution factor is 500. This is done to calculate how many plastids there were, per µL.

Lastly, multiply this value by the amount of the total amount of µL in the whole sample, to get a value that represents the total number of plastids.

This calculation was now done a total of three times, with 10 µL each, so that a mean plastid value could be determined. This is more representative of the total plastid sample. This calculation was done each time a plastid isolation was conducted. This makes everything easier as it shows it to be safe to work with later on for other procedures. The reason behind this being increased accuracy. A calculation example is shown below:

$$261 \times 4 = 1044\ (plastids\ for\ 16\ cells) \rightarrow 1044 \times 10 = 10440\ \left(\frac{plastids}{\mu L}\ dilution\right) \rightarrow\ 10440 \times 500$$

$$= 5{,}220{,}000\ \left(\frac{plastids}{\mu L}\ original\ sample\right) \rightarrow 5.22 \times 10^{6} \times 200 = 1.044 \times 10^{9}\ (\frac{plastids}{sample})$$

2. Absorbance spectroscopy and TLC-analysis:

The plastid measurement in 80% acetone can be seen in figure 7, and the raw data used in Appendix A:

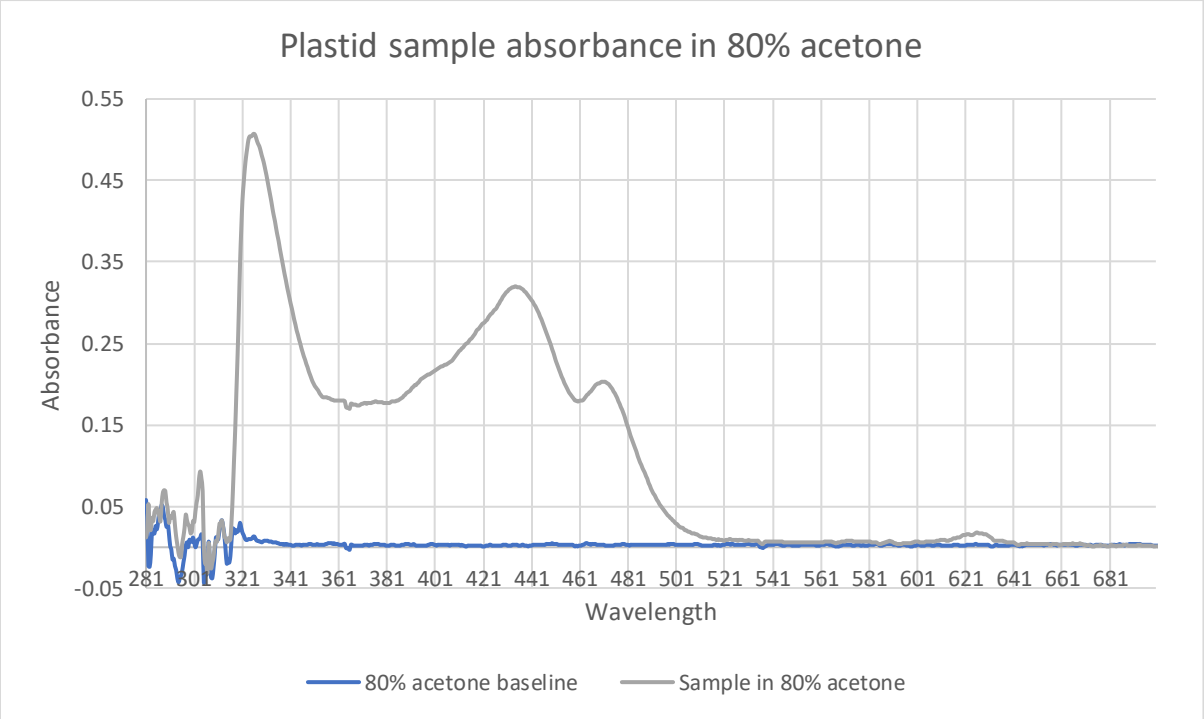

Fig. 7 | Plastid sample absorbance graph in 80% acetone. This graph represents an absorbance spectrum for the whole plastid sample in the range 281 nm to 700 nm. The blue graph represents the baseline while the grey graph represents the plastid sample.

The spectrum was measured between 281 nm and 700 nm. This was done for two reasons. The first reason being that the spectrum for the different plastid pigments lies in between 340 nm and 700 nm. Since carotenoids and xantophylls absorb between 400 nm and 500 nm (Lichtenthaler & Buschmann, 2001), then the two maxima recorded at 437 nm and 474 nm represent exactly that. Also, since

protochlorophyllide absorbs at 627 nm and 633 nm respectively (Ignatov & Litvin, 1996), then the maxima recorded at 627 nm represents that.
The second reason for the spectrum being measured between 281 nm and 700 nm is because under 281, there were huge interferences that were caused by the 80% acetone solution. This ended up creating very huge peaks that would go up to an absorbance value of 5. This, over time, can cause damage to the spectrophotometer. All of this can be seen in the full data values in Appendix A.

Next up the TLC was ran, followed by the scanning of the TLC-plate, using the Typhoon. This gave 4 scans using different emission filters and lasers, as shown in figure 4. Here are the two clearest scans compared side by side:

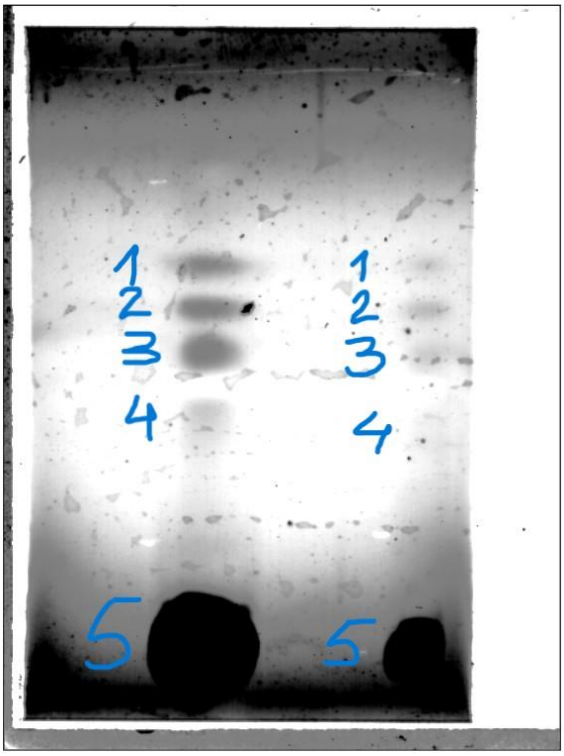

Fig. 8 | TLC-plate scan with red laser. This scan represents the TLC-plate, after the TLC was ran, under the exposure of a red laser. The 5 different bands represent different pigments.

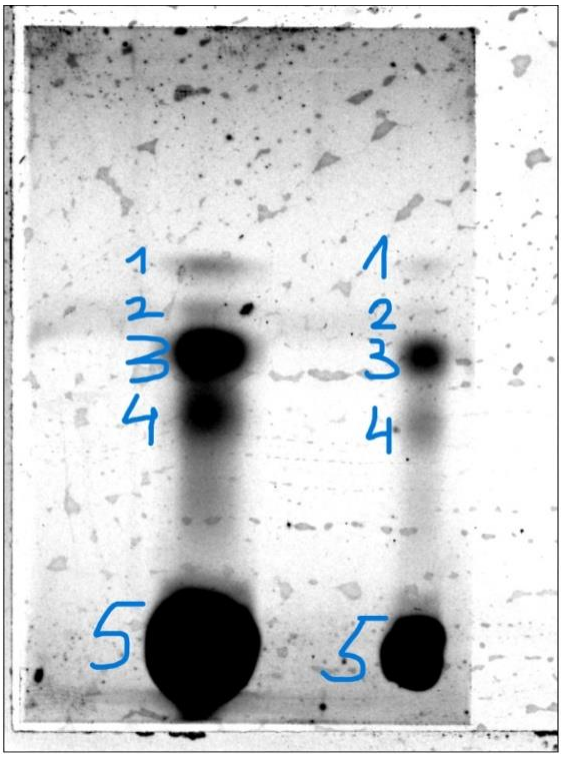

Fig. 9 | TLC-plate scan with blue laser. This scan represents the TLC-plate, after the TLC was ran, under the exposure of a blue laser. The 5 different bands represent different pigments.

Figures 8 and 9 are the third and fourth scans done by the Typhoon, and as stated, they were exposed to a red (633 nm) and blue (488 nm) laser respectively. The differences in lasers used can be observed in bands 2 and 4. In figure 8, band 2 is more visible than band 4, whereas in figure 9 the opposite is true. This is due to the differences in the absorbances of the different pigments that make up these two bands.

The main question that would arise after viewing these two figures is, “what pigments do the different bands represent?”.

The answer lies in a study recently published in June 2023 by a group of researchers from the Prayoga Institute of Education Research in India. The researchers used a mixture of hexane, ethyl acetate, acetone, isopropyl and water in a 6:1:1:1:1 respective ratio as the mobile phase for the TLC. The results ended up giving a TLC-plate that looks like this:

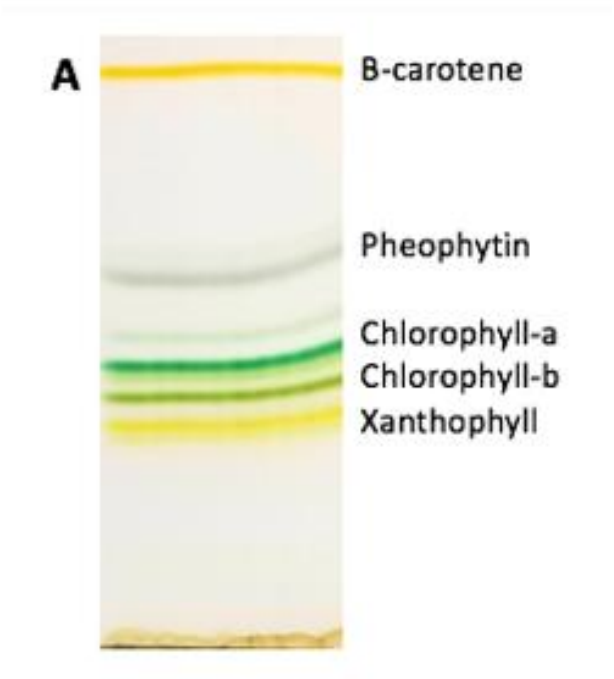

Fig. 10 | TLC images of deetiolated green leaf pigment extracts. The 6 pigment bands that are shown represent the 6 main pigments that help a green leaf with photosynthesis. Source: (Vishwajit, Samanyu, Varnika, Chinmaya, & Subhadip, 2023)

The mobile phase used for the TLC in figure 10 is the closest mobile phase to the one used in this thesis. Ethyl acetate, acetone, isopropyl and water represent the polar part of this solvent, making up 40% of it, while the hexane represents the non-polar part of this solvent, making up 60% of it. In this experiment, acetone (polar) makes up 40% of the solvent, while petroleum ether (non-polar) makes up 60% of it.

