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Cloning and reconstitution of PORA from *Arabidopsis thaliana*

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Master thesis in Biological Chemistry

University of Stavanger

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I. Abstract

Protochlorphyllide oxidoreductase, POR, is an enzyme found in the prolamellar bodies in etioplasts. Three isoforms have been detected so far; PORA, PORB and PORC. PORA is found in the etiolated seedlings, PORB is present at all times and PORC is thought to have a photoprotective role. POR is a light dependent enzyme that catalyzes the transformation from protochlorphyllide (pchlide) to chlorophyllide (chlide). This step is the only light regulated step in the biosynthesis of chlorophyll.

In this thesis a mature product of *PORA* from *Arabidopsis thaliana* has been cloned into a pET151 vector. PCR sequencing has confirmed that the 1065 nucleotide long sequence is correct. The mature product of PORA (AU2), has been expressed by *E. coli* and purified by centrifugation, filtration and metal chelate affinity chromatography. Expected size of AU2 is 40kDa where 2 of the kDa is a histidine-tag. Mass spectrometry (MS) analysis confirmed that AU2 is PORA from *Arabidopsis thaliana.* SDS PAGE revealed several protein bands of the purified AU2. The band of highest molecular weight was PORA, the other lower band is probably AU2 of shorter length, or degraded AU2.

Ratio between POR and pigments in extracted etioplasts from 4.5 day dark grown barleys were determined to be $2.7x10^6$ etioplasts per 1ng of POR. Reconstitution with AU2 and pigments in that ratio with NADPH in excess were performed, but in the absorbance spectra AU2 did not convert pchlide into chlide.

Future use of cloned POR products will be in reconstitution experiments. And possible protein-protein interactions to itself and Lil3 protein.

II. Acknowledgements

I don't know how many gels I have casted, how many times I have pipetted, how many hours I've spend in the lab, how many mistakes I've made or how much knowledge I have gained for these last 10 months. But I do know that I have enjoyed doing all these things. I have learned many new techniques, to work independently, use the resources around me and not to give up when results honor me with their absence. And I have been blessed with great people to work with in the lab. I could not ask for better colleagues.

First of all I would like to thank professor Lutz Eichacker for giving me this wonderful opportunity and letting me take part of your research. Your team has been nothing but welcoming and helpful. Chimuka and Ann Kristin have been sitting next to me and answered all of my questions. Astrid taught me to be structured and the importance of sterile technique. Clemens followed me through the whole process and he has helped me with my million questions, methods and thesis writing. Not to mention the daily philosophical discussions when we were pipetting away. Dimitry guided me through MS analysis as we solved world problems regarding religion and Napoleon.

I would also like to thank my friends and family for being so patient with me and believing in me. Especially in the last part of the thesis writing when I needed you the most.

''*If you really want it you can make it happen*''

Stavanger, 15th June 2012

Elisabeth Håland

III. Abbreviations

1^{o}AB:	Primary antibody
2^{o}AB:	Secondary antibody
APS:	Ammonium persulfate
AM:	PORA full sequence
AL:	Mature PORA. Signal sequence predicted from article (1)
AU:	Mature PORA. Signal sequence predicted from ChloroP
AU2:	Positive cloned transformant of mature PORA (colony number 2)
BLAST:	Basic local alignment search tool
BM:	PORA full sequence
BL:	Mature PORA. Signal sequence predicted from article (1)
BU:	Mature PORA. Signal sequence predicted from ChloroP
BSU:	Bovine serum albumin
CBB:	Coomassie Brilliant Blue
Chlide:	Chlorophyllide
ddH_2O:	Double distilled water
DNA:	Deoxyribonucleic acid
DTT:	Dithiothreitol
ECL:	Enhanced chemiluminescence
E.coli:	Escherichia coli
EDTA:	Ethylenediaminetetraacetic acid
GC%:	Guanine and cytosine content
His-tag:	Histidine-tag consisting of 6 histidines
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
LB-medium:	Luria-Bertani medium
Lil3	Light harvesting like protein called Lil3
NADPH:	Nicotinamide adenine dinucleotide phosphate
NaOH:	Sodium hydroxide
MS:	Mass Spectrometry
MS/MS spectrum:	Daughter spectra of a MS spectra

OD_{600}:	Optical density at 600nm
PCR:	Polymerase Chain Reaction
Phlide:	Protochlorophyllide
POR:	NADPH:protochlorophyllide oxidoreductase
RED:	Family of reductases, epimerases and dehydrogenases
RT:	Room temperature
SDS LB:	2X SDS loading buffer
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Speed vac:	Speed vacuum centrifuge
T7 primer:	Primer binding to the T7 promotor
TAE:	Buffer containing TrisHCl, Acetic acid and EDTA
TBS:	Tris-buffered saline buffer
TEMED:	Tetramethylethylenediamine
TTBS:	TBS with Tween

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1. Introduction

1.1. Photosynthesis

The role of POR and the formation of chlorophyll are related to photosynthesis, the process where electromagnetic energy is converted into chemical energy. In all organisms, it involves the capture of the energy of a photon by a substance pigment. This process of absorption will lead to the formation of an electronic excited state. This excited electron can be used by an acceptor substance which is reduced and forms an energy rich molecule. In plants water is used to supply the excited electrons and carbon dioxide is reduced. By the help of chlorophyll sugars are produced and oxygen is released as summarized by Equation 1-1. (2)

$$6\,CO_2 + 6\,H_2O \xrightarrow{light} \xrightarrow[chlorophyll]{} C_6H_{12}O_6 + 6\,O_6$$

Equation 1-1

Photosynthesis occurs in plants, algae and many species of bacteria but not in archaea. Organisms that perform photosynthesis are called photoautotrophs. These organisms are vital for all aerobic life on earth. They maintain the oxygen levels in the atmosphere and are the source of energy for nearly all life on earth, with exception of chemoautrophic organisms (2).

Figure 1-1. Photosynthetic activity occurs daily in plants.

1.3. Etioplasts

Photosynthetic activity takes place in chloroplasts. The theory of endosymbios explains where chloroplast originated from and how it developed into a organelle. According to the theory cyanobacteria were taken up by primitive eucaryotic cells 1-2 billion years ago. The symbiotic metabolism between the engulfed cyanobacteria and its host finally lead to evolution of the chloroplast. Today the chloroplasts have their own genome but most of their genes are located within the nucleus. Chloroplasts are surrounded by a double lipid membrane layer. The membrane consists of an inner envelope membrane that originates from the cyanobacteria, and an outer envelope membrane originating from the host cell. Within the organelle there are membrane structures organized in a disk shape called thylakoid, the membrane is the site of photosynthesis. The volume between the envelope membrane and the thylakoid is called stroma(3).

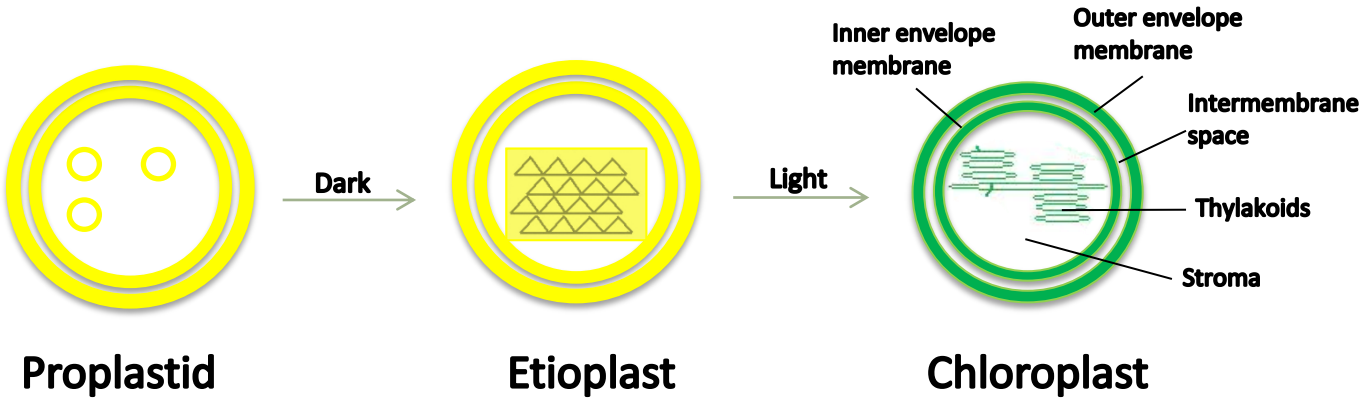


Figure 1-2. Development from proplastid to chloroplast. Proplastid contain vacuoles and vesicles. Etioplasts contain prolamellar bodies that have a crystalline arrangement. In the chloroplast the prolamellar bodies have transformed into thylakoids connected by tubules. The transformation from proplastid to etioplasts takes place in the dark while the developing to chloroplast requires light. Proplastid and etioplast have a faint yellow color while the chloroplasts are green.

The complete thylakoid membrane structure that is found in chloroplasts develops during biogenesis of a plant from progenitor organelles. In the light proplastids can differentiate into chloroplasts, chromoplasts, leucoplasts and amyloplasts. Proplastids have a diameter of 1-1.5 µm whereas chloroplasts in higher plants have a diameter of 3-10 µm (2). If the plants are grown in the dark,

proplastids develop into etioplasts, upon light exposure they develop into chloroplasts (Figure 1-3). Etioplasts are an intermediate stage of chloroplast development and they are devoid of chlorophyll. They don't have a thylakoid membrane, instead they have a membrane precursors, termed prolamellar bodies. Plant development often starts with germination in the darkness of the soil (3).

The development from etioplast to chloroplast takes place in several steps (Figure 1-3). In the etioplast the main protein within the prolamellar bodies is POR. When the etioplasts are exposed to light they will start the transformation into chloroplasts. POR catalyzes the light dependent reaction from protochlorophyllide to chlorophyllide. The chlorophyllide is then eventually transformed into chlorophyll. The prolamellar bodies loses the crystalline layer and forms first a primary layer, granas and stroma is formed, and then the thylakoid is assembled.