Since the ratio of polar to non-polar is the same, then it can be imagined that the pigments in etiolated leaves will move similarly to the pigments in deetiolated leaves. With that said, it can be concluded that the pigments that show up numbered 1 through 5 are as follows: Number 1 is β-carotene. Number 2 is pheophytin, because it absorbs at a wavelength between 630 and 670 nm (Marker, 1977), and it can be seen that the second pigment shows a stronger band under a red laser. Number 3 is protochlorophyll, which is much more non-polar than protochlorophyllide due to its

long chain of phytol (Kurdziel, Kruk, & Strzałka, 2013). It absorbs between 630 and 640 nm under the red laser (Kurdziel, Kruk, & Strzałka, 2013), but under the blue laser the chlorins in protochlorophyll absorb between 488-496 (Ruban, Pascal, Robert, & Horton, 2001). Number 4 is xantophyll which absorbs between 466-495 nm (Study.com, n.d.). Finally number 5 is protochlorophyllide which is mostly polar so it barely moves. It also absorbs at 633 nm for the red laser (Ignatov & Litvin, 1996).

3. Pigment determination and spectra analysis:

The five tubes with pigments dissolved in 80% acetone were analysed and graphed from 850 nm to 190 nm each. This was done for two reasons. To show the entire spectra and to be able to give a better overview of the different pigments' absorption properties. The graphs made can be seen below in figure 11, and the complementary raw data in Appendix B:

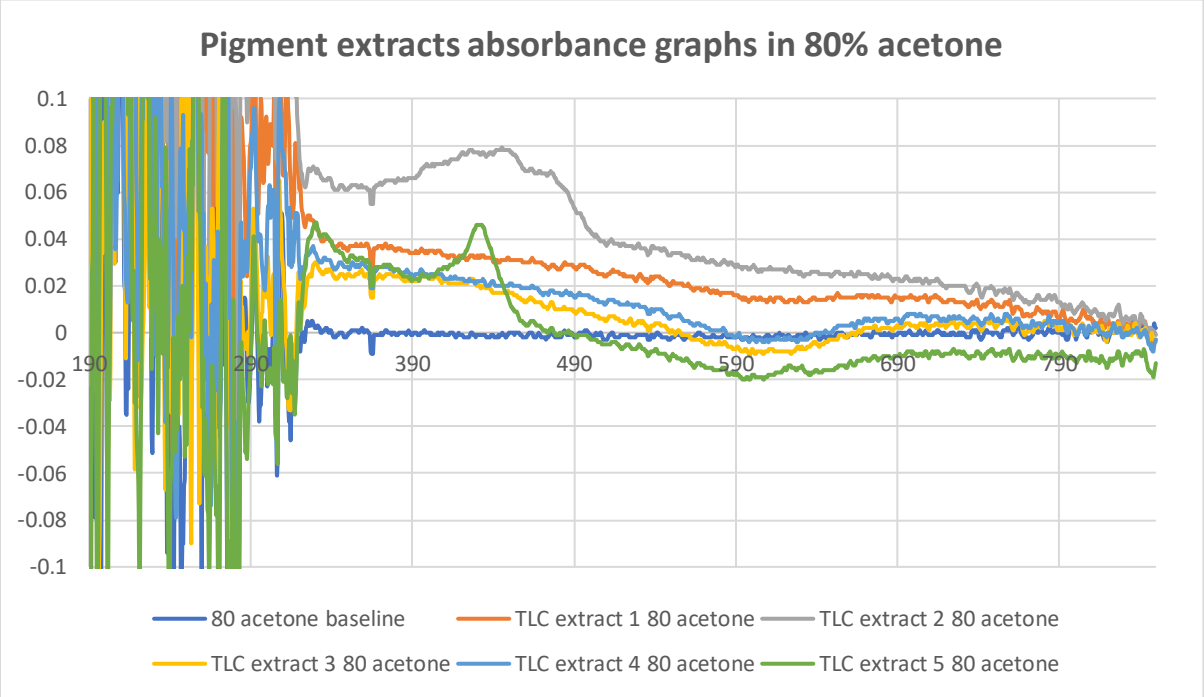

Fig. 11 | Pigment extracts absorbance graphs in 80% acetone. This graph represents 5 absorbance spectra of the different bands from the TLC plate together with the 80% acetone baseline.

Under circa 340 nm, a lot of disturbances start to occur from both the baseline and the other extract graphs. This is likely due to the acetone absorbing at around 300 nm, causing a lot of scattering in return. To counteract that, the graph can be made more restricted to get a clearer view, narrowing down the x-axis from 340 nm to 700 nm. Most pigments absorb in this range as shown in part 2 of the analysis section.

The corrected graph can be seen below in figure 12:

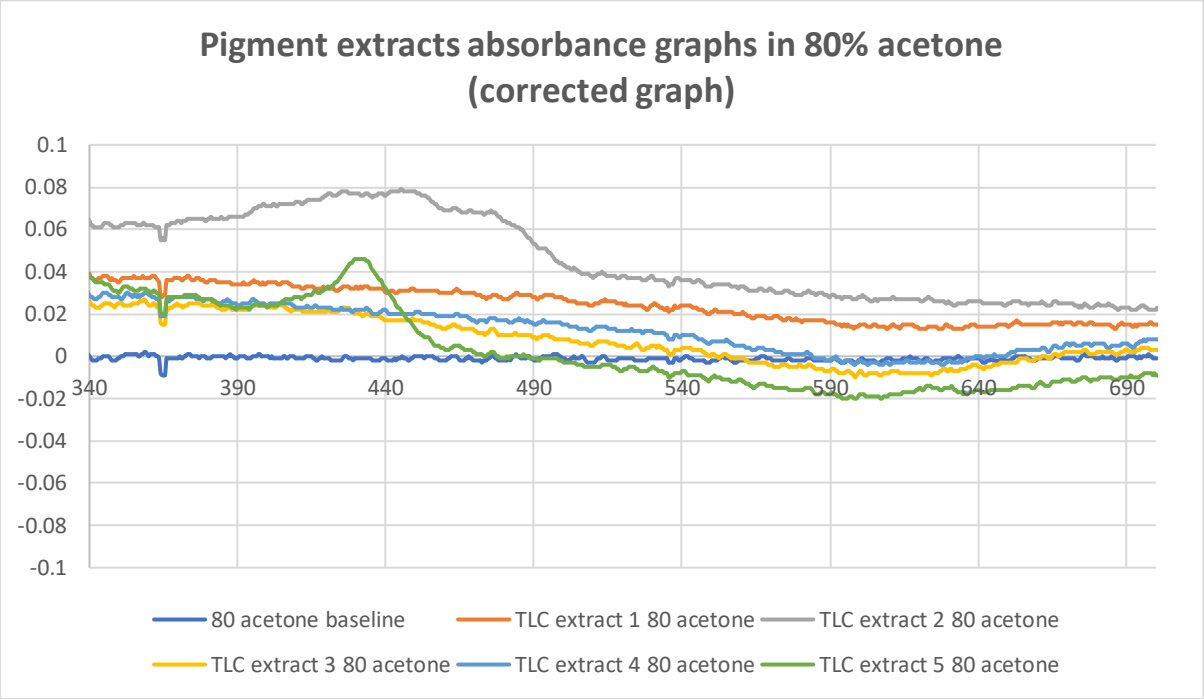

Fig. 12 | Pigment extracts absorbance graphs in 80% acetone (corrected graph). This graph represents 5 absorbance spectra of the different bands from the TLC plate together with the 80% acetone baseline. This graph has been corrected and narrowed down in order to assure a better view of the different variations in absorbance between the different extracts.

According to part 2 of the analysis section and to part 5 of the methods section, the extracts 1 through 5 should contain β-carotene, pheophytin, protochlorophyll, xanthophyll and protochlorophyllide respectively. But the absorbance values of the extracts are very low and do not show any significant peaks throughout. The only two peaks that could be taken into consideration and discussed are the second and fifth extract.

The second extract shows an increase in absorbance from around 510 nm, until a peak at 476 nm and then again until a peak at 446, increasing from an absorbance of 0.037 to 0.068 and then to 0.078 respectively. Part 2 of the analysis section says that the second extract is supposed to be pheophytin, but pheophytin absorbs in between 630 nm and 665 nm (Marker, 1977). There is also no absorption peak in between 630 nm and 665 nm for pheophytin. Then what could it be, causing the peak from 476 nm and 446 nm?

The fifth extract shows an increase in absorbance from around 473 nm, until 432 nm, increasing from an absorbance of 0.000 to an absorbance of 0.043. Part 2 of the analysis section says that the fifth extract is supposed to be protochlorophyllide, but protochlorophyllide absorbs at 633 nm, and its enzyme POR absorbs at 488 nm. But there is no absorption peak at either 633 nm or 488 nm for those two. Then what could it be causing this peak at 432 nm?

If the cuvette has any scratches or impurities like dust, sweat, or fat particles, the spectrophotometer will pick it up through its light scattering pattern. This input is relative to the solvent and the output is the amount of scattering detected that gives an absorbance value. According to an application experiment, water absorbs at around 750 nm regardless of purity. This can be seen in figure 13:

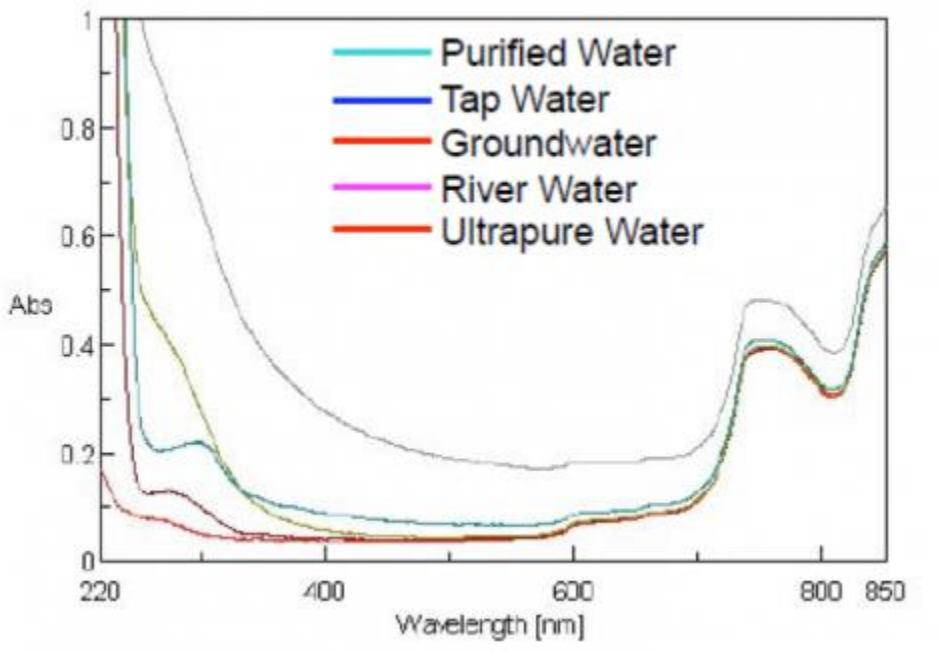

Fig. 13 | Water absorbance graphs. This graph depicts different types of water with different degrees of purity, and their respective absorbance spectra, showing a common absorbance peak at around 750 nm. Source: (Uchiyama, 2021)

There is a common peak for all the water samples, starting from around 720 nm and continuing until around 780 nm. This experiment was performed in a 30 cm cuvette cell. In a 30 cm cell, the differences between the water samples become more apparent than in a normal 10 mm cell. But nevertheless, the absorption peak of water is way off compared to the peaks recorded in the extracts' graphs. So it is safe to conclude that water is not the cause of the peaks.