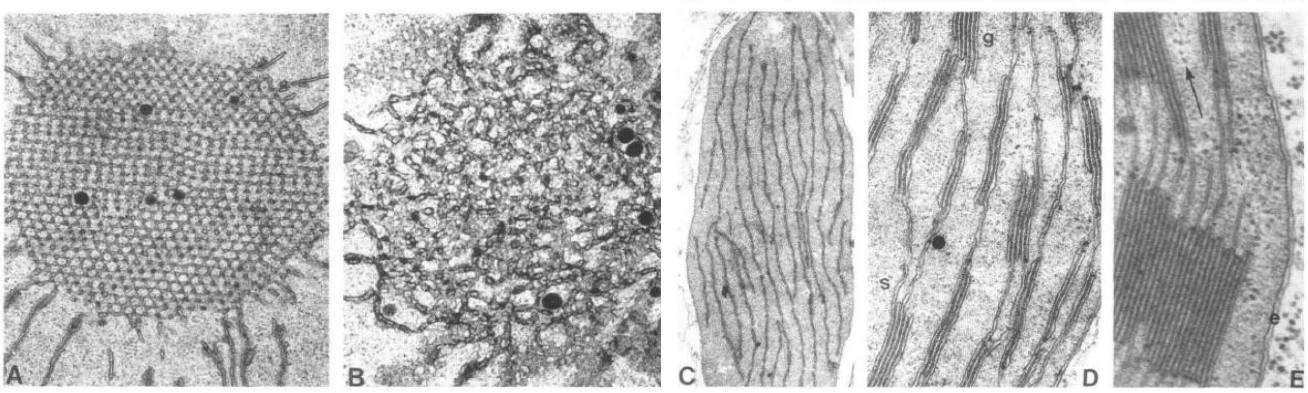


Figure 1-3. Membrane structure of A) a 7 day old dark grown etioplast that contains crystalline prolamellar bodies, B) light exposure and reduction of pchlide to chlide makes the membrane tubes of the prolamellar bodies lose their ordered arrangement, C) formation of primary layer, D) grana (g) and stroma (s) thylakoids are assembled, E) complete structure of thylakoid stacks connected by tubules (4).

Chloroplasts harbor chlorophyll in the photosynthetic machinery, localized in the thylakoid membrane. Chloroplyll is a pigment that absorbs energy from photons within the range of 330 – 1050 nm with an exception of the 'green gap' at around 500 nm (5). Normally the green color of plants are caused by the chlorophylls, although there can be other pigments in the vacuole and within the membrane, like the anthocyanins and xhantophylls, that masks the green color (3).

To capture photons, a plant concentrates the chlorophyll in so called antennas. The antenna delivers the photon energy to a core complex and a photosynthetic reaction centre to enable the transition into chemical energy. (2)

1.3. Biosynthesis of chlorophyll

Chlorophyll and tetrapyrrole are built from glutamate. Chlorophyll is a magnesium containing tetrapyrrole that can interact with electromagnetic energy. The last step to complete the tetrapyrrole structure is regulated by the light dependent enzyme NADPH:protochlorophyllide oxidoreductase, more commonly called POR, which catalyzes the reaction from protochlorophyllide (Pchlide) to chlorophyllide (Chlide). In this thesis, the emphasis will be on the light dependent POR that is found in all angiosperms.

1.4. Protochlorophyllide transformation to chlorophyllide

Light dependent transformation of protochlorophyllide (Figure 1-4a) to chlorophyllide (Figure 1-4b) structure is achieved via the reduction of the double bond between carbon atom C_{17} and C_{18} at pyrrole-ring D (6). The reduction leads to change in the three dimensional arrangement of the methyl group bound to C_{18} and the propionic acid group bound to C_{17}. The reduction increases the asymmetry of the delocalized electronic structure of the π- orbitals among the four pyrrole rings, the vinyl extension (C_3, C_8) and the keto-group at C_{13}' in ring E. Protochlorophyllide and chlorophyllide therefore exhibits different spectroscopic properties which is briefly discussed in sub chapter 1.6.

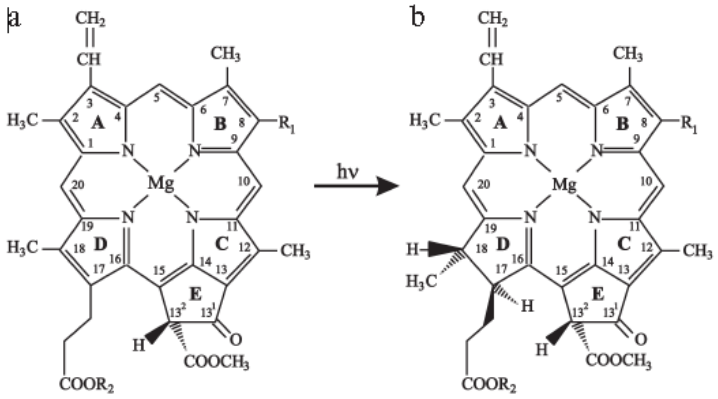


Figure 1-4. Structure of protochlorophyll(ide) a (a) and chlorophyll(ide) a (b). $R1_1$ is CH_2-CH_3 for the monovinyl pigment form and CH=CH_2 for the divinyl form. For protochlorophyll and chlorophyll R_2 is $C_{20}H_{39}$, for the protochlorophyllide and chlorophyllide R_2 it is a hydrogen atom (7).

1.5. Protochlorophyllide oxidoreductase

The enzyme POR catalyzing the *trans* addition of proton and hydride to the C17-C18 double bond of pyrrole ring D of pchlide (Figure 1-5) (6) belongs to the RED (reductases, epimerases, dehydrogenases) superfamily) (8). A family of single-domain oxidoreductases that catalyze proton and hydride transfer reactions with $NAD(P)^+$ or NADPH as a cofactor. The hydride is transferred from the *pro-S* face of the nicotinamide ring to the C17 position of the Pchlide molecule (9) (10). The proton is donated from a conserved tyrosine residue to the C18 position. The close proximity of a conserved lysine is proposed to be necessary to lower the apparent pK_a. of the phenolic group which will allow deprotonation to occur (6).

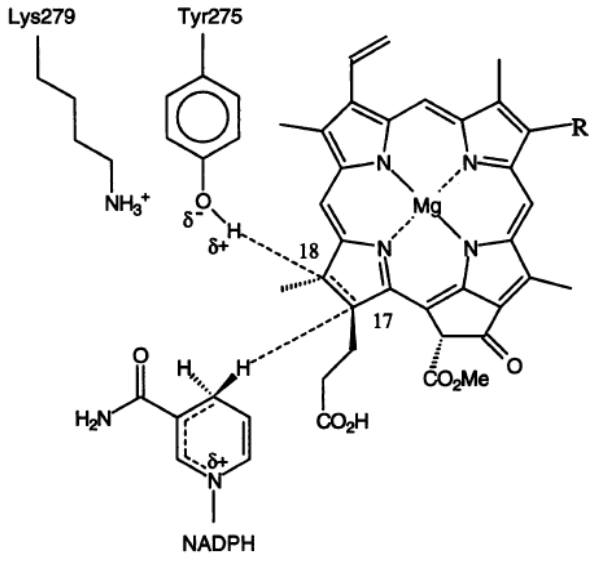


Figure 1-5. Proposed mechanism by Wilks *et a.l* (6). The model proposes that the proton at the C18 position of protochlorophyllide is derived from Tyr-275 and the hydride is transferred to the C17 position from the *pro-S* face of NADPH. R is CH_2=CH_3 or CH=CH_2. The position of lysine and tyrosine residues are from the sequence of *Pisum sativum L*.

Under low temperatures it has been measured that transferal of the proton takes 3 ps and the hydride is transferred within 400 ps (11). Figure 1-6 illustrates the mechanism that can take place in either a concerted mechanism where proton and hydride is transferred in one step, or the sequential mechanism where the proton and hydride are added in two separate steps (Figure 1-6).

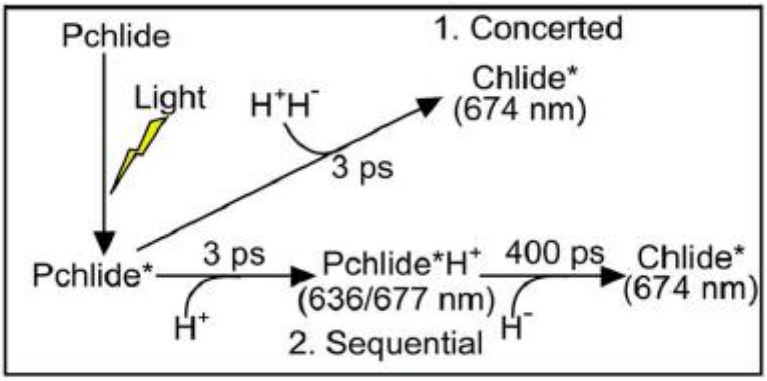


Figure 1-6. Transfer of proton and hydride can happen in a concerted mechanism occurring in one step, or in a sequential mechanism where the proton and hydride are transferred in two separate steps. Pchlide*H^+ is the intermediate product consisting of the excited pchlide with a newly received proton.

Models of POR present the enzyme with the shape of a globular, soluble protein. Townley *et al.* created a model of POR from *Synechocystis* where the sequence was compared to 7α-hydroxysteroid dehydrogenase from *E.coli,* a template chosen because it belonged to the tyrosine-dependent oxidoereductases of the NAD(P)-binding Rossmann-fold superfamily (8). It has nine alpha helices and seven beta flakes making up a hydrophobic core whereas the outside is covered with hydrophilic and

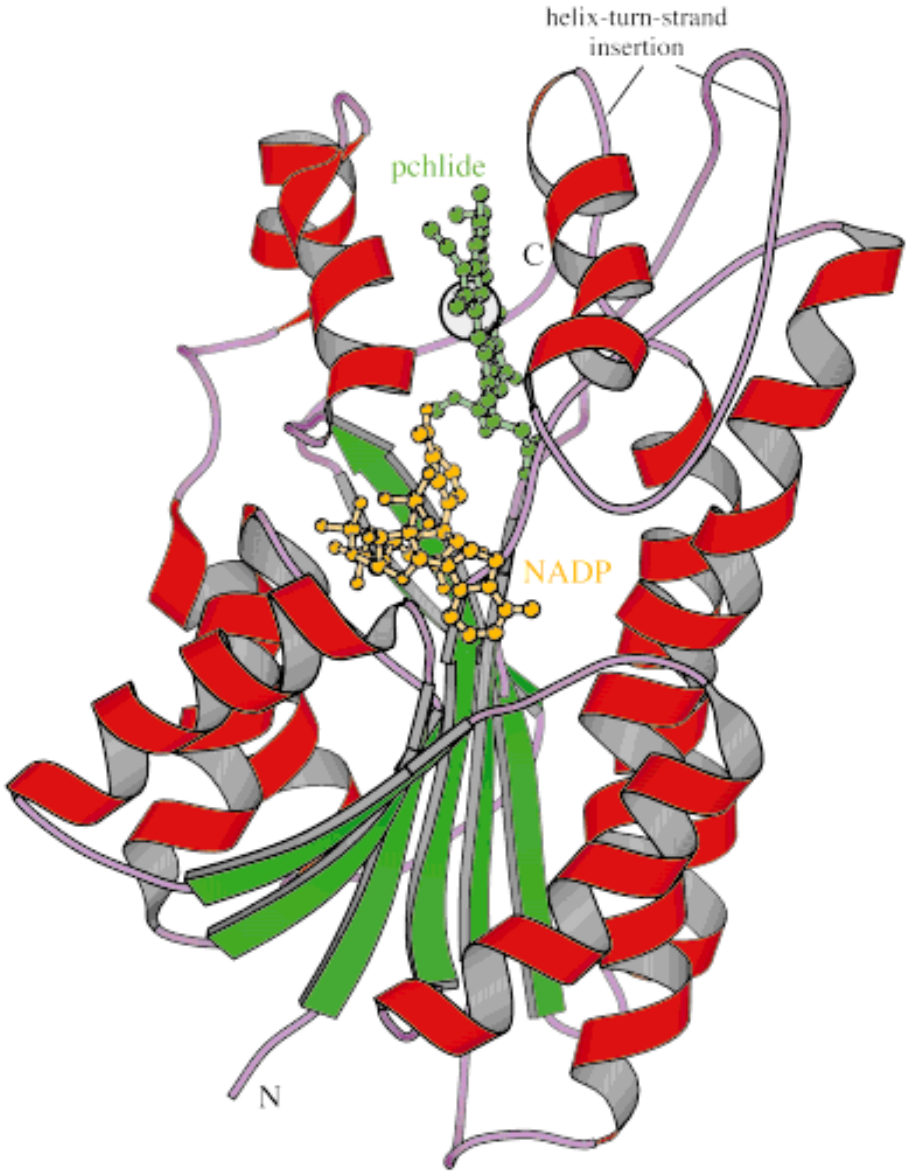


Figure 1-7. Proposed model of POR from *Synechocystis* sp. Betaflakes are coloured in red, beta flakes in green, NADPH in orange, pchlide in dark green and magnesium as a black circle. Townley *et al*. (8)

charged amino acids (Figure 1-7).

In 1995 Armstrong *et al.* published his discovery of a second POR protein that were found in *Arabidopsis thaliana* (12). The two POR versions were designated the names POR A and POR B. The same year Holtorf *et al.* reported that two forms of POR were also found in barley (13). The interesting part was

that both groups found that the two POR versions had different expression patterns. In etiolated seedling mRNA's for both *PorA* and *PorB* were present, but only few hours after exposure to light, *PorA* mRNA could not be detected. Runge *et al* proposed that the different expression patters could explain how chlorophyll biosynthesis were maintained in mature, fully greened angiosperms, where PORA is needed for greening and PORB is sustaining chlorophyll biosynthesis (14). Recent studies indicates that PORA might have a photoprotective role during the greening process (15). In 2001 a third POR was identified in *Arabidopsis thaliana*, this one was called PORC (16). In contrast to PORA the expression of PORC was up regulated in the presence of light and down regulated if put back in the dark.

Reinbothe *et a.l* claimed to have identified that POR A binds specifically to pchlide *b (*a pchlide with a formyl group at C7*)* and that POR B to binds specifically to Pchlide *a*. Through reconstitution experiments PORA, PORB and their substrates would form a complex of PORA:Pchlide*b*-PORB:Pchlide*a* in a ratio of 5:1. This was thought to act as a light-harvesting-complex (17). However this is not scientifically acknowledged based on several reasons. Armstrong *et al.* discuss the controversial views on this proposed model in a review (18). Arguments starts with how the model would require large amounts of Pchlide b, even though Pchlide *b* have never been detected in etiolated angiosperms (Koski et al 1948, Scheumann et all 1999). Second, the substrate specificity of PORA and PORB would lead to a unnatural high number of nonphotoactive pchlide of around 85%. Experiments also show that PORA and PORB can sustain their functions in etioplast formation and photoreception interchangeable (14) (19) (20) (21).

1.6. Absorption spectra

The catalytic mechanism of the reaction has only been possible in the recent years to understand and resolve due to the availability of large quantities of pure recombinant protein. The substrate and the products are rich in optical signals, so various spectroscopic techniques can be used to probe the catalytic mechanism. Although the phototransformation of pchlide to chlide is easy to detect and measure with absorbance and fluorescence spectroscopy, in vivo and in vitro photoconversion of pchlide to chlide results in different transformation rates and maxima. Reconstitution of in vitro synthesized POR is therefore still a challenge to establish and characterize.

POR is an excellent model to study the mechanism and time-scales of enzymatic proton and hydride transfers. Since it is light dependent it can be pre formed in the dark, and initiated with only a short pulse of light (22). Both substrate and product possesses spectroscopic properties which make it possible to easily detect the depletion of substrate and the formation of both the intermediate product and the final product.

Spectroscopic experiments have revealed the existence of many forms of pchlide, where there are three main forms (23). The three active pchlides characterized by their absorption spectra have absorbance maxima at around 630nm, 637 nm and 655nm. Pchlide 630 is thought to be photoinactive while the other two are photoactive. Phclide 655 are typically found when pchlide, POR and NADPH are incubated in the dark, where pchlide 637 is the main form before the incubation time. Pchlide 655 is the common form in *in vivo*. A diploma thesis proposes a model where POR acts as a dimer, giving the absorbance maxima at 655 and therefore indicates that this is what happens *in vivo (24).* Enzymes within the RED family are known to appear in dimers or tetramers.

Fluorescence and absorbance spectrophotometry will show the depletion of pchlide as a decrease of the peak at 637 and 650nm. The formation of chlide will be seen as an increasing peak at around 680 nm (25).

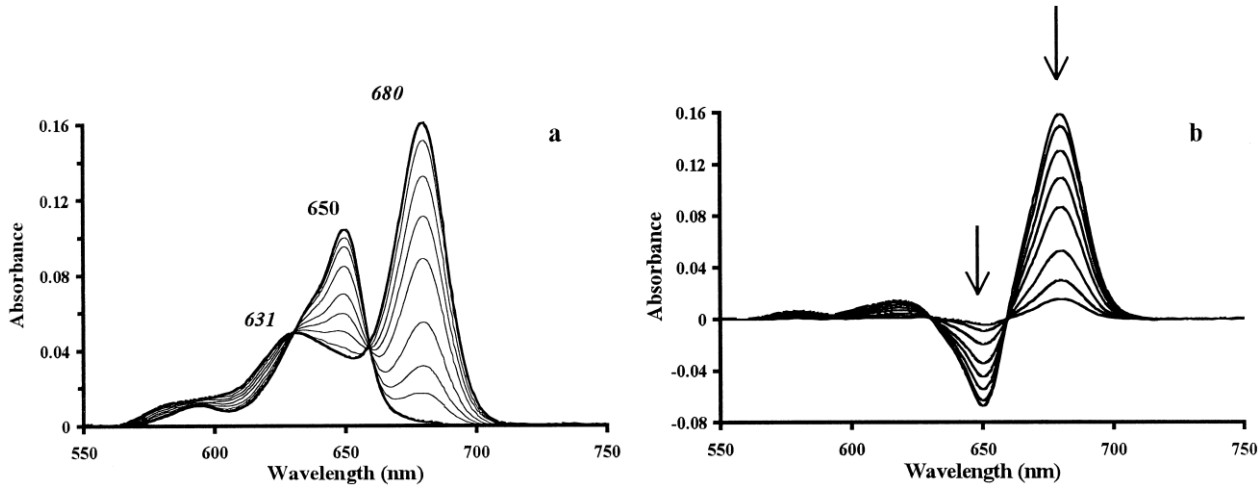


Figure 1-8. Klement *et a.l (25)* ***shows the photoconversion of pchlide to chlide. Absorption spectra are recorded after different time lengths of irradiation, as shown in a). The decrease of the peak at 650 nm, and the increase of the peak at 680 is shown in b).***

1.7. Reconstitution

Reconstitution of POR involves the reconstruction of the *in vivo* complex with pchlide and NADPH. Many experiments have been done of this enzyme so far and it is well proved that POR binds to its substrate pchlide and its cofactor NADPH.

The strategy in this thesis starts with the cloning of PORA and PORB from *Arabidopsis thaliana*, expression and purification. To check the function of the cloned POR reconstitution will be made with pigments extracted from *Hordeum Vulgare* and NADPH with absorbance spectrometry.

Future experiments would involve protein-protein interaction studies with other proteins from the light harvesting complex like protein Lil3. The function of Lil3 is not yet determined; however, other members of the research team work on the interaction of Lil3 with other enzymes regulating the biosynthesis of chorophyll. POR is one of the candidate interaction partners. Therefore, expression was established and reconstitution of POR was reinvestigated in this work.

2. Material

2.1. List of chemicals

- Acetic acid 96% — AppliChem
- Acetone — VWR
- AcTEV™ Protease — Invitrogen
- Acrylamide 2K — Applichem
- Agarose SERVA for DNA electrophoresis — SERVA
- Agar-agar ultrapure (for agar plates) — MERCH
- 2° antibody (goat anti-mouse – HRP) — Santa cruz biotechnology
- Ammonium persulfate, APS — MERCK
- Bromophenolblue sodium salt — SIGMA
- Coomassive Brilliant Blue R-250 — SERVA
- p-Coumaric acid — SIGMA
- 1,4 Dithiothreitol, DTT — BIOMOL
- 1 kb plus DNA ladder — Invitrogen
- EDTA disodium, Na_2EDTA — SERVA
- Absolutt alcohol prima, ethanol — Kemetyl
- GelRed 10 000X in water — Biotium
- Glycine — AppliChem
- Glycerol — SERVA
- Hydrogen peroxide, H_2O_2, 30% — VWR
- HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid — VWR
- Hydrogen chloride, HCl — VWR
- Imidazole — Fluka
- IPTG — VWR
- Kaliumchloride, KCl — ROTH
- diKaliumhydrogenphosphate, K_2HPO_4, anhydrous — MERCK
- Kaliumdihydrogenphosphat, KH_2PO_4 — ROTH

• Kaliumhydroxide, KOH	MERCK
• Kanamycin disulfate	MERCK
• KODAK developer	KODAK
• KODAK fixer	KODAK
• Luminol	(unknown)
• Lysozyme from chicken egg white	SERVA
• Magic Mark™ XD Western Protein Standard	Invitrogen
• Methanol	MERCK
• Milk powder	(unknown)
• Percoll™	GE healthcare
• SeeBlue® Plus 2 Prestained Standard	Invitrogen
• Sodium chloride, NaCl	Merck
• Sodium dodecyl sulphate, SDS	MERCK
• Sodium hydroxide, NaOH	MERCK
• D(-) Sorbitol	VWR
• N, N, N', N'- Tetramethyletylendiamine, TEMED	Fluka
• Tris	MERCK
• Triton® X-100	VWR
• Bacteriological peptone, peptone	USB
• Tween® 20	AppliChem
• Yeast extract granulated	MERCK

2.2. List of solutions according to method

Concentrations are given as a molarity, M, or in weight percent, w/v %. If the reagent is given as a volume percent, v/v %, this will be specified. For simplicity for some of the solutions, the exact volumes and weights are specified for each reagent rather than the end concentration.