But what about sweat? Sweat is mostly made up of water, but a lot of salts and different electrolytes too. According to a study from 2014, sweat absorbs at around 450 nm, regardless of concentration. This can be seen in figure 14:

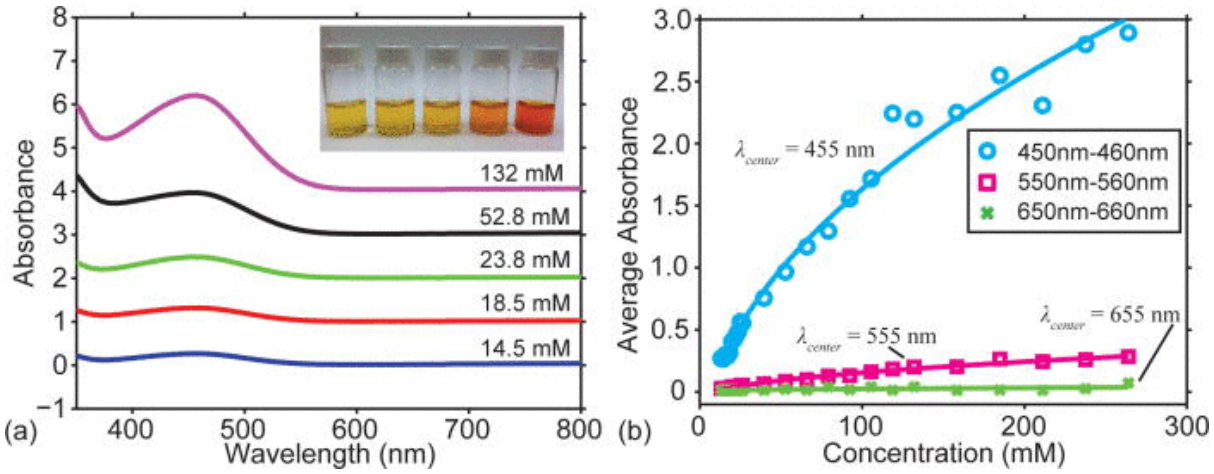

Fig. 14 | UV-vis spectra of artificial sweat and average absorbance analysis. These graphs show a) Spectra of artificial sweat at 5 different constituent concentrations and b) Average absorbance of the different artificial sweat solutions versus concentration at the centre wavelengths of 455 nm, 555 nm and 655 nm to which the averaging was done over a 10 nm bandwidth. Source: (Alomari, Liu, Mueller, & Mock, 2014)

From these graphs it can be observed that the absorption peak is around 455 nm regardless of the sweat concentration, ranging from around 450 nm to 460 nm. To check that the absorption value is correct, a plot was made of the average absorbance of the solutions against the concentration. This, showed that the absorbance increases exponentially in relation to increasing concentrations in between 450 nm and 460 nm, showing that sweat perfectly absorbs in this range.

The two absorbance peaks in the TLC-extracts graph are at 446 nm and 476 nm for the second extract and at 432 for the fifth extract. It can be observed that the graphs resemble somewhat the sweat absorption graphs shown in figure 14. The reason the peaks might be situated at either a higher or a lower wavelength is perhaps due to other kinds of impurities getting caught up in the sweat molecules. This is causing a deviation of about 20 nm on each side. The best answer is that the peaks were caused by small sweat molecules on the outside of the cuvettes light pathway, making it a systematic error.

The question remains as to why the pigment extraction failed and gave an extremely weak, almost inexistant absorbance spectrum. There could be a good reason as to why that could have happened. The pigment determination experiment was done 2 days after the plastid isolation and 1 day after the TLC-analysis. That means the isolated plastids were no longer fresh and had been on ice for around 48 hours. Even on ice, the plastids do not get frozen down, they just get cooled until 4°C at best. So, this means the integrity of the plastids could have, and probably have suffered greatly over the course of those 48 hours. This is especially considering the total volume of the isolated plastids was only 1 mL. So, the best explanation as to why there were no significant detectable traces of any pigment is because the pigments had deteriorated over the 48 hours. Hence, the experiment has per definition, failed. For future reference, it is recommended that the extraction should be done on the same day as the plastid isolation. This in turn, allowing for more

accurate measuring and categorization of the different existent pigments, while ensuring the freshness of the sample.

4. Protein determination and spectra analysis:

The protein determination graph and the different respective spectra can be seen in figure 15, and its subsequent raw data in Appendix C:

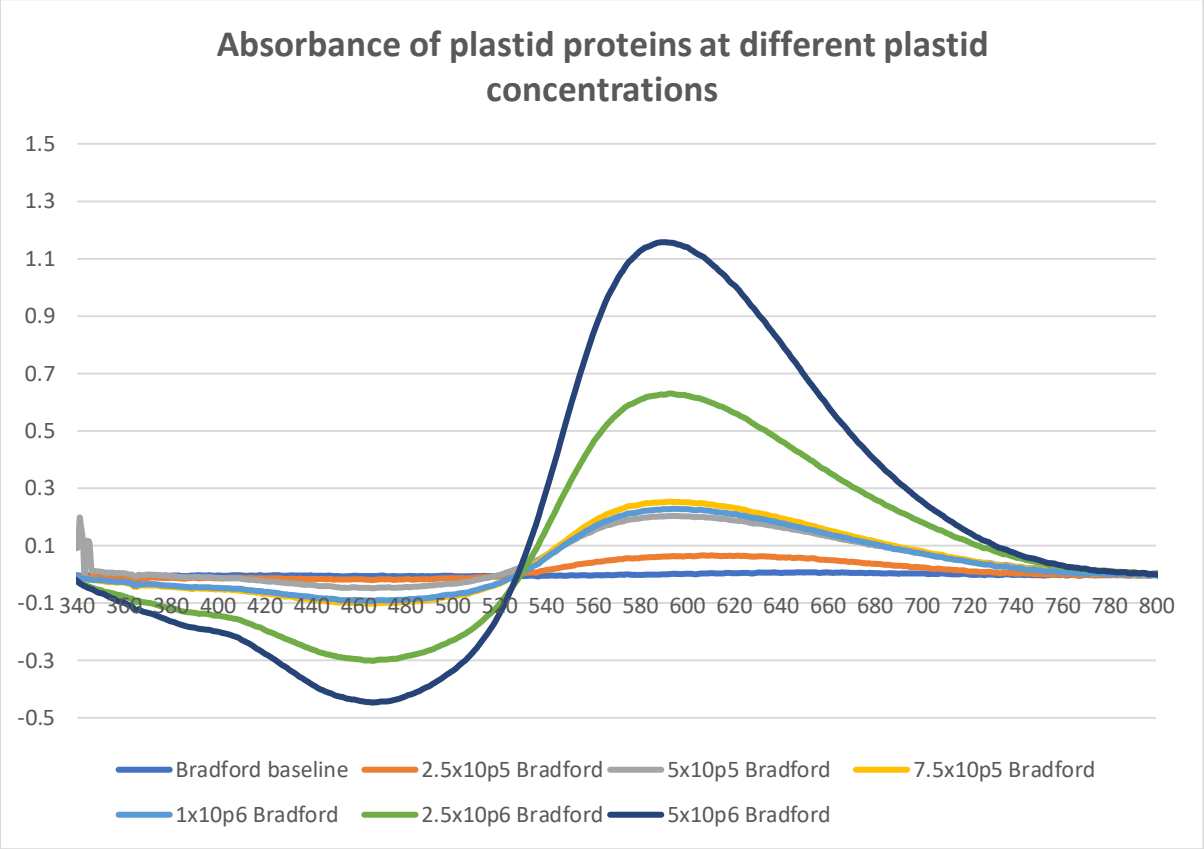

Fig. 15 | Absorbance of plastid proteins at different plastid concentrations. This graph shows the different absorbance values for the different plastid concentrations using the Bradford protein determination assay as a baseline for the measurements.

The spectra were measured in between 340 nm and 800 nm. There are a couple reasons for this, first one being when the 5x10p5 measurement was taken. A big mistake was made during this, mistakenly opening the spectrophotometer. This in turn made the absorbance value to shoot up to 5, causing a lot of disturbance in the graph. The second reason being that the most important part of the graph resides in between 520 nm and 800 nm. Zooming in to get a better view of that region helps showing if the ratios between the different concentrations match up.

Now, looking why there is a peak around 595 nm and what it means, the explanation lies in the Bradford assay. The Bradford assay is a colorimetric protein assay and is based on an absorbance shift of the dye “Coomassie brilliant blue G-250”. The dye exists in three forms: anionic (blue), neutral (green) and cationic (red). Under acidic conditions, the red form of the dye is converted into the blue form, causing it to bind to the protein that is being assayed. The cationic, or the unbound form of the dye has

an absorption spectrum maximum at 465 nm. The absorbance shift happens when the cationic form, transforms into the anionic, or bound form of the dye, causing the peak to shift from 465 nm to 595 nm. The increase of absorbance at 595 nm is directly proportional to the amount of bound dye. Meaning that it is directly proportional to the concentration of protein present in the sample (Wikipedia, 2024).

Now, that the Bradford assay has been explained, a closer look can be taken at the graph, and further evaluated. In the graph, there can be observed that there is a minimum at 467.5 nm with an absorbance reading of -0.446. This matches up with the explanation from earlier about there being a signal at around 465 nm. It can be concluded that the minimum observed at 467.5 nm is due to the unbound Coomassie dye, that being the Bradford assay.

Lastly, there can be observed that there is a maximum at 593 nm with an absorbance reading of 1.155. This maximum also matches up with the explanation earlier. It can also be observed that the increase in absorbance almost always follows the trend of the increase in concentration over the different measurements. The maximum absorbance readings over the different concentrations from lowest to highest at 593 nm are: 0.063 (for 2.5x10p5), 0.203 (for 5x10p5), 0.253 (for 7.5x10p5), 0.227 (1x10p6), 0.63 (for 2.5x10p6) and 1.155 (for 5x10p6) respectively. What can be deduced, is that the increase in absorbance values was proportional to the increase in plastid protein concentration. This is showing how many dye particles were bound, with the only anomaly being the decrease in absorbance from 7.5x10p5 to 1x10p6. The anomaly can be explained by human errors such as incorrect plastid sample pipetting amounts and not cleaning the cuvette thoroughly before use.