2.2.1. Cloning:

❖ Agarose gel (0.7%), 100 ml

- 0.7 g Agarose gel for DNA electrophoresis
- 100 ml TAE buffer
- Store at RT

❖ 1 kb plus DNA ladder, 90µl

- 7 µl 1 kb plus DNA ladder
- 20 µl 6X Loading buffer (LB)
- 63 µl TE buffer
- Store at -20^{o}C

❖ 0.5 M EDTA pH 8.0, 50ml

- Dissolved in dH_2O
- pH adjusted with NaOH
- Autoclave, store at RT

❖ GelRed 1:50, 50 µl

- 1 µl GelRed
- 49 µl autoclaved ddH_2O
- Store at -20^{o}C

❖ GelRed/LB, 120µl

- 20 µl GelRed 1:50
- 100 µl 6xLB
- Aliquot and store at -20^{o}C

❖ 6X Loading buffer (LB) (for agarose gel electrophoresis), 1 ml

- 30 % glycecol
- 70 % autoclaved dH_2O

- 2.5 mg bromophenol blue
- Solubilize in sterile ddH_2O
- Aliquot in 5x200µl and store at -20^oC

❖ Luria Brettoni (LB) plates with antibiotics (Kanamycin and ampicillin), 1l

- 1 % Tryptone
- 0.5 % yeast extract
- 1 % NaCl
- 1.5 % agar
- Solubilize in dH_2O and autoclave it. Antibiotic is added afterwards, in this thesis kanamycin and ampicillin to an end concentration of 50µg/ml (kanamycin) and 100µg/ml (ampicillin).
- Plates are stored at +4^oC

❖ 1 kb plus DNA ladder with loading buffer, 90µl

- 7 µl 1 kb plus DNA ladder
- 20 µl 6XLB
- Store at -20^oC

❖ SOC medium, 15 ml

- 2 % Tryptone
- 0.5 % yeast extract
- 0.05 % NaCl
- 10mM $MgCl_2$
- 10mM $MgSO_4$
- 20mM glucose
- Solubilize in dH_2O (15 ml), aliquot and store the medium at -20^oC

❖ Tris-EDTA (TE) buffer pH 8.0, 50ml

- 5 ml Tris HCl pH 7.5 (end concentration 100mM)
- 1 ml EDTA pH 8.0 (end concentration 10mM)

- ddH_2O up to 50 ml
- autoclave and store at RT.

❖ 50X TAE buffer, 1l

- 24.2 % Tris
- 50 mM EDTA pH 8.0
- ddH_2O up to 1l
- Store at RT

2.2.2. Expression:

❖ IPTG

- 100mM (aq)
- Premade aliquots of 1.5ml stored at -20^{o}C

❖ Luria-Bertania (LB) media, 500ml

- 1 % Tryptone
- 0.5 % yeast extract
- 1 % NaCl
- Solubilize in ddH_2O
- Autoclave and store at +4^{o}C

❖ 1 M K_2HPO_4, 100 ml

- 17.4 g K_2HPO_4
- ddH_2O up to 100 ml
- Autoclave and store at +4^{o}C

❖ 1 M KH_2PO_4, 100ml

- 13.6 g KH_2PO_4

- ddH_2O up to 100 ml
- Autoclave and store at +4^oC

❖ Lysis buffer, 100ml,

- 3mM KH_2PO_4
- 47mM K_2HPO_4
- 2.3 % NaCl
- 0.75 & KCl
- 10 % (v/v) glycerol
- 0.5 % Triton X-100
- 68 mg imidazole
- Autoclaved dH_2O up to 100 ml. Adjust pH to 7.8 with HCl

❖ Lysozyme solution, 15 ml

- 0.1 gram lysozyme
- 0.15 ml Tris pH 8.0 (stock concentration 1M)
- 9.85 ml dH20
- 5 ml glycerol
- Premade aliquots of 7.5 mg/ml stored at -20^oC

2.2.3. SDS PAGE

❖ 10 % APS (aq)

- Premade stock of 1.5 ml aliquots stored at -20^oC

❖ 10% SDS

- 10 % SDS
- Solubilize in ddH_2O
- Store at RT

❖ SDS gel, 12 %, 5 gels

- 7.5 ml Tris HCl pH 7.5 (stock solution is 1.5M)

- 9.9 ml ddH_2O
- 300 µl SDS (stock concentration is 10%)
- 12 ml Acrylamide
- 300 µl APS (stock concentration is 10 %)
- 12 µl TEMED
- Mix together the reagents in the order they are given (APS and TEMED must be added at last). Afterwards cast the gel immedialtely and finish with a layer of butanol on top, 0.5 ml per gel.
- The gels can be stored at +4^{o}C for two weeks if wrapped up in wet paper.

❖ 2X SDS sample buffer, 10 ml

- 125 mM Tris HCl pH 6.8
- 20 % (v/v) Glycerol
- 0.2 % Bromophenol blue
- 2 % DTT
- 4 % SDS
- Autoclaved ddH_2O up to 10 ml, make aliquots of 1.5ml
- Store at -20^{o}C

❖ 1 X SDS sample buffer

- Same reagents as in 2X SDS sample buffer but half the amount.

❖ SDS running buffer for SDS page, 1l

- 3 % Tris
- 14.4 % Glycine
- 10 % (v/v) (stock solution is 10%)
- Solubilize in ddH_2O

❖ 0.5 M Tris HCl pH 6.8, 500 ml

- Solubilize with dH_2O, pH adjust with HCl
- Autoclave and store at RT

❖ 1.0 M Tris HCl pH 7.5.

- Solubilize with dH_2O, pH adjust with HCl
- Autoclave and store at RT

❖ 5M NaOH, 500 ml

- Dissolve in ddH_2O
- Autoclave and store at RT

2.2.4. Western Blot

❖ Primary (1°) antibody solution for histidine, for 2 gels, 40ml

- Premade solution in 5% milk powder, dissolved in 1X TBS solution
- Antibody added in a 1:3000 dilution.
- The 1° can be reused for each time. Store at -20°C.?

❖ Secondary (2°) antibody solution, for 2 gels, 40 ml

- 5% milk powder
- 40 ml 1X TBS solution
- 8 µl 2° antibody (goat anti-mouse). 1:5000.

❖ Blocking solution, 40 ml

- 5% milk powder
- 1X TBS
- Mix together right before usage

❖ 40 mM p-Coumaracid (DMSO)

- Premade stock of 1 ml aliquots stored at -20^{o}C

❖ ECL1, for 1 membrane, 5 ml

- 0.1 M TrisHCl pH 8.3
- 2.5 mM Luminol
- 400 µM p-coumaracid
- dH_2O up to 5 ml

❖ ECL2 (1 membrane), for 1 membrane, 5 ml

- 0.1 M TrisHCl pH 8.3
- 0.061 % v/v H_2O_2 (stock concentration is 30%)
- ddH_2O up to 5 ml

❖ 250 mM Luminol (DMSO)

- Premade aliquots of 1 ml stored at -20°C

❖ Transfer buffer/Towbin buffer for Western Blot, 900 ml

- 96mM Glycin
- 10 nM Tris
- ddH_2O
- Store at RT

❖ 2.0 M Tris HCl pH 8.3, 500ml

- Solubilize with dH_2O, pH adjust with HCl
- Autoclave and store at RT

❖ TTBS

- 10 % 10XTBS solution
- 90 % dH2O
- 5 % Tween

❖ Methanol transfer buffer

- 10 % methanol

90 % transfer buffer

❖ 10X TBS buffer, 1 l

- 100 ml 1M Tris pH 7.5
- 150 ml 5M NaCl
- 250 ml dH_2O

2.2.5. Coomassive staining

❖ Coomassive brilliant blue, 500 ml

- 1 g coomassive brilliant blue
- 37.5 % (v/v) acetic acid
- 50% (v/v) ethanol[1]
- dH_2O up to 500 ml
- Store at RT

❖ Destaining CBB solution, 500 ml

- 0.75 % (v/v) acetic acid
- 10% (v/v) ethanol[2]
- ddH_2O
- Store at RT

2.2.6. Desalting

❖ Hepes solution, 3l

- 25 mM Hepes
- 300 mM NaCl
- pH adjusted to 7.5 with NaOH
- Make it fresh for each experiment. Discard afterwards.

[1] The correct reagent is methanol, not ethanol
[2] The correct reagent is methanol, not ethanol.

2.2.7. Purification with His-tag coloumn

❖ Buffer A for protein purification, 1l

- 25 mM Hepes pH 7.5
- 300 mM NaCl
- 10 mM imidazole
- ddH_2O
- Filtrate with Supor®-200, 0.2µm, 47 mm membrane filter (PALL Life Sciences P/N 60301)
- Degass by magnetic stirring with vacuum for 15 minutes.
- Store at $+4^oC$

❖ Buffer B for protein purification, 1l

- 25 mM Hepes pH 7.5
- 300 mM NaCl
- 250 mM imidazole
- ddH_2O
- Filtrate with Supor®-200, 0.2µm, 47 mm membrane filter (PALL Life Sciences P/N 60301)
- Degass by magnetic stirring with vacuum for 15 minutes.
- Store at $+4^oC$

❖ Buffer C for protein purification, 1l

- 25 mM Hepes pH 7.5
- 300 mM NaCl
- 500 mM imidazole
- ddH_2O
- Filtrate with Supor®-200, 0.2µm, 47 mm membrane filter (PALL Life Sciences P/N 60301)
- Degass by magnetic stirring with vacuum for 15 minutes.
- Store at $+4^oC$

❖ 20 % ethanol for protein purification, 1l

- 20 % ethanol

- ddH_2O
- Sterile filtrate with the same filter used for Buffer A, B, C
- Store at +4°C

❖ Degassed water for protein purification, 1l

- ddH_2O
- Sterile filtrate with the same filter used for Buffer A, B, C
- Degass by magnetic stirring with vacuum for 15 minutes.

2.2.8. Plastid isolation

❖ Isolation medium, 1l

- 400 mM sorbitol
- 50 mM Hepes/KOH
- 2 mM EDTA pH 7.5
- Store at -20°C. Thaw right before usage.

❖ 1 M Hepes/KOH, pH 8.0, 250 ml

- 1 M Hepes
- 0.2 M KOH

❖ 40 % Percoll solution, 120 ml

- 40% Percoll
- 50 mM Hepes/KOH
- 400 mM Sorbitol
- 1 mM EDTA pH 7.5
- dH_2O

❖ 80 % Percoll solution, 60 ml

- 80 % Percoll
- 50 mM Hepes/KOH

- 400 mM Sorbitol
- 1 mM EDTA pH 7.5
- dH_2O

2.2.9. Reconstitution

❖ 1 M Hepes, 250 ml

- 1 M Hepes
- Sterile ddH_2O
- Sterile filtrate with the same membrane as in Buffer A, B and C for protein purification
- Store at RT

❖ 1 M NADPH, 27.65ml

- 1mM NADPH
- Sterile ddH_2O
- aliquot and store at -20^{o}C

❖ 150 mM NADPH, 922.8µl

- 150 mM NADPH
- Sterile ddH_2O
- aliquot and store at -20^{o}C

2.3. List of kits

- *Kit for cloning.* Champion pET Directional TOPO Expression Kits. Five minute, directional TOPO Cloning of blunt-end PCR products into vectors for high-level, inducible expression in *E.coli*. Product number K151-01.
- *Kit for plasmid isolation.* QIAprep® spin miniprep kit. Catalogue number 27106. Qiagen.
- *Kit for extraction of DNA in agarose gel.* QIAquick Gel Extraction Kit. Catalogue number 28704. Qiagen.