5. Excitation and Emission graphs (fluorescence checking):

The excitation and emission graphs and their spectra can be seen below in figure 16 and the raw data present in Appendix D:

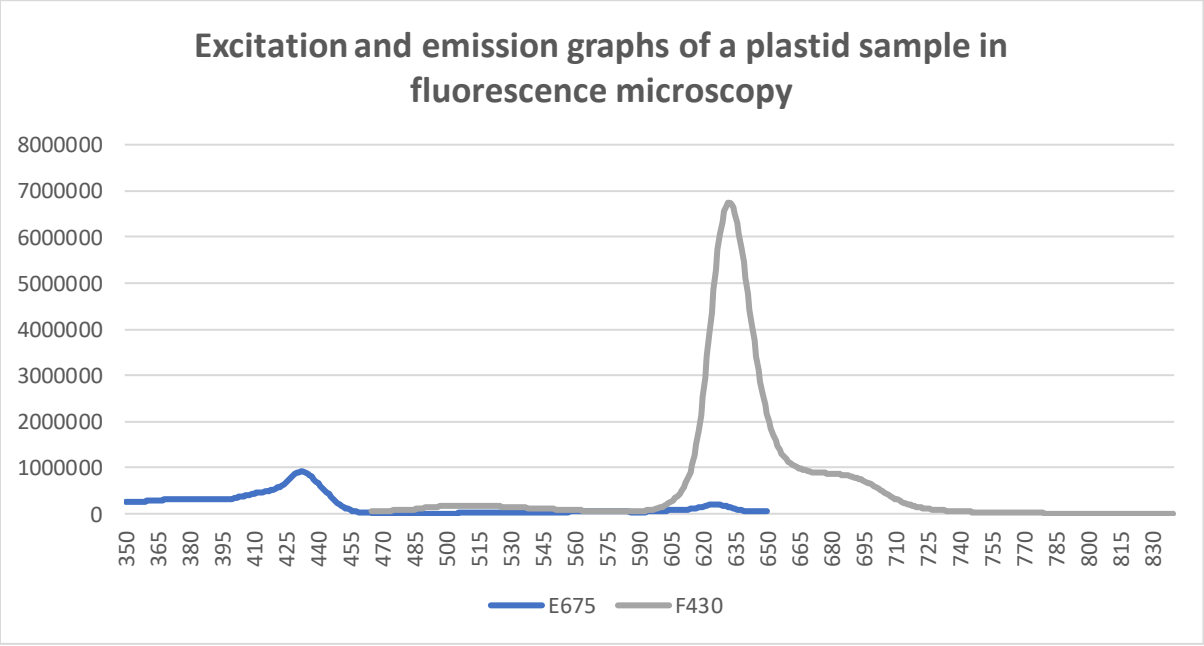

Fig. 16 | Excitation and emission graphs of a plastid sample in fluorescence

microscopy. This graph showcases the different excitation and emission graphs recorded on the fluorolog for a freshly isolated plastid sample.

In this graph, there can be seen multiple curves that depict the different peaks and ranges of the curves. These give some information about which wavelengths were used for either excitation or emission checks.

Excitation curve: Represents the efficiency of a fluorophore at different wavelengths of light. It shows how effectively the fluorophore can absorb photons at each wavelength. When a fluorophore is exposed to light, it can absorb photons if the energy of the photons matches the energy difference between the ground state and an excited electronic state. By measuring the fluorescence intensity at a fixed emission wavelength while varying the excitation wavelength, an excitation spectrum is obtained. This spectrum peaks at the wavelengths where the fluorophore absorbs light most efficiently.

Emission curve: Represents the fluorescence emitted by the fluorophore after it has been excited. This curve shows the intensity of light emitted at different wavelengths. After absorbing light, the fluorophore reaches an excited state. It then relaxes to a lower energy excited state through non-radiative processes (internal conversion). Eventually, it returns to the ground state by emitting a photon, which is observed as fluorescence. By measuring the fluorescence intensity at different wavelengths while exciting the fluorophore at a fixed wavelength (typically at the peak excitation wavelength), an emission spectrum is obtained. This spectrum peaks at the wavelengths where the fluorophore emits light most efficiently.

The first measurement taken, the F430 is an emission spectrum measured from 465 nm until 840 nm but it has an excitation check at 430 nm. This is also called a fluorescence check, hence the F in F430. It was done to see if there is any absorption being recorded at that specific wavelength before the fluorolog starts measuring.

Following that, an excitation graph was recorded, as seen in E675. The measurement was taken from 350 nm to 650 nm, but it has an emission check at 675 nm. This is also hence the E in E675. It was done to see if there is any more light that the pigments emit after being excited, before the measurement ends.

Now as for the graph, the thing being tested and observed, is the isolated plastid sample, more specifically, the pigments in the isolated plastid sample. So what pigments can be recognized in the different curves? Well, there are 2 main defined peaks that are of interest when it comes to this graph. The first is at 627 nm in the E675 graph, and the second is at 633 nm in the F430 graph.

There is only one candidate for the peak at 627 nm and that is protochlorophyllide. This was discussed about earlier in the second section of the analysis (Ignatov & Litvin, 1996). There is also only one candidate for the peaks at 633 nm and that is

also a form of protochlorophyllide that was also discusses about earlier in the second section of the analysis (Ignatov & Litvin, 1996).

6. Protochlorophyllide concentration determination:

The spectra used for the concentration determination can be seen in figure 17, and the raw data used can be seen in Appendix E:

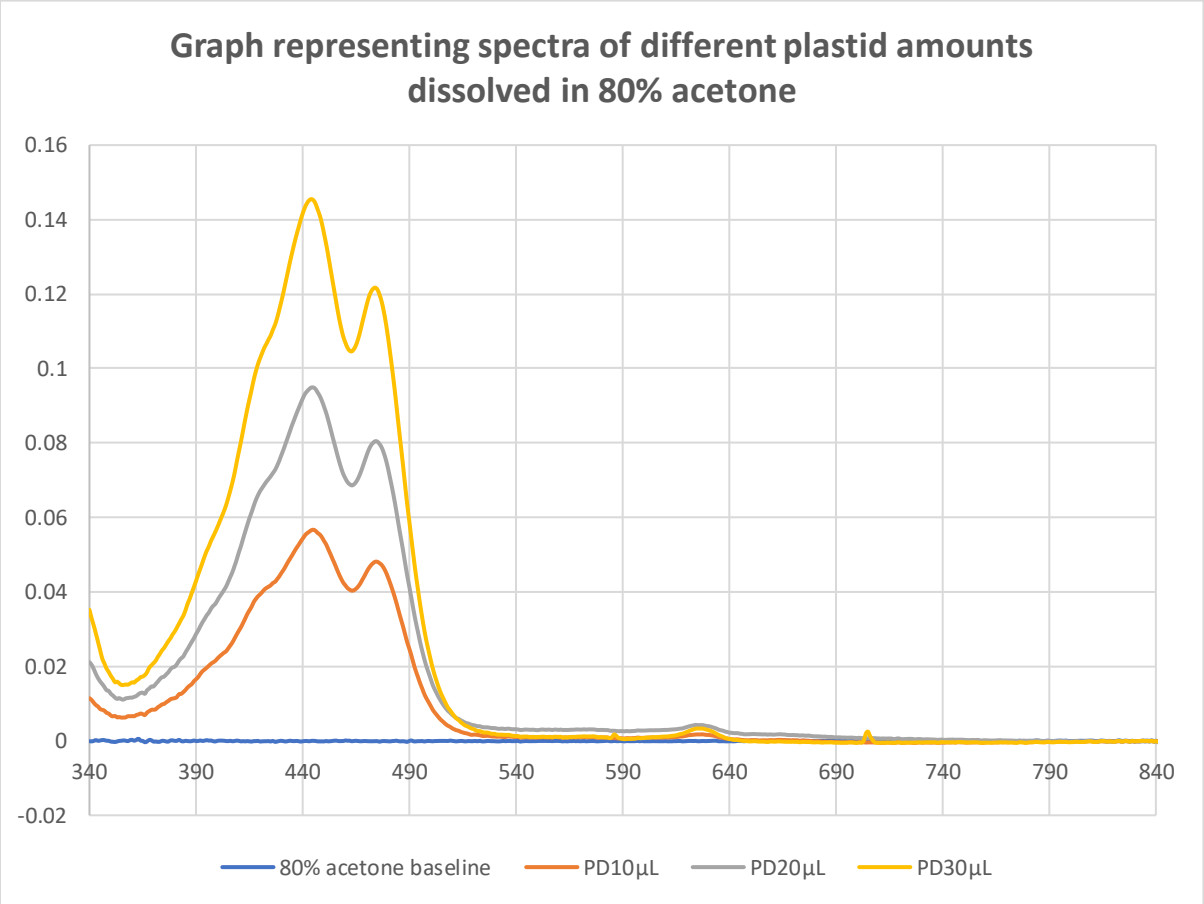

Fig. 17 | Graph representing spectra of different plastid amounts dissolved in 80% acetone. This graph showcases the different pigments existent in a plastid sample. The most important one, protochlorophyllide being displayed at 627 nm. The abbreviation PD refers to protochlorophyllide followed by the amount of sample tested.

The carotenoid and xanthophyll signals in between 440 nm and 490 nm act like a control. This shows that the absorbance ratio between the different plastid amounts match up. For example, the absorbance value of PD10µL at 447 nm is 0.05583, for PD20µL its 0.14267 and 0.09354 for PD30µL. Although the ratios between PD10µL with respect to PD20µL and PD30µL are not exactly 2X or 3X respectively, it does show a close enough representation of how the protochlorophyllide absorbance values should look like.

But looking at the protochlorophyllide, at 627 nm, two things become evident. Problem number one is that the absorbance values of protochlorophyllide across all spectra are very low almost non-existent. Problem number two is that the protochlorophyllide signal is lower for PD30µL than for PD20µL and vice-versa, despite having a higher concentration of plastid.

Before calculating and determining the concentration of protochlorophyllide, the possible causes of these two problems, need to be discussed. For both problems, the reasons as to why the absorbance value of protochlorophyllide is very low, can be tied to either the plastid isolation process or to the pigment isolation process. Firstly, the percoll plastid isolation could have either been inefficient or just failed, leaving no plastids to be detected and no protochlorophyllide to be detected. The problem here would be that despite the very low protochlorophyllide concentrations, there is still a pretty defined carotenoid and xanthophyll reading. This is despite it having a pretty low absorbance reading between 0.05 and 0.15. Overall, it would be impossible to record a carotenoid spectrum if there weren't any plastids in place already.

The second process, the acetone pigment isolation process is more likely to have gone wrong. The reason being that a simple pipetting or measuring mistake of can disturb the ratio between the solvent and the solute and change the concentration solution. This can result in a lower absorption value, as seen between PD20µL and PD30µL. Since the volumes of the sample tested are so small, every single drop is significant to the analysis. A drop missing could be leading to large errors in the analysis. The conclusion to why the absorption of the protochlorophyllide is low is because the pigment isolation failed. This can be due to a combination of faulty pipetting of the plastid sample and accidental addition of either extra 80% acetone or water to the cuvette. The results would then be a very low peak and an uneven ratio between the different sample amounts.

Now, for the actual calculations of the protochlorophyllide concentration at 627 nm for each of the three samples, Lambert-Beer's law will be used to calculate it by manipulating the formula like so: $\frac{A}{\varepsilon \times l} = c$. The calculations that follow are summarized in Table 1.

For PD10µL: $\frac{0.00174}{30400 \times 1} \times 100 = 5.7237 \times 10^{-6} M$

For PD20µL: $\frac{0.00424}{30400 \times 1} \times 50 = 6.9737 \times 10^{-6} M$

For PD30µL: $\frac{0.00330}{30400 \times 1} \times 33.33 = 3.6181 \times 10^{-6} M$

The A parameter in the equation is the absorbance value and is read directly from the raw data at 627 nm. The ε parameter is the extinction coefficient for protochlorophyllide (source). Finally, the l is the light pathway that the light has to traverse before hitting the cuvette, which is always 1 cm.

Table 1 | Table summarizing the protochlorophyllide concentration calculations and variables.

Sample	Absorbance value	Ratio	Concentration
PD10µL	0.00174	1:100	5.7237×10^{-6}M

PD20µL	0.00424	1:50	1.39474×10^{-5}M
PD30µL	0.00330	1:33.3	1.08553×10^{-5}M

7. In vivo photoconversion of protochlorophyllide to chlorophyllide followed by chlorophyll synthesis:

In this experiment, the amount of data recorded spanned 205 data sets, so for the purpose of clarity, the most important events will be summed up in three data sets. Those datasets are, the dark spectrum, the spectrum of the illuminated chlorophyllide after 6 hours, and the spectrum of the illuminated chlorophyllide recorded 18 hours after illumination. This can be seen in figure 18, together with its respective raw data in Appendix F:

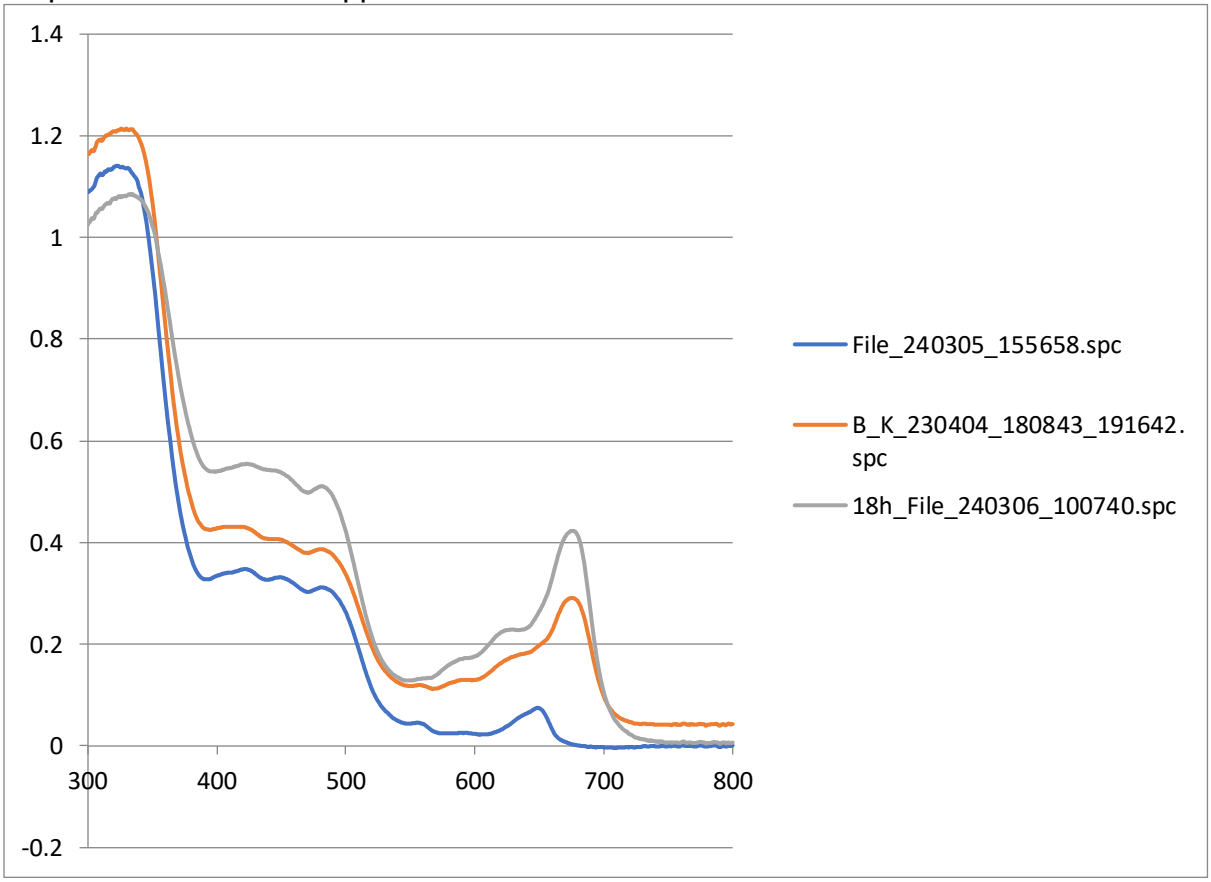

Fig. 18 | Graph representing the evolution of in vivo production and conversion of POR and NADPH bound protochlorophyllide to POR bound chlorophyllide. This data recorded in this graph shows the in vivo dark spectrum (represented in blue), the 6 hour chlorophyllide transformation (represented in orange), and the 18 hour chlorophyllide transformation (represented in grey).

It can be observed, the peaks of the different curves are a bit different than what has been presented so far. So far, protochlorophyllide has been recorded at either 627 nm or 633 nm, but now is shifted to 651 nm. All the experiments up until this point have been using isolated plastids, meaning, all the experiments have been in vitro. This signifies that the plant is not self-sustaining. Now that there are etiolated leaves being used, the setup is in vivo, meaning that the plant is alive and it is self-

sustaining as long as the optimal conditions are being met. At the same time, chlorophyllide has been recorded at around 663 nm, but also at 677 nm.

There are three peaks of interest in these three graphs, one for the dark spectrum at 651 nm, and two for the 6 hour and 18 hour illumination graphs respectively at 677 nm. The following paragraphs will try to explain what compounds might cause these absorbance shifts.

According to a couple scientific studies by a group of researchers at the University of Göteborg and a group of French researchers, the peak recorded at 651 nm is a form of non-phototransformable protochlorophyllide intermediate. This intermediate is used by the system to regenerate the phototransformable version that is detected at around 657 nm (Kovacheva, Ryberg, & Sundqvist, 2000) (Schoefs, Bertrand, & Funk, 2000). The way that was discovered is through adding different ATP concentrations and seeing if either the phototransformable or non-phototransformable version of protochlorophyllide get affected in any way. No effect on ATP was observed in the phototransformable version, regardless of concentration. But forthe non-transformable version, its reformation was inhibited, indicated by a steady decrease in concentration of this form, over time (Kovacheva, Ryberg, & Sundqvist, 2000) (Schoefs, Bertrand, & Funk, 2000). The conclusion reached after reading both publications is that this is a non-phototransformable version of Pchlide is linked to the oxidized form of NADPH, that being $NADP^+$ and to the POR enzyme.

Now about the chlorophyllide peak, a look needs to be taken at a couple of scientific studies from a group of Hungarian and Swedish and Australian researchers. The peak recorded at 677 nm is a form of chlorophyllide intermediate that is still bound to the POR enzyme. But this type is not bound to NADPH or $NADP^+$, and will end up transforming into chlorophyllide over time during constant exposure to light (Solymosi, et al., 2007) (Henningsen, Thorne, & Boardman, 1973). The reason why the peaks for the two illuminated graphs stay at around 677 nm even after 18 hours, is because constant illumination on the leaves is necessary for the chlorophyllide:POR macrodomain to shift and detach. This in turn gives chlorophyllide and POR separately and later the chlorophyllide binds to chlorophyll synthase (Solymosi, et al., 2007) (Henningsen, Thorne, & Boardman, 1973).

Now that the different peaks have been described and established, a correction of the graph showcased in figure 18 needs to be done. This means, that the graph needs to be normalized with respect to the orange graph, that being the 6 hour illumination graph. This is because it starts at a higher absorbance value than the other graphs, which start at the baseline, building their way up as the different wavelengths illuminate the sample. The new corrected and normalized graph can be observed in figure 19 and its raw data in Appendix G:

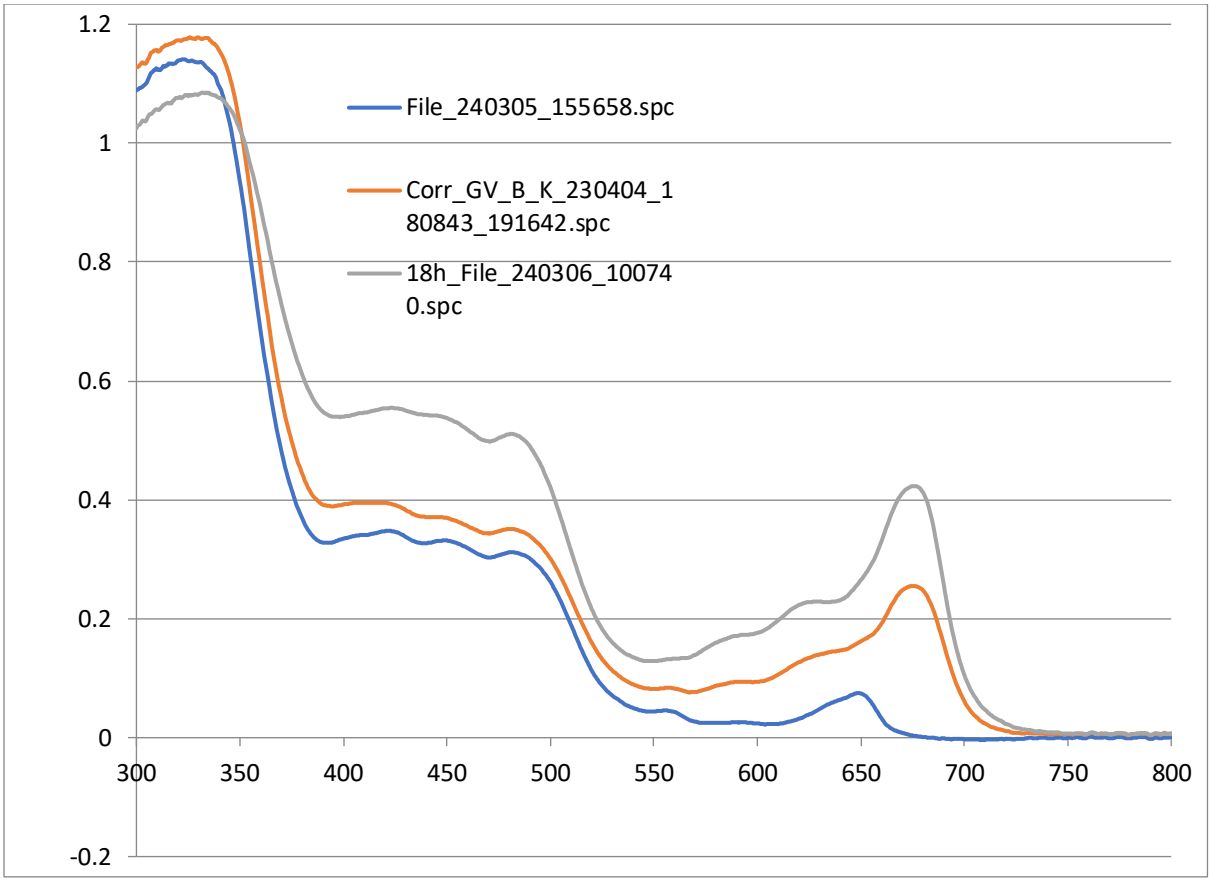

Fig. 19 | The normalized graph representing the evolution of in vivo production and conversion of POR and NADPH bound protochlorophyllide to POR bound chlorophyllide. This data recorded in this graph shows the in vivo dark spectrum (represented in blue), the corrected version of the 6 hour chlorophyllide transformation (represented in orange), and the 18 hour chlorophyllide transformation (represented in grey).