- *Kit for measuring protein consentration.* Pierce® BCA Protein assay kit. Product number 23227. Thermo Scientific.

3. Methods

3.1. Experimental strategy

First the full length and mature sequences of *PORA* and *PORB* (*Arabidopsis Thaliana)* will be cloned. The ones that are successful will be further expressed in *E.coli* and purified. Pigments (pchlide) will be provided by extraction of etioplastids from 4.5 day old dark grown barley (*Hordeum Vulgare)*. From the etioplastid extract, proteins will be extracted and quantified. Reconstitution will be performed by absorbance spectroscopy using the same ratio of POR and pigments as was found in the etioplast extract.

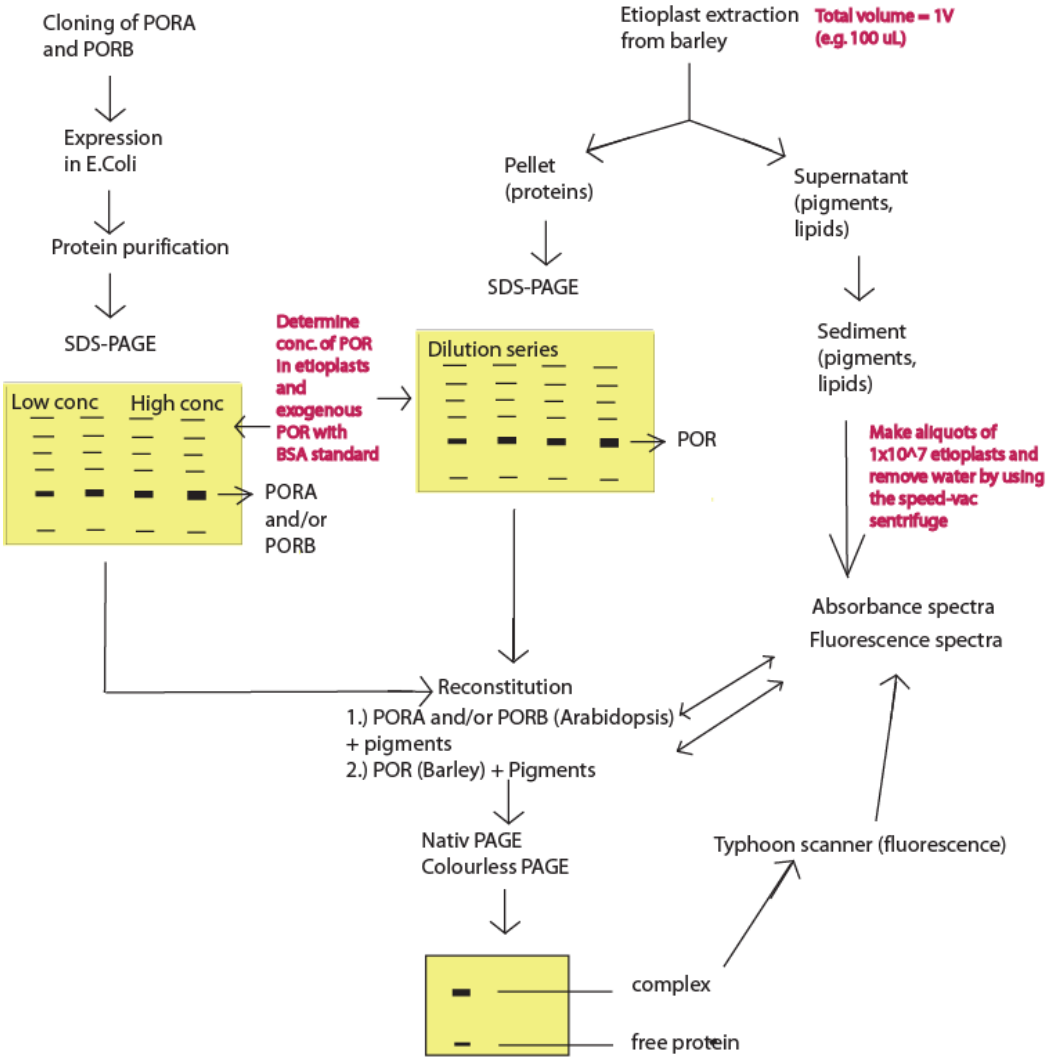


Figure 3-1. Experimental outline for this thesis involve cloning of *PORA* and *PORB*, extraction of plastids from dark grown barley, determination of POR:pigment ration in plastids and finally the reconstitution of POR with pigments (pchlide) and NADPH using absorbance spectroscopy.

3.2. Cloning of PORA and PORB

3.2.1. POR A and POR B template

DNA template with the sequence for *PORA* and *PORB* was ordered from The Arabidopsis Information Center from the website www.arabidopsis.org (Chicago, USA). The template was received in a pUNI51 vector and pENTR™/SD/D-TOPO vector inside the two strains (PIR1 and Top-10) of the host *E.coli*. To retrieve the vector, the host strain was cultured on a LB plate containing 50 µg/ml Kanamycin, then cultivated in 10 ml of LB medium overnight, followed by plasmid isolation with QIAquick Gel Exctraction Kit from Invitrogen. The concentration and the purity of the plasmid were measured spectrophotometrically with NanoDrop 2000.

3.2.2. Primers for PCR amplification

Primers were ordered from Eurofins mwg|operon (Eurofins MWG GmbH, Ebersberg, Germany). The sequences are listed in Table 3-1. The primers were added TE buffer according to the instructions from the producers, giving a primer concentration of 100 µM. A tenfold dilution was made from this stock. Parameters like GC content, nucleotide length, 3' overhang, annealing temperature and self end complementarity were important to make primers of good quality. The online primer design program Primer3 was used (http://frodo.wi.mit.edu/) (26).

Table 3-1 Primers for PCR amplification. T_m and GC% are data from the manufacturer.

Name of primer	***Description***	***Primer sequence***	***T_m (sequence minus CACC)***	***GC %***	***Length (nucleotides)***
PORAmsfp	Forward primer for full length PORA	CACCATGGCCCTTCA AGCTG	61.4 (55.5)	60	20
PORAusfp	Forward primer for mature PORA, predicted by Chloro P	CACCTGCAAGAGGGA ACAGAGC	64.0 (52.7)	59.1	22

PORAlutzfp	Forward primer for PORA, chemically predicted (1)	CACCGCAATCGCGACTTCAA	67.73 tm *(55.0)*	52	20
PORArp	Reverse primer for PORA	TTAGGCCAAGCCTACGAGCT	59.4	55	20
PORBmsfp	Full length	CACCATGGCCCTTCAAGCTG	61.4 (55.4)	60	20
PORBusfp	Chloro.P predicted mature sequence	CACCTCCGAACATGGATCTTCCT	62.4 (58.0)	52.2	23
PORBrp	Reverse primer for PORB	TTAGGCCAAGCCCACGAG	58.2	61.1	18

3.2.3. Prediction of signal sequence length

The sequence lengths were predicted by using the website ChloroP (http://www.cbs.dtu.dk/services/ChloroP/)(27) and by comparing the determined mature sequences of PORA and PORB in barley (1).

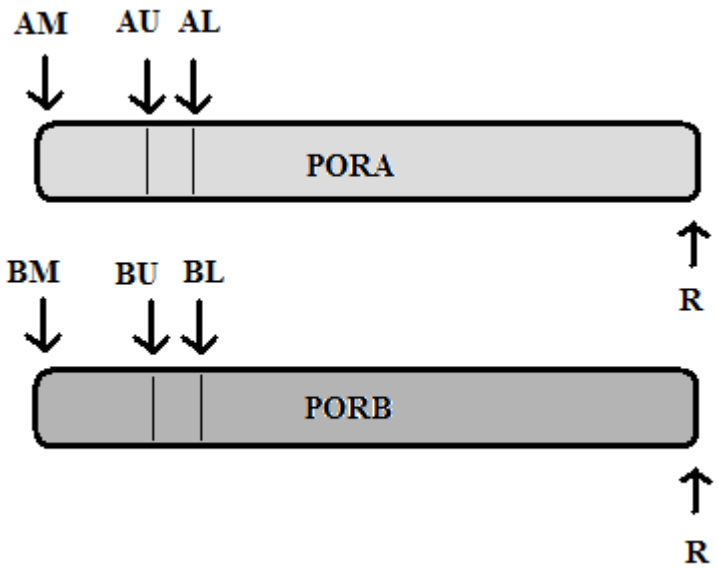


Figure 3-2. Primers binding at different parts of the gene to produce the different products. AM and BM are products of full length. AU, BU, AL, BL are mature products which length are explained in subchapter 3.2.3. Abbreviations are in norwegian meaning that AM is PORA with (*med*) signal sequence, AU is without (*uten*) signal sequence, AL is named after professor Lutz Eichacker due to the article where signal sequence of PORA and PORB in barley was determined(1).

3.2.4. PCR amplification

PCR amplification (28) was performed with VENT polymerase with the reagent composition listed in the set-up from Table 3-2. A mastermix with all the common reagents were made where the VENT polymerase was added as the last reagent. Then primers and templates were pipetted into each PCR tube before the mastermix to a total volume of 50 µl was added. As the temperature in the PCR machine reached 80 °C, the PCR tubes were inserted and run on a temperature cycle according to Table 3-3. 6 different PCR products were produced, products illustrated in Figure 3-2. VENT polymerase was used because it produces blunt end products, which is required to clone into the pET151 vector (Invitrogen). According to the producer the VENT polymerase produces 95% blunt end products. All these steps were performed on ice.

Table 3-2 Reagents of PCR reaction with VENT polymerase

Reagent	Volume	End concentration
VENT polymerase	1 µl	2 units
10 mM dNTP	1 µl	0.2 µM
10 µM Forward primer	1 µl	0.2 µM
10 µM Reverse primer	1 µl	0.2 µM
10 ng/µl template	1 µl	0.2 ng/µl
10X PCR buffer	5 µl	1 X
Autoclaved dH20 up to	50 µl	

Table 3-3 PCR temperature syklus

Step	*Procedure*	*Temperature*	*Duration*
1	Initial denaturation	95 °C	5 minutes
2	Repeat step 2-4 25 times	95 °C	30 seconds
3		60 °C	30 seconds
4		72 °C	1 minute 10 seconds
5	Final extension	72 °C	5 minutes
6	Storage	4 °C	∞

3.2.5. Agarose gel electrophoresis and spectrometry

The concentration of the PCR products was determined spectrophotometrically with NanoDrop. The quality of the PCR products was analyzed by running agarose gel electrophoresis.
50 – 100 ng/µl of DNA were diluted with sterile ddH_20 up to 5 µl. 1 µl of GelRed (diluted 1:50) was added and then loaded into a well. 6µl of 1 kb DNA ladder from Invitrogen was used as a DNA size marker. 1XTAE was used as anode and cathode buffer. The gel was run on 170V for 1 hour. DNA bands were visualized under UV light and pictures taken.