Now that the graph has been corrected, one thing remains to be explored in this section. That is looking at what the ratio is between the 18 hour and the 6 hour chlorophyllide transformation is. This could give an idea of the relationship between the rate at which the different graphs increase in value. The ratio was added to a secondary axis to present the curve more clearly. The graph with the previous curves plus the ratio can be seen in figure 20, with the full raw data present in Appendix H:

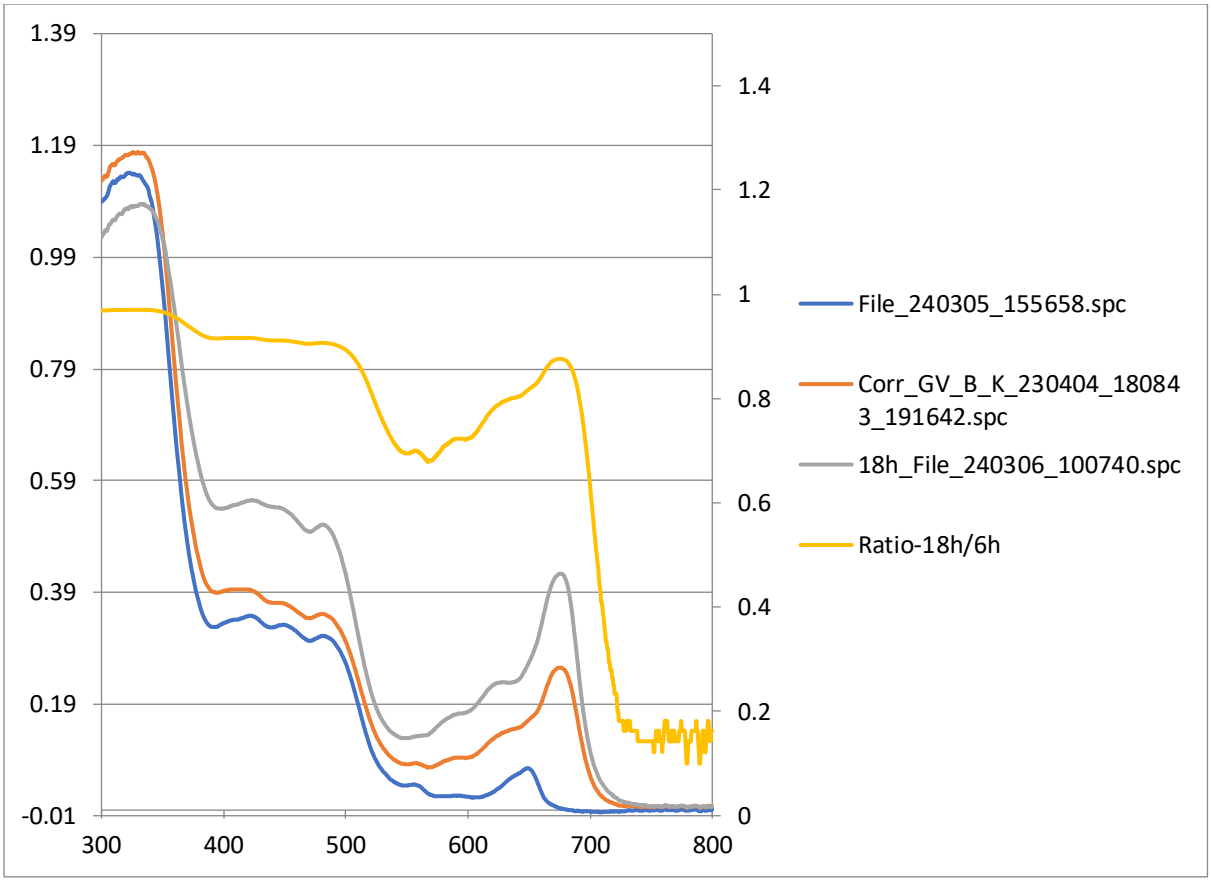

Fig. 20 | The normalized graph representing the evolution of in vivo production and conversion of POR and NADPH bound protochlorophyllide to POR bound chlorophyllide together with the ratio between the 18 hour and the 6 hour chlorophyllide accumulation. This data recorded in this graph shows the in vivo dark spectrum (represented in blue), the corrected version of the 6 hour chlorophyllide transformation (represented in orange), and the 18 hour chlorophyllide transformation (represented in grey) and the ratio between the 18 hour chlorophyllide transformation and the 6 hour chlorophyllide transformation (represented in yellow).

What can be observed in this graph is that the ratio between the two graphs increases, as both the absorbance difference and the absorbance itself increases. Also, the closer the graphs are to each other in absorbance value, the straighter the line becomes, hinting to a better correlation between the two graphs.

8. In vitro addition of NADPH to isolated etioplasts & subsequent photoconversion of protochlorophyllide to chlorophyllide followed by evidence of chlorophyll synthesized through TLC:

In this experiment, the amount of data recorded spanned 30 data sets, so for the purpose of clarity, the most important events will be summed up in multiple graphs with at most four data sets each. Firstly, the dark spectrum with NADPH added at 0 hours and the dark spectrum at with NADPH at 1.5 hours will be shown. This can be seen in figure 21, together with the raw data in Appendix I:

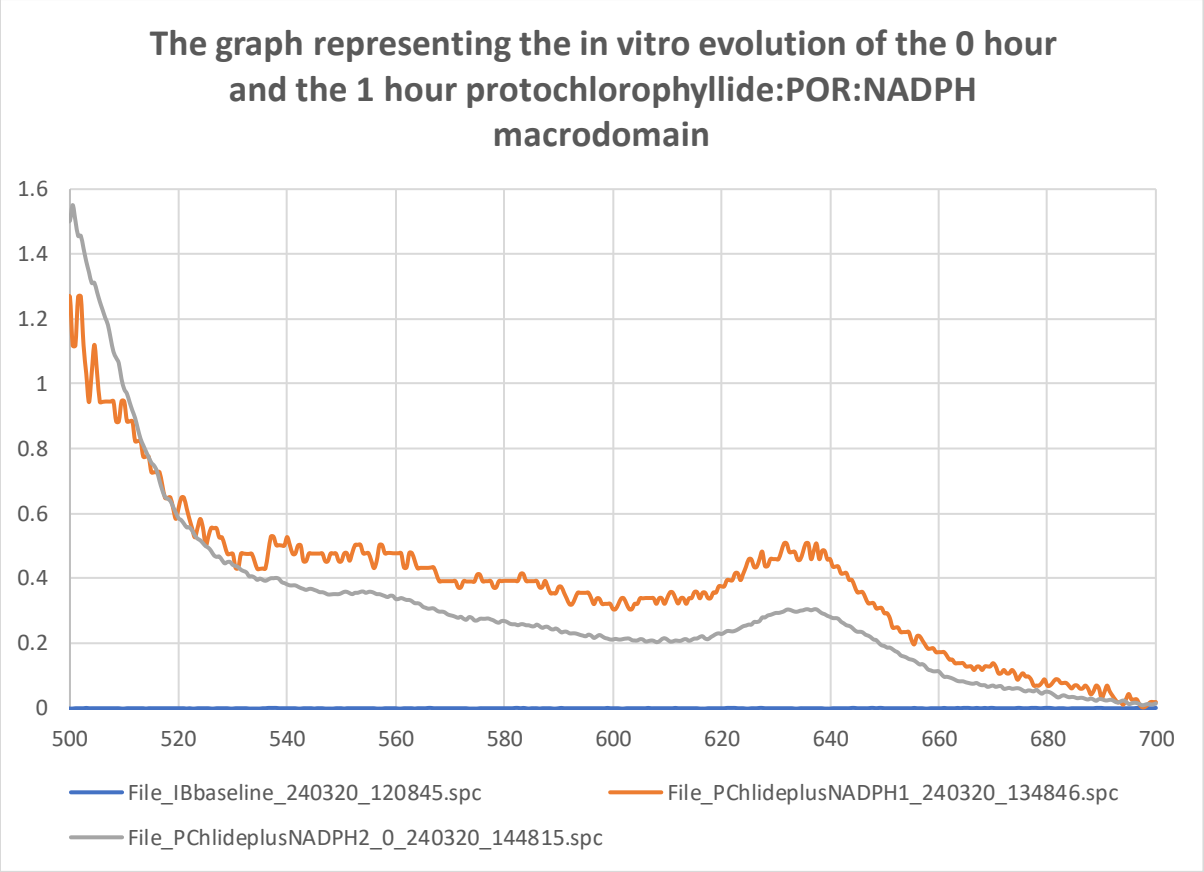

Fig. 21 | The graph representing the in vitro evolution of the 0 hour and the 1 hour protochlorophyllide:POR:NADPH macrodomain. This data showcases three data sets: the isolation buffer baseline (represented in blue), the protochlorophyllide bound to POR and NADPH, right after NADPH was added at 0 hours (represented in orange), and the protochlorophyllide bound to POR and NADPH, 1 hour after NADPH was added (represented in grey).

This graph was made because this experiment is an in vitro experiment, meaning that the reactions are not self-sustaining like they were in the live leaves. The only things existing in an in vitro environment of isolated plastids are the different pigments, including protochlorophyllide, together with the different enzymes. So naturally, NADPH has to be added exogenously in order to make chlorophyllide through POR, and GGPP has to be added exogenously in order to make chlorophyll through chlorophyll synthase.

Technically according to section 7 of the analysis, the protochlorophyllide peak should shift from 633 nm to 650 nm over time, so this somewhat shows that. The reason for using “somewhat” is because a cuvettes’ volume is very limited, having used only 385 µL of plastid sample+NADPH solution for these measurements. Meaning that, over a short amount of time, the liquid inside the cuvette, exposed to the high temperature that is naturally occurring in the spectrophotometer, will evaporate. This, leading to fast decreases in absorbance and concentration of the sample, because without the isolation buffer, the plastids will go bad and decompose over a short period of time.

At first glance, it does not look like the peak shifted at all even after 1 hour, but looking at the raw data in Appendix I it can be seen that the highest point of the 0 hour graph is at 632 nm with an absorbance value of 0.507 while the highest point of the 1 hour graph is at 637 nm with an absorbance value of 0.304. So the maxima has indeed shifted, but just by 4-5 nm. The shift from 633 nm to 650 nm in the in vivo experiment was very quick and took only around 1-2 hours. That is normal, because as stated, in an in vivo experiment, the organism regulates every reaction on its own. Meaning that the NADPH gets attached to the Pchlide:POR macrodomain faster due to the different plant mechanisms that force the NADPH to bind. But since this is an in vitro experiment, the NADPH will bind to the macrodomain by chance, making the process take a longer time. The sample was not left for longer than that due to evaporation, slowly compromising the overall quality of the measurements.

Now that it has been shown that the exogenous NADPH addition influenced the sample, it is time to look at the holistic change of the protochlorophyllide, all the way to the synthesis of chlorophyll. The graph depicting this can be seen in figure 22, with its raw data in Appendix J:

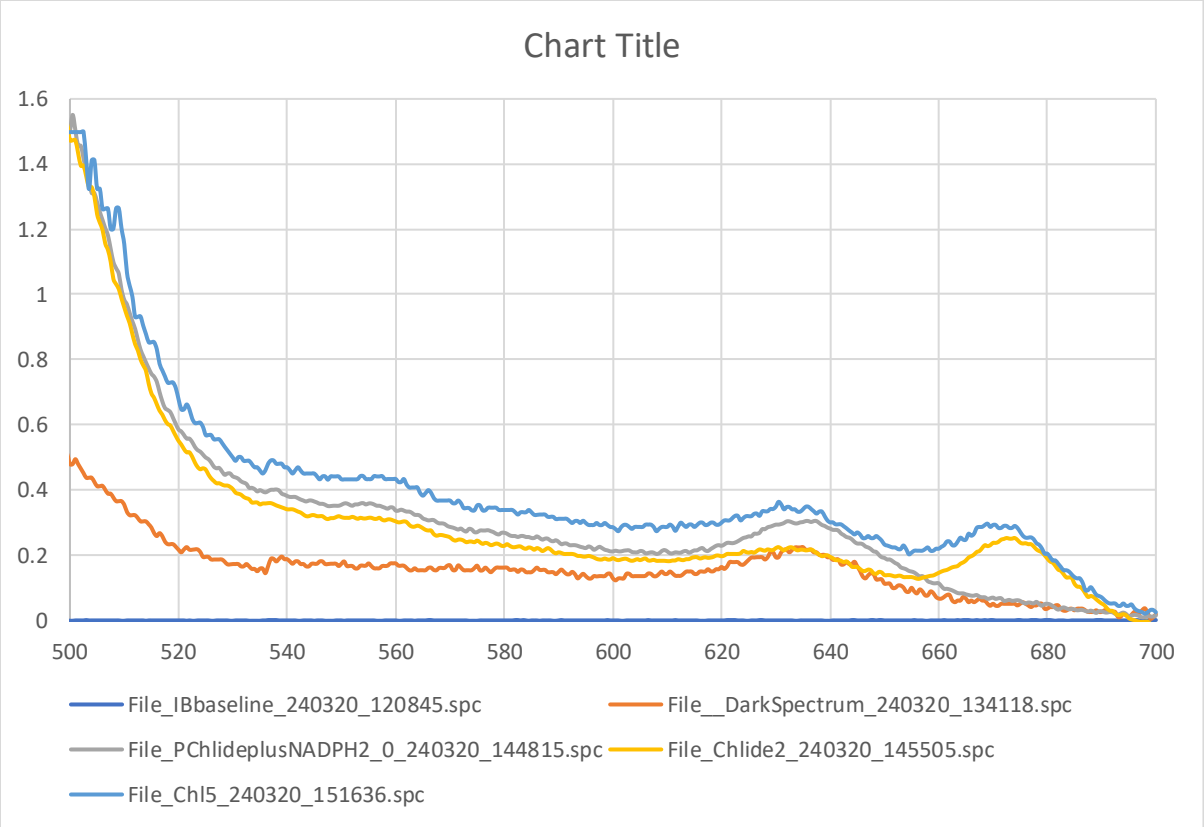

Fig. 22 | The graph representing the in vitro evolution of the 0 hour and the 1 hour protochlorophyllide:POR:NADPH macrodomain. This data showcases three data sets: the isolation buffer baseline (represented in dark blue), the protochlorophyllide bound to POR spectra (represented in red), the protochlorophyllide bound to POR and NADPH, right after NADPH was added at 1 hours (represented in grey), the chlorophyllide bound to POR 8 minutes after illumination (represented in yellow), and the chlorophyll spectrum after 20 minutes of GGPP addition (represented in light blue).

The most important information that could be observed and gathered here are the transitions between protochlorophyllide to chlorophyllide, and thereafter from chlorophyllide to chlorophyll. Since the protochlorophyllide shift was discussed in the last paragraph, the dark spectrum and the 1 hour NADPH bound protochlorophyllide readings will be absent from further discussions. They are rather, used as a reference point for discussing around the other readings.

Taking a look at the difference between the 1 hour protochlorophyllide reading and the chlorophyllide reading after the 10 second burst of white light, a couple things become apparent. The most obvious change is that there is now a peak that has formed at 675.5 nm with an absorbance value of 0.259. This means that the conversion of protochlorophyllide to chlorophyllide worked. This chlorophyllide at 675.5 nm is most probably the chlorophyllide intermediate that is still bound to the POR, as discussed in section 7 of the analysis.

The second change, is that at the same time as the chlorophyllide peak appeared, the protochlorophyllide peak decreased substantially. It went from a peak at 637 nm of 0.304, to a peak back at 633.5 nm with an absorbance of 0.218. This means that some, but not all, of the protochlorophyllide had been used up to make chlorophyllide. This serves as a bit of extra evidence to the theory stating that not all the protochlorophyllide was bound to the NADPH. This means that there is not a system that can regulate and ensure that all NADPH is to be bound to the protochlorophyllide, making the binding random.

Furthermore, comparing these graphs to the graphs in section 7, where the experiment was in vivo, it is seen that in the in vivo graphs, post-illumination, there were no more immediate recorded traces of protochlorophyllide present. This hints at the fact that in an in vivo setting the systems appear to be regulated leading to a holistic and continuous change. This causes almost all the protochlorophyllide to bind to NADPH, being converted into chlorophyllide on a much larger scale than in an in vitro setting.

For the last step, the conversion of chlorophyllide to chlorophyll through the addition of exogenous GGPP. There can be observed a shift from the peak at 675.5 nm to 668.5 nm with an absorbance value of 0.292. This means that the graph started shifting towards the usual absorbance value of chlorophyll of 663 nm. At the same time, a small anomaly can also be seen when it comes to the absorbance value of protochlorophyllide over time, but that can be easily explained by the addition of extra plastid sample, due to a lot of liquid evaporation.

The whole experiment was re-done but this time in a mini cuvette, as illustrated in section 10 of the methods section, to see if the total amount of volume makes any difference in terms of clarity to the graphings. This graph can be seen in figure 23, and the full raw data in Appendix K:

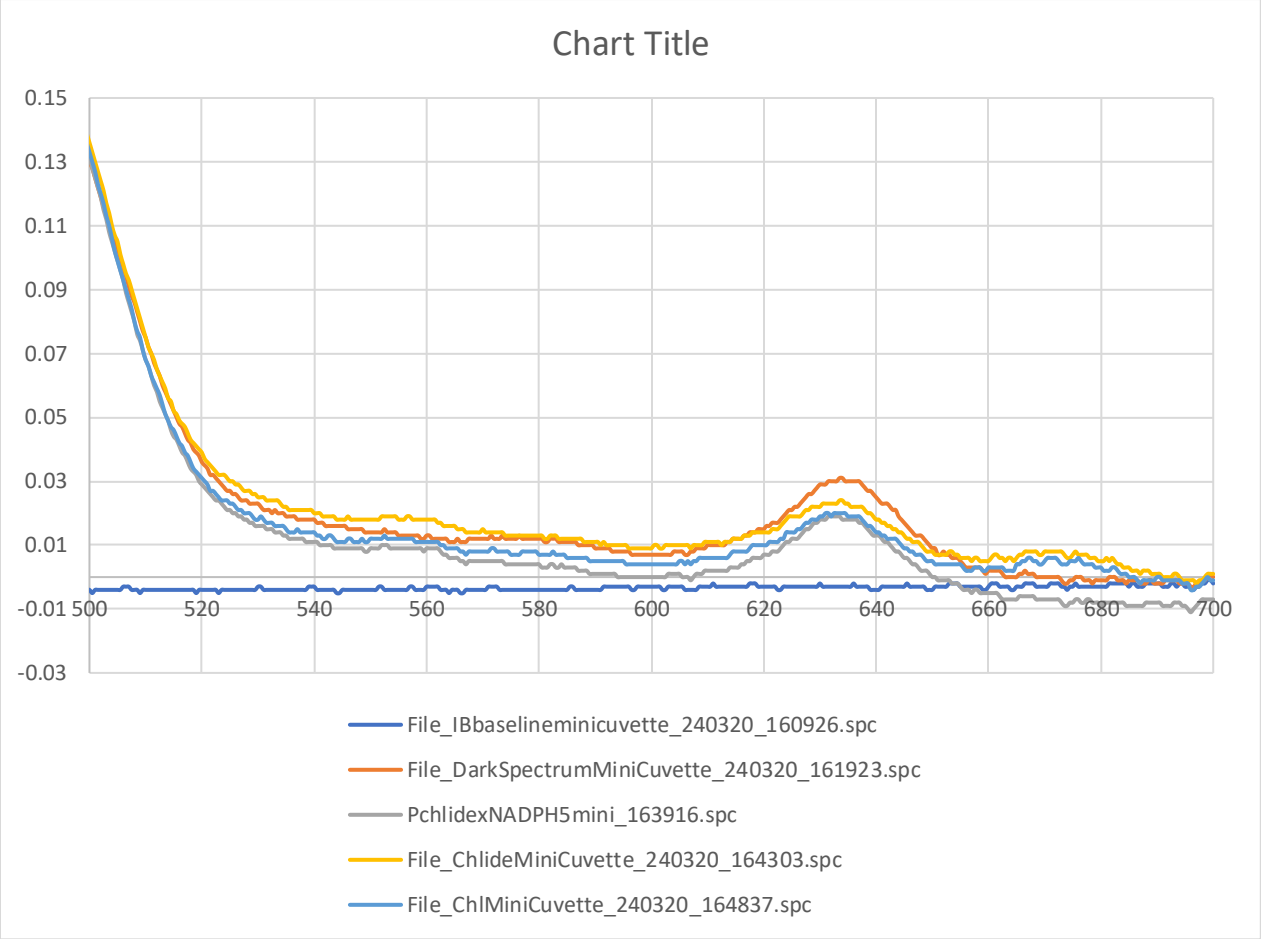

Fig. 23 | The graph representing the in vitro evolution of the 0 hour and the 0.5 hour protochlorophyllide:POR:NADPH macrodomain using a 50µL mini cuvette. This data showcases three data sets: the isolation buffer baseline (represented in dark blue), the protochlorophyllide bound to POR spectra (represented in red), the protochlorophyllide bound to POR and NADPH, right after NADPH was added at 20 minutes (represented in grey), the chlorophyllide bound to POR 4 minutes after illumination (represented in yellow), and the chlorophyll spectrum after 4 minutes of GGPP addition (represented in light blue).