3.2.6. TOPO cloning and transformation into competent cells

The PCR product was cloned into a pET 151 vector followed by transformation into TOP10 competent *E.coli*. Vector and competent cells are all components from the Champion pET Directional TOPO Expression Kit. The protocol from the kit was followed.

4 µl of PCR products together with 1 µl salt solution and 1 µl of TOPO vector were mixed gently (softly stirred with pipette tip) in a sterile eppendorf tube, and left for incubation at room temperature. After 30 minutes of incubation it was placed back on ice. 3 µl of the TOPO cloning reaction was added into one vial of One Shot TOP10 chemically competent *E.coli*, mixed gently and put on ice. After 30 minutes of incubation the reaction was heat shocked at 42 °C in water bath for 30 seconds, then placed directly back on ice again. 250 µl of SOC medium was added and another incubation period of 1 hour at 37 °C with shaking followed. 100µl + 250 µl of bacterial culture was spread on a prewarmed LB plate containing 100g/ml ampicillin and left for overnight incubation at 37 °C.

3.2.7. PCR screening identifies positive transformants

Once the transformed *E.coli* had incubated on LB plates containing ampicillin, they had to be PCR screened. The method identifies the colonies that have been transformed with an insert containing vector, without having to purify plasmids from the colony first. Forward and reverse primers that are specific to the T7 promoter region were used. If the colony contained a vector without the insert, empty vector, the PCR products would be very small, about 160 bp. If the vector contained the insert, the size of the product would that of the cloned sequence + 160 bp.

The reagents were mixed together with the composition from Table 3-4 and pipetted into PCR tubes. An LB plate with ampicillin was divided into 10-15 areas. The colony to be PCR screened was first picked up

with a pipette tip, the colony streaked on the LB plate on its dedicated area, and then the dipped into the PCR screening reagent composition. This was repeated with every colony. A negative control was also run, this contained sterile water instead of a colony. As the PCR reaction was run following specifications in Table 3-5, the streaked plate was incubated on 37 °C for 4-6 hours.

Table 3-4. Reagents for PCR screening

Reagents	Volume	Final concentration
5X GoTaq Green/Flexibuffer	4 µl	1X
5U GoTaq DNA polymerase	0.1 µl	0.5 U
25 mM $MgCl_2$	1.6 µl	2mM
10 mM dNTP	1 µl	0.2mM
0.5 µM forward primer specific to the T7 promotor region	1 µl	0.5 µM
0.5 µM reverse primer	1 µl	0.5 µM
ddH_2O up to	20 µl	-

Table 3-5. Temperaure cycle for PCR screening.

Step	Temperature	Duration	
1	95°C	5 minutes	
2	95°C	30 seconds	Repeat step 2-5 35 times
3	60°C	30 seconds	
4	72°C	60 seconds	
5	72°C	5 minutes	
6	4°C	∞	

3.2.8. Isolation of plasmids from positive transformants

After the PCR reaction, the samples were run on an agarose gel using the 1 kb DNA ladder as a standard. 1XTAE buffer was used as anode and cathode buffer. 170V was applied for 1 hour. The positive transformants were easily visualized as clear bands of high molecular weight, while the negative ones would appear to have stronger lower bands of about 160 bp. The expected product size of positive transformants will be 'PCR product + 160 bp', e.g. 1222bp + 160bp= 1382bp. A colony from the positive PCR screened transformants were grown overnight in 5 ml of LB medium with 100µg/ml ampicillin. After

inoculation the plasmid was isolated with QIAquick Gel Extraction Kit and the concentration measured with NanoDrop.

3.2.9. Sequencing

The sequence of the clone is determined by sequencing. 1 µl primer (only forward or reverse in each tube, never both) together with 1 µl of BigDye v3.1, 2 µl 5XBuffer, 150-250ng of plasmid and autoclaved H20 up to 10 µl. For one sample two reactions will have to be made; one with forward primer, and one with reverse primer. Four primers were used as shown in Figure 3-3. 'T7' binds to the T7 promoter region, 'T7-50' binds 50 nucleotides before T7, 'T7 rev' binds to the T7 reverse priming site and 'T7-rev-50' binds 50 nucleotides after T7-rev. T7-rev-50 had to be used because T7-rev was not working well. The samples are then run on a PCR program shown in Table 3-7. Afterwards 10 µl of autoclaved water was added to the samples and sent to "Medisinsk Genetisk avdeling" at Universitetssykehuset Nord Norge HF (Tromsø, Norway).

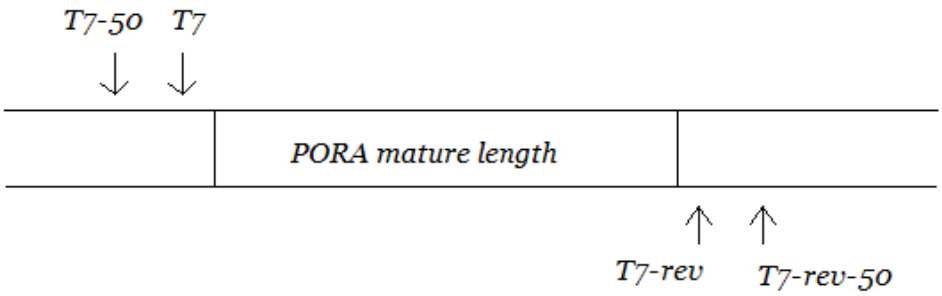


Figure 3-3. ***Four primers used to sequence the cloned product, illustrated as to where they bind around the gene in the pET151 vector. T7 binds to the T7 promoter region, T7-50 binds 50 nucleotides before T7, T7 rev binds to the T7 reverse priming site and T7-rev-50 binds 50 nucleotides after T7-rev.***

Table 3-6 Reagents for sequencing

Reagent	Volume	Final concentration
214.7 ng/µl POR A mature plasmid	1 µl	21.47 ng/µl
5X BigDye sequencing Buffer	2 µl	1X
3.2 µM Primer (reverse or forward)	1µl	0.32 µM
BigDye	1 µl	
autoclaved dH_2O	6 µl	-
autoclaved dH_2O	*10 µl*	*Added after PCR.*

Table 3-7 Temperature cycle for sequencing

Step	Temperature	Duration	
1	96°C	3 minutes	
2	96°C	10 seconds	Repeat step 2-5 25 times
3	50°C	5 seconds	
4	60°C	4 minutes	
5	4°C	∞	

3.2.10. Analysing the sequencing results

Results were received as sequence chromatogram. The files were opened and processed in the plasmid editor program ApE (http://biologylabs.utah.edu/jorgensen/wayned/ape/). The program allows visualizing of the chromatogram and translating it into a DNA sequence. One file can consist of 1200 bases but only the first part will be of good quality. Therefore the two files belonging to one sample (one with forward primer and one with reverse primer) were put together into what is called a contig. The contig was used to do a BLAST search. The BLAST search will show if the cloned gene is the correct gene, and how many basepairs are correct.

3.3. Expression of PCR product

Only one desired PCR product was successfully cloned, POR A mature (Chloro.P predicted). Therefore only this clone was proceeded with. Once the sequence was confirmed the PCR product were expressed with Champion pET Directional TOPO Expression Kit from Invitrogen.

3.3.1. Small scale expression

A small scale culture expression shows if the a protein of interest can be expressed and in which quantities. It also indicates the solubility of the protein. Following vectors listed in Table 3-8 were used, where 1, 4 and –K are used as controls.

1 µl of a vector was added to a vial of BL21 competent cells. This was done separate for the four vectors listed in Table 3-8. The vials were then incubated on ice for 30 minutes before it was spread on ampicillin containing LB plates. The plates were left for incubation overnight at 37 °C.

Table 3-8. Vectors used in small scale expression

Name	Vector
1	Lil3-2 full length
4	Lil3-2 mature
AU2	POR A mature (Chloro.P. predicted cleavage site)
-K	Empty p151 vector

Precultures were made by adding 1 colony to 10 ml of LB medium with 100µg/ml ampicillin. This was incubated overnight with shaking (250rpm) at 37 °C. Next day expression cultures were made out of the precultures. 0.5 ml of preculture was added to 9.5 ml LB medium with 100µg/ml ampicillin. Two expression cultures per vector were made. This was left shaking at 250 rpm and 37°C until optical density at 600nm (OD_{600}) were 0.8. 1mM IPTG was added to the precultures followed by incubation at 16 °C with shaking at 250rpm overnight.

3.3.2. Harvesting the small scale expression products

The expression cultures were transferred from the erlenmeyer flasks to falcon tubes and spun down for 5 minutes at 5200g. The supernatant were discarded and the pellets solubilized in 500 µl of lysis buffer,

followed by freeze-thaw treatment consisting of freezing the samples for 5 minutes at -80°C and then thawing it at 42 °C in water bath for two minutes. The freeze-thaw treatment was repeated three times. 70 µl of 7.5 mg/ml lysozyme was added before the samples were sonicated. Sonication was performed with 30 % amplitude for 3x10 seconds. During sonication the samples were kept on ice. The samples were transferred to 1.5 ml eppendorf tubes and centrifuged at 10 minutes at 16100g at 4^{o}C. The centrifugation separates the soluble fraction (supernatant) from the insoluble fraction (pellet). The supernatant that contained the soluble fraction were transferred to a new eppendorf tube.

3.3.3. Analyzing the expressed products by SDS PAGE followed by coomassive staining and immunoblotting

The harvested proteins from the cell cultures were run on of sodium dodecyl sulphate (SDS) polyacrylamide agarose gel electrophoresis (SDS PAGE) to separate the proteins according to size. The proteins are given a negative charge by loading of the SDS. The proteins can then move to the positive anode. Polyacrylamide is added to gel which together with APS creating pores that the proteins can move through. The larger the protein the harder it will be for it to move. Ultimately the larger the protein, the slower they travel, and that will separate proteins in a mix of proteins. SDS gels contain 12% of acrylamide.

The samples were prepared for SDS-PAGE in the following way. 25 µl of 2XSDS loading buffer was added to 25 µl of supernatant in an eppendorf tube. The pellet was added 500 µl of 1XSDS buffer and solubilized in an eppendorf tube. The samples were boiled at 95 °C for 4 minutes. Pre stained standards for SDS gels are SeeBlue, Magic Marker and Mark12. 5 µl of standard and 10µl of prepared sample were loaded into a well on the gel. SDS running buffer was used as anode and cathode buffer. The gels were run at 15mA/gel for 1 hour and 15 minutes.