Practically though, the absorbance wavelength of both chlorophyllide and chlorophyll is the same, that being 663 nm, so realistically, there is no way to discern between these two without additional methods of analysis. Therefore, a TLC was performed to see if the TLC-plate UV-data matches the results illustrated in figure 10. The subsequent blue and red laser scans can be seen in figures 24 and 25:

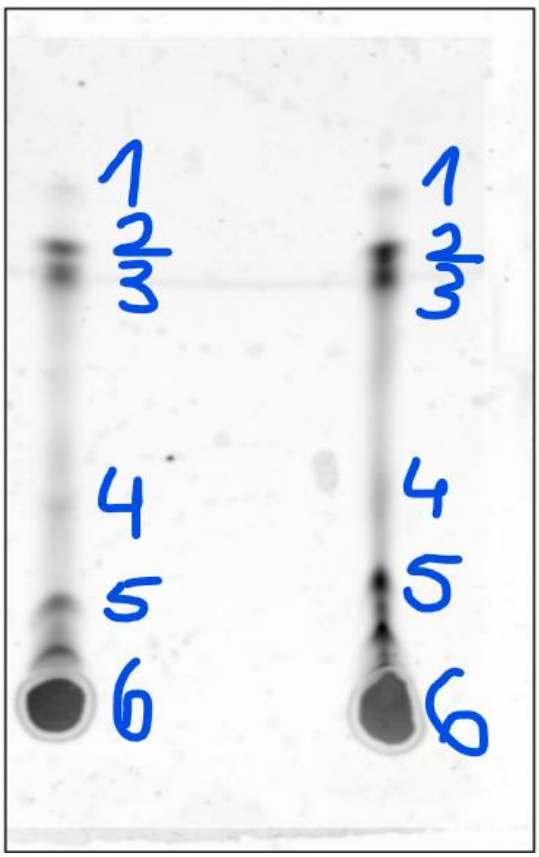

Fig. 24 | TLC-plate scan with red laser. This scan represents the TLC-plate, after the TLC was ran, under the exposure of a red laser. The 6 different bands represent different pigments.

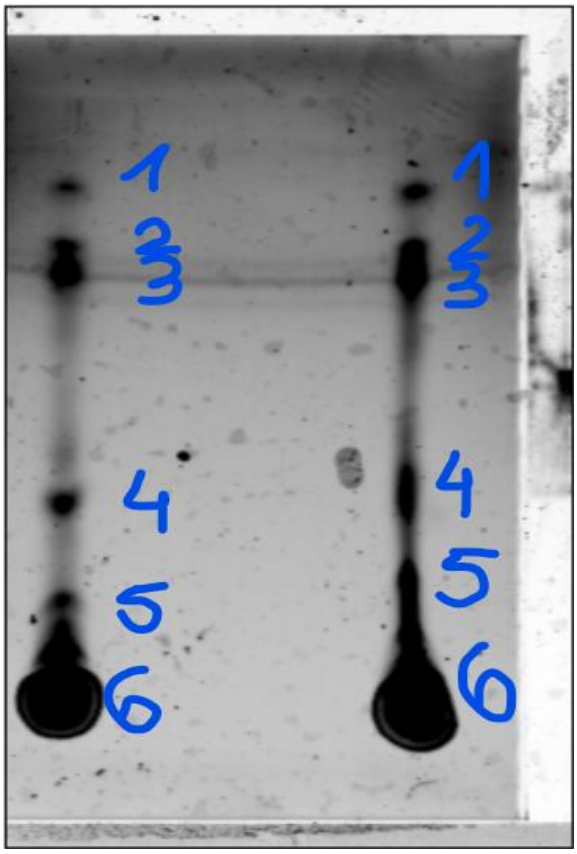

Fig. 25 | TLC-plate scan with blue laser. This scan represents the TLC-plate, after the TLC was ran, under the exposure of a blue laser. The 6 different bands represent different pigments.

Figures 24 and 25 are the third and fourth scans done by the Typhoon, and as stated, they were exposed to a red (633 nm) and blue (488 nm) laser respectively. This can be observed in band 4, where it is more observable under the blue laser than the red one. This is due to the differences in the absorbances of the different pigments that make up these two bands.

Since the ratio of polar to non-polar is the same, then it can be imagined that the pigments in etiolated leaves will divide similarly to the pigments in deetiolated leaves. With that said, it can be concluded that the pigments that show up numbered 1 through 5 are as follows: Number 1 is β-carotene. Numbers 2 and 3 are chlorophyll a and b, because it absorbs at a wavelength between 630 and 665 nm (Vishwajit, Samanyu, Varnika, Chinmaya, & Subhadip, 2023). It can also be seen that the second pigment shows a stronger band under a red laser. Number 4 is pheophytin, because it absorbs at a wavelength between 630 and 670 nm (Marker, 1977). It can also be seen that the second pigment shows a stronger band under a red laser. Number 5 is xanthophyll which absorbs between 466-495 nm (Study.com, n.d.). Number 6 is the remaining protochlorophyllide which is mostly polar so it barely

moves and it also absorbs at 633 nm for the red laser (Ignatov & Litvin, 1996).

Comparing this data with data from figure 10, the number of bands counted are six, which exactly matches the number of bands from the figure. There are two chlorophyll signals, signified by the two very close bands at the top. The only anomaly detected in the TLC was the xanthophyll, or pigment number 5, which should have absorbed better under blue laser than the red laser, meaning that there should not have been that much absorbance under the red laser.

Discussion:

Now that all the results have been presented regarding all the of the experiments, the discussion must be held exploring as to what can be taken away from all of this. In other words, what has been learned, what more could have been learned and how to ensure possible better results for an eventual future deep dive into this topic.

What has been learned? Well to find that out, a holistic look at the thesis structure needs to be taken. Parts 1 through 8 of the methods section and parts 1 through 6 of the analysis section are so-called “General preparatory experiments”. While parts 9 through 10 of the methods section and parts 7 through 8 of the analysis section are so-called “Specific and focused experiments”. The difference between these two types of experiments is that the former are utilized to develop an understanding of theory and methodology behind a scientific method. Regardless of it being qualitative or quantitative, the purpose of why it is important to conduct any experiment is to gain understanding. All these different experiments together aim to deepen ones work experience and understanding of the research field one has immersed oneself into. The latter though, is more focused on using ones gathered experience to try and solve a more complex problem or situation through trial and error, using the already existing knowledge as a stepping stone for future breakthroughs and innovations.

Then, responding to the question of what has been learned from all this, first thing, is of course hands-on experience on how to conduct scientific research not only in terms of both practical and theoretical experience, and also in terms of safety and procedural knowledge. Second, and probably the most important thing is the result and realization that chlorophyll cannot be synthesized in the dark in flowering plants. This is despite the exogenous addition of the different components like NADPH and GGPP, due to the light-dependent step of chlorophyllide synthesis. Regardless though, the mechanism worked after a 10 second burst of strong white light, which indicates that the system only needs a push in order to start.

The difference between the in vivo and the in vitro process is the fact that when the barley leaves were illuminated, the light-dependant mechanism kept going for a while. While in the isolated plastids, the process stopped almost as soon as the burst of light was over. The difference is most likely because in a whole, living leaf, the different processes leading to the synthesis of chlorophyll, are internalized and controlled by gene expressions. This is in turn leading to a cascade of reactions that

is otherwise impossible in isolated plastids due to the reaction lacking lots of components essential to keep the systems alive and going.

There is one important implication to take away from this, and that is the fact that the 10 second burst of white light was enough to keep the light-dependant system going on for a while without stopping. This is important to consider and further researched, because then the findings could be eventually implemented into e.g. greenhouses. Then, instead of needing to constantly shine light on the plants to grow, they would just need a 10 second burst of strong white light, separated by specific time intervals. The consequences of this would be immense, as the amount of electricity required to grow crops would drop dramatically. This would inadvertently lead to a large decrease in carbon emissions and over time, would make a very good impact on the Earth.

Another thing that can be further researched, is seeing if light-dependent plants, can be genetically modified to be able to naturally synthesize chlorophyll in the dark. This can be done by for example, taking a gene from a plant that is able to synthesize chlorophyll in both light and dark conditions, e.g. a pine tree, and trying to splice that gene (or genes) into the flowering plant. This can lead to the mutant being able to synthesize chlorophyll regardless of light being present or not. Of course, this is easier said than done, as extensive research would have to be done over the course of years to analyse and categorize which genes play a major role. At the same time other secondary genes could interact with those primary genes, so instead of only one gene, maybe 10 genes would need to be individually spliced in order for the reactions to take place.

The combination of decreased electrical energy usage together with a plant capable of independently being able to autonomously synthesize chlorophyll, would invertedly lead to a higher increase in yield and diversity over shorter amounts of time. This is because summer plants could then be grown in winter and vice-versa, leading to colder climate countries like Norway, to be able to supply itself with a more varied selection of fruits and vegetables. At the same time, the countries carbon footprint goes down, aiding in the current global warming situation as well as in the current upcoming global food crisis. Of course, these innovations would not only help Norway, but also every other country in the world, leading to a much better quality of life, and overall better economical situations.

Conclusion:

To conclude, the goal of this paper was to try and synthesize chlorophyll in the dark, and depending on the way one looks at it, it is either a success or a failure. The isolated plastids were taken from etiolated leaves, meaning they were grown in light deprived conditions, and from those, the chlorophyll was then synthesized by applying light at the end. If one takes into consideration the fact that the plants were grown in the dark, then yes, chlorophyll was synthesized successfully in the dark. But if one only looks at the isolated plastids, then technically the chlorophyll was

synthesized using light, rendering the experiment unsuccessful. However, that is not to say that the experiments conducted throughout were in vain, because they helped build up an understanding of the different methods. Whether that being extraction/isolation methods or just the scientific analysis methods themselves as well as building a better understanding of the way plants work on an atomic level, building the groundwork for future innovations and potentially ending certain crises such as world hunger.

The take home message should be that plants are much more complicated than they seem, leading to us as humans taking what they do for granted, producing the one element needed for us to survive, that being oxygen. In reality, lots of things have to be in the right place and at the right time, both at a macroscopic and microscopic level in order for us to be able to live. Leading to plants being one of the most important organisms that should be thoroughly investigated, since they are the source of all existing life.

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Appendices:

Appendix A:

https://docs.google.com/spreadsheets/d/17ol9mUm82GXRljCtXcbaoZrBJrljgodp/edit#gid=703442846

Appendix B:

https://docs.google.com/spreadsheets/d/1QrrQM-jyiMAg9xDYbdEALsKo8vPV6grl/edit#gid=354605331

Appendix C:

https://docs.google.com/spreadsheets/d/1DUGxDiajYtrBJKlV_41tEi1A0ZRPCDDK/edit#gid=154907025

Appendix D:

https://docs.google.com/spreadsheets/d/1_gtk1N-l3pnRYj1gT64MiqUcjpMdoRko/edit#gid=1331800993

Appendix E:

https://docs.google.com/spreadsheets/d/1ldbw5eJ0M5tvLTecSqhvpkwaGgD4dy1V/edit#gid=1432777362

Appendix F:
https://docs.google.com/spreadsheets/d/1fye6LZfuhiQ9JC-fRXrD95nymKLRdJDp/edit#gid=1409690501

Appendix G:
https://docs.google.com/spreadsheets/d/1-Q-NSs2BA7nb4bWiQrjimbTxdMRqdbsy/edit#gid=1359372102

Appendix H:
https://docs.google.com/spreadsheets/d/1RHWTWMej6RptUKmhmKbx2UzatB6D4rak/edit#gid=1199709645

Appendix I:
https://docs.google.com/spreadsheets/d/1SAbqrfCfJyhMgrOl9w04VC0biP9ST8bH/edit#gid=2091517684

Appendix J:
https://docs.google.com/spreadsheets/d/1rez7ZQKkqkHFjK-1W1hlRBzAb_KD4c01/edit#gid=1262402529

Appendix K:
https://docs.google.com/spreadsheets/d/173139cPXo9KVISpFMb_YHSi1lDNZ-dy0/edit#gid=804939699