3.3.4. Coomassive staining

The SDS gel intended for staining were released from its surrounding plates. The stacking layer was removed and the gels were soaked in coomassive staining solution for 45 minutes in room temperature whilst being shaken. 3x5 minutes of washing with dH_2O removed the staining solution. Then the gels were left in destaining solution for at least one day, where the solution was changed regularly 2-3 times. This had the purpose of removing the dye absorbed by the gel and thus giving clearer bands and better photos with a clearer background.

3.3.5. Western Blot

The other set of gels were transferred to a nitrocellulose membrane by Western Blot. The gels from the SDS-PAGE were released from their cover, the stacking layer removed and the gels placed between filter papers and a nitrocellulose membrane. The filter paper, the nitrocellulose membrane (6x8cm), and gel were soaked in 10% methanol in Towbin buffer before being stacked together into a sandwich. The sandwich composition is; 3 filter paper on the bottom, 1 nitrocellulose membrane, 1 gel, 3 filter papers. On top of the sandwich, a glass pipette is used to roll over the sandwich to press out the bubbles that could be hiding inside. When this is done the 10% methanol in Towbin buffer is poured over the sandwich to ensure it is wet enough. Before starting the transfer, the lid of the device was loaded with heavy objects. The transfer was performed with 400mA for 1 hour.

The membranes were blocked by incubating them in 5% milk in TTBS solution for 1 hour at room temperature, followed by washing for 3x5 minutes in TTBS at RT with shaking. Then it was incubated with primary antibody solution for 1.5 hour at RT with shaking. The membranes were washed again with TTBS for 3x5 minutes and incubated with secondary antibody for 2 hours at RT with shaking. Before photos could be developed the membranes were washed 2x5 minutes with TTBS and 1x5 minutes with TBS. In a dark room with red light the gels were placed in a solution with ECL1 and ECL2 solutions for 1 minute. These solutions were mixed together right before usage. Developer and fixer solution were all provided from Kodak. Afterwards the membranes were placed in a small sealable plastic bag together with 3 ml of TBS solution and stored at +4°C.

3.3.6. Large scale expression of mature POR A

A large scale expression was performed in order to isolate the mature POR A proteins. 1 µl of isolated mature POR A plasmid was added to 100 µl of BL21 culture and incubated on ice for 30 minutes and then heat shocked at 30 seconds in waterbath at 42°C. A preculture was made by adding this mixture to 9.7 ml of LB medium containing 100 µg/ml of ampicillin. This was incubated overnight with shaking at 37°C. Next day the 7.5 ml of the preculture was added to 400 ml of LB medium containing 100µg/ml ampicillin. The large scale culture was incubated at 37 °C until OD_{600} between 0.4-0.6 was achived, before 1mM IPTG was added and the incubation continued at 16 °C with shaking overnight. The cultures were divided in two centrifuge bottles and spun down at 7464g at 16 °C for 15 minutes. Supernatant was discarded. The pellets were stored at -20°C for 2 months before thawed and solubilized in 2x20 ml lysis buffer.

Followed by three freeze-thaw treatment where freezing period is 12 minutes and thawing period is 5 minutes. 0.05 mg/ml lysozyme was added to the mixture followed by incubation on ice for 1 hour and 40 minutes before sonication. Sonication was performed on ice with 30 % amplitude for 6x30 seconds.

3.3.7. Filtering, purification and desalting.

The culture was filtered afterwards with a 0.45 µm cellulose acetate membrane (VWR) and a sterile 20 ml syringe (BD Plastipak). The filtered solution was purified using metal chelate affinity chromatography where stationary phase is nickel resins that retain the histidine tagged (his-tagged) proteins. Imidazole competes with the nickel resins and causes the His-tagged proteins to be eluted. Column used was His-Trap HP Column (GE healthcare). Extract, flow through and the collected samples from buffer B and C was run on SDS-PAGE. Afterwards coomassive staining and western blot was done following the same procedure as described in sub chapters 3.3.3, 3.3.4 and 3.3.5.

The samples with the highest concentration of expressed protein was collected in an activated dialysis tube (pore size: 3kDa) and placed it in a 25 mM Hepes and 300 mM NaCl solution, pH 8.0 overnight. The pores in the dialysis tube allow the imidazole to diffuse out but keep molecules larger than 3kDa inside the tube.

3.3.8. Removal of His tag by TEV protease treatment

A mixture of 50 µl of TEV protease, 125 µl of 20X TEV buffer, 45 µl of 0.1M DTT, 4000 µl mature POR A extract and 180 µl of autoclaved dH_2O were mixed together in a falcon tube and left at 4 °C overnight. The low temperature is to prevent the protein of degradation.

3.3.9. Mass spectrometry analysis

Mass spectrometry, MS, is a method used to analyze proteins by sequencing the amino acid sequence. The protein sample is treated with trypsin (or similar enzymes) to cleave the protein on the carboxy-terminal side of arginine and lysine residues. This efficiently breaks up the protein into smaller peptides that are within the preferred mass range for sequencing and contains a basic residue at the carboxyl terminus of the peptide. After the protein sample is prepared it can be injected onto a microscale capillary high performance liquid chromatography (HPLC) column, or just be placed directly into the

needle. As it flows to the tip of the needle the liquid is vaporized and the peptides are ionized by the action of a strong electric potential (electrospray ionization). The peptide ions are guided and manipulated by electric fields into a mass analyzer. The ionized peptides are separated by mass according to charge (m/z) which is recorded in a mass spectrum. MS is sufficient for protein identification but not for complete protein characterization (29).

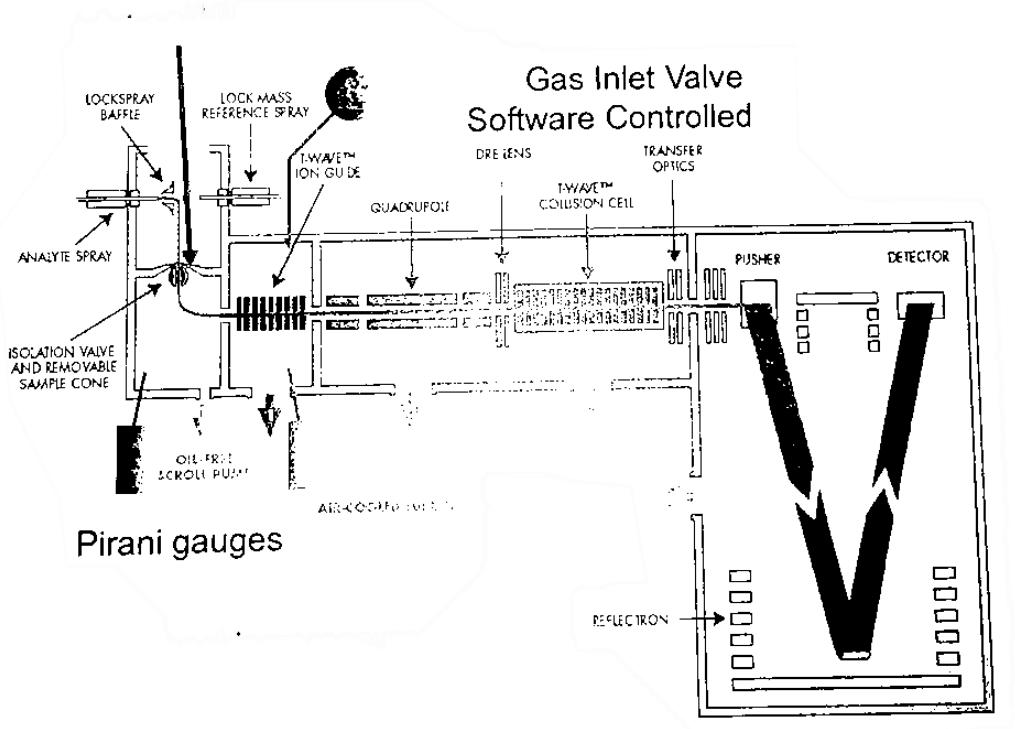


Figure 3-4. The setup of Waters Micromass Q-TOF Premier Mass Spectrometer. Picture is taken from the Q-tof premier touble shooting guide.

From a 12% SDS gel, bands from three collected samples of purified protein were cut out with a sterile scalpel and brought for MS analysis. The small gel fragment was put in a sterile eppendorf tube. The coommassive brilliant blue was removed by washing with a solution containing 25% acetonitrile and 25 mM NH4HCO3, before placing it on a clean glass plate to dry it. When dry it was transferred to an OMX-S tube according to the manufacturers protocol. The gel was incubated in 0.2 µg Trypsin for 1 hour and 15 minutes. A stage tip is activated with a solution of 80% acetonitrile, 5% formic acid and 15% water. 2x20µl of 5% formic acid is added. The peptide sample were desalted and concentrated on the 20µl StageTip C18-RP microcolumn (Thermo Fischer, Stockholm, Sweden) and were eluted in 2-4 µl of 65% CAN, 1% 2-propanol, 0.1% formic acid.

For electrospray ionization, peptides were loaded into borosilicate nano ES emitters (Proxeon, Stockholm, Sweden), and sprayed at 0.8-1.5 kV (ESI +) and a cone voltage of 40 V in a nano-ESI source. MS and MS/MS analysis of peptides was performed using a Waters Q-Tof Premier mass spectrometer (Waters Corporation, Milford, MA, USA). MS spectra were recorded between 400-2000 m/z for at least 30 s (1 s/scan). MS/MS spectra were acquired using argon at collision energies between 26 and 40 eV. De novo sequence analysis35 was performed using MassLynx/Biolynx 4.2 software and the b- and y-ion series of spectra interpreted manually. Mascot (http://www.matrixscience.com/search_form_select.html) (30) was used to run the MS/MS spectra against the database to find matching sequences. Sequences were obtained from 3 bands of one SDS-PAGE separation. The sequence coverage calculated as the ratio of the number of amino acids in identified peptides divided by the number of amino acids in the complete protein sequence.

3.4. Extraction of pigments and endogenous POR from *Hordeum Vulgare*

3.4.1. Prepapration of Barley plants

4.5 half days old barley plants grown in the dark were used to plastids. The plants were never exposed to light and therefore most of the plastids are etioplasts.

3.4.2. Isolation of plastids

The protocol for plastid isolation was described by Klein et al. (31) and modified by Eichacker et al (32,33). Isolation of plastids was performed in the darkness except for a faint green light source. The plants were cut off 2 cm from the root and homogenized in isolation medium with the use of an ultra thorax. The homogenate was filtrated using a pore size of 22µm, and then centrifuged at 4°C, 4100xg for 6 minutes. The supernatant was discarded and the pellet resuspended in the little bit of supernatant that was left. The resuspended pellet was carefully added to a percoll gradient and centrifuged for 8 minutes at 4100 g. From the layer between 80 % Percoll and 40 % Percoll, intact plastids could be pipetted out. The plastids were placed in a light sealed box. 2 µl of isolated plastids were diluted with 998 µl of washing buffer and counted in a Thoma counting chamber.

3.4.3. Separation of pigments and proteins from isolated plastids

The isolated plastids were added to 80 % of acetone and left overnight at -20°C. The following day the plastids were centrifuged at max speed for 15 minutes. The supernatant now contained pigments and

lipids. The supernatant was poured into a new eppendorf tube and centrifuged in a speed vacuum centrifuge (speed vac) to concentrate it. The pellet was left to dry in the fume hood until the acetone had evaporated. Both proteins and pigments were stored in the freezer until they were resuspended in 50 mM Hepes buffer according to the original concentration of plastids. The protein pellet was resuspended in 1XSDS LB. The proteins were run on a SDS gel to check the presence for POR proteins.

3.4.4. Establishing approximate concentration of endogenous POR and exogenous PORA with BSA standard on SDS gel

The purified mature PORA (AU2) was concentrated down to 100 µl to an unknown concentration. The amount of POR in the protein sample from the isolated plastids was also unknown. To establish the concentration of the concentrated PORA and the relationship between plastids and POR in barley etioplasts, they were run on SDS gel with a BSA as a standard.

BSA from Pierce® BCA Protein assay kit (Thermo Scientific) has a stock concentration of 2 mg/ml. 15 µl of BSA was mixed with 15 µl of 2XSDS LB in an eppendorf tube. 30 µl of concentrated PORA was mixed with 30 µl of 2XSDS LB in an eppendorf tube. The protein sample from isolated plastid was resuspended in 200 µl of 1XSDS LB (to its original volume). They eppendorf tubes were boiled at 94°C for 5 minutes before loaded on the 12% SDS gel. Following amounts were loaded onto the gel:

- BSA: 1 ng, 2 ng, 5 ng, 10 ng
- Concentrated PORA + 2XSDS LB: 1µl, 2µl, 5µl, 10µl. Half of the volume is the concentrated PORA.
- Protein sample + 2XSDS LB: 1µl, 2µl, 5µl, 10µl. Half of the volume is the protein sample.

They were run on SDS PAGE with SDS buffer as anode and cathode buffer at 15mA/gel for 1 hour and 15 minutes. Afterward followed coomassive staining for 45 minutes and destaining of the CBB according to the method described in sub chapter 3.3.4.

The amount of PORA and POR from protein sample was determined by finding a band matching one of the bands of the BSA standard.

3.5. Reconstitution

A specialized cuvette (engineered by professor Lutz Eichacker to measure low volumes of maximum 50µl) was used for the reconstitution part. UV-2401 PC UV-Vis recording spectrophotometer (Shimadzu)

with was used to measure the samples by absorbance spectrophotometry. The program Hyper UV (Shimadzu) was used to interpret the results.

A total volume of 50µl was used for all the spectrophotometric measurements. NADPH concentration was either 2µM or 6mM. Amount of pigments for each experiment was $1x10^7$. Glycerol content was either 0 or 10%. Volume of concentrated PORA was either 3.7µl or 44µl, or 44µl from an unconcentrated stock. 50mM Hepes solution was added up to 50µl.

Reference solution was either 50mM Hepes with 10% glycerol, or only 50mM Hepes, or 25mM Hepes with 300mM NaCl. Reference solution was selected according to which sample was measured.

First the reference was pipetted into two cuvettes, one for sample and one for reference cuvette. A baseline was first made from either 200-750 nm, or from 600-700nm. The spectrophotometer was auto zeroed before making the measurement which were named ‘baseline’. Then the reagents were mixed together, the volume pipetted into the sample cuvette and inserted into the spectrophotometer. Before every measurement the spectrophotometer had to be auto zeroed.

The absorbance spectra from the samples were copied from the program and pasted into another program (e.g. Microsoft Excel) where the data could be organized and analyzed into graphs.

4. Results

4.1. Purification of template-containing vectors of PORA and PORB

Templates of *PORA* and *PORB* from *Arabidopsis thaliana* were ordered from The Arabidopsis Information Resource (Ohio, United States of America) (34). They were received in a pUNI51 and pENTR/SD/D-TOPO vector inside host strain *E.coli* PIR1 and *E.coli* Top-10 (Table 4-1).

The host strains were spread on an LB-plate with kanamycin 50 µg/l and left for incubation at 37°C overnight. Following day a colony from each clone were added to 10 ml of LB-medium with 50 mg/l kanamycin and grown overnight at 37°C with shaking at 250rpm. The vectors were retrieved by plasmid isolation according to the manual from QIAprep® Miniprep Handbook (details about the kit in sub chapter 2.3). The concentration of the purified plasmids was measured with NanoDrop spectrophotometer (Table 4-1). Concentration for PORA is 259.5 ng/µl and for 220.6 ng/µl for PORB.

Table 4-1. Clone number and vector type for PORA and PORB templates. Concentration and absorbance 260/280 are from the purified vectors.

DNA template	Clone number	Vector	Host strain	Concentration, ng/µl	ABS 260/280
PORA	U20856	pUNI51	*E.coli* POR1	259.5	1.98
PORB	U15710	pENTR/SD/D-TOPO	*E.coli* Top-10	220.6	1.97

4.2. Checking plasmid quality with agarose gel electrophoresis

50 ng of the purified plasmids were run on an agarose gel to check the quality(Figure 4-1). PORB displays one strong band and two weaker bands. The strong lower band is a plasmid in the supercoiled form, and the two weaker bands are linearized form (middle band) and open circular form (upper band).

PORA shows a weak lower band (supercoiled form) and a strong upper band (open circular form) but some of the plasmid is also in linearized form (middle band).

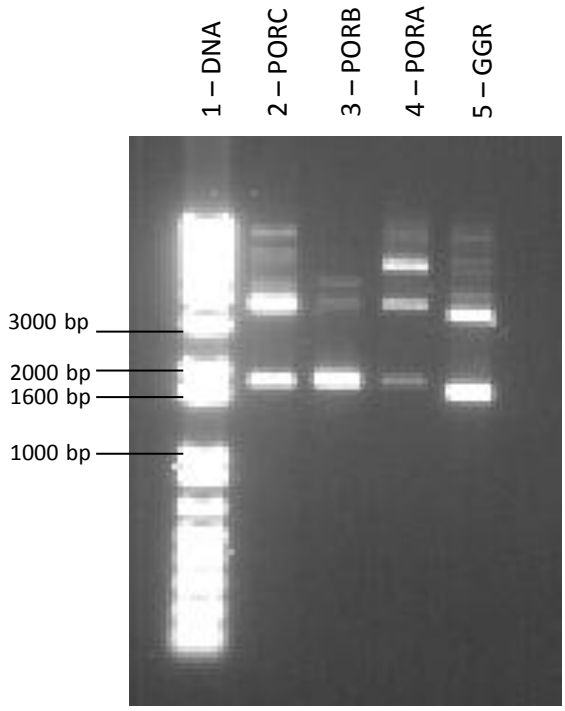


Figure 4-1. Agarose gel electrophoresis of the purified plasmids PORA (lane 4) and PORB (lane 3). The plasmids were run together with purified plasmids of PORC (lane 2) and geranyl geranyl reductase (GGR) (lane 5).

4.3. PCR products of *PORA* and *PORB*

With primers from Table 3-1 and reagent composition from Table 3-2, PCR products were synthesized by running the temperature cycle from Table 3-3. 2.5µl of each PCR product was run on an agarose gel to verify the expected size of the products. Expected size according to its product is; AM: 1218bp, AU: 1065bp, AL: 1008, BM: 1210bp and BU: 1081bp. These sizes do not include the CACC which is a requisite to have before the cloned sequence when expressing it in the pET151 vector.

Figure 4-2 shows the PCR product sizes of AU, AM, BU and BM. AM and BM are a little bit higher than their smaller products; AU and BU, just like they are supposed to. The bands are clear and within the size they are expected to have. PCR product of AL is also within the expected size (Figure 4-2).

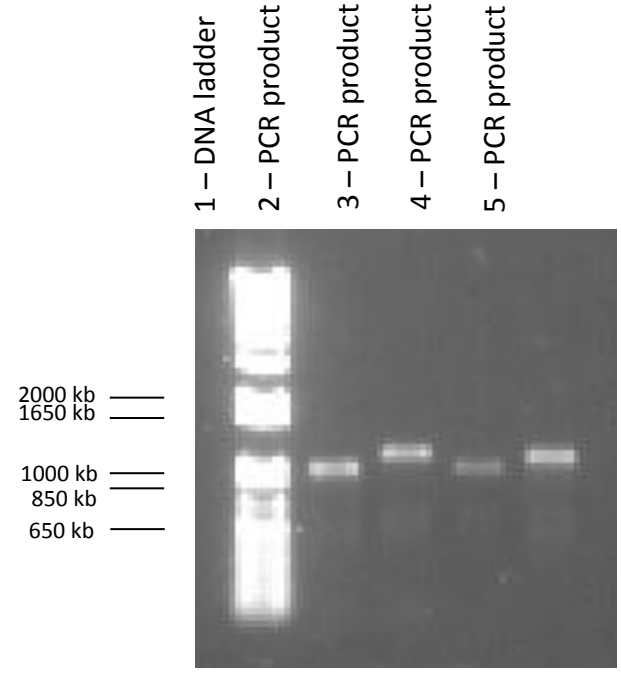


Figure 4-2. PCR products of AU, AM, BU and BM.

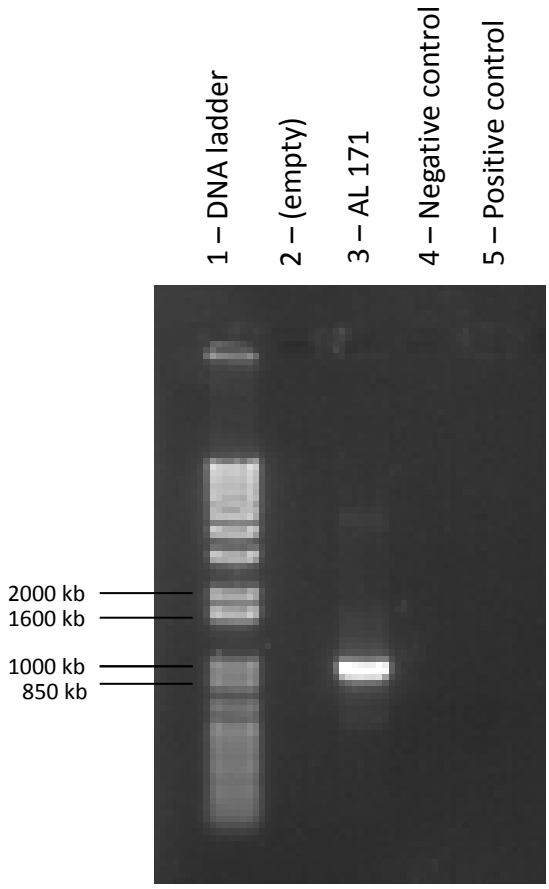


Figure 4-3. PCR product of mature PORA sequence based on where the cleavage site is predicted after the publication by Lutz et al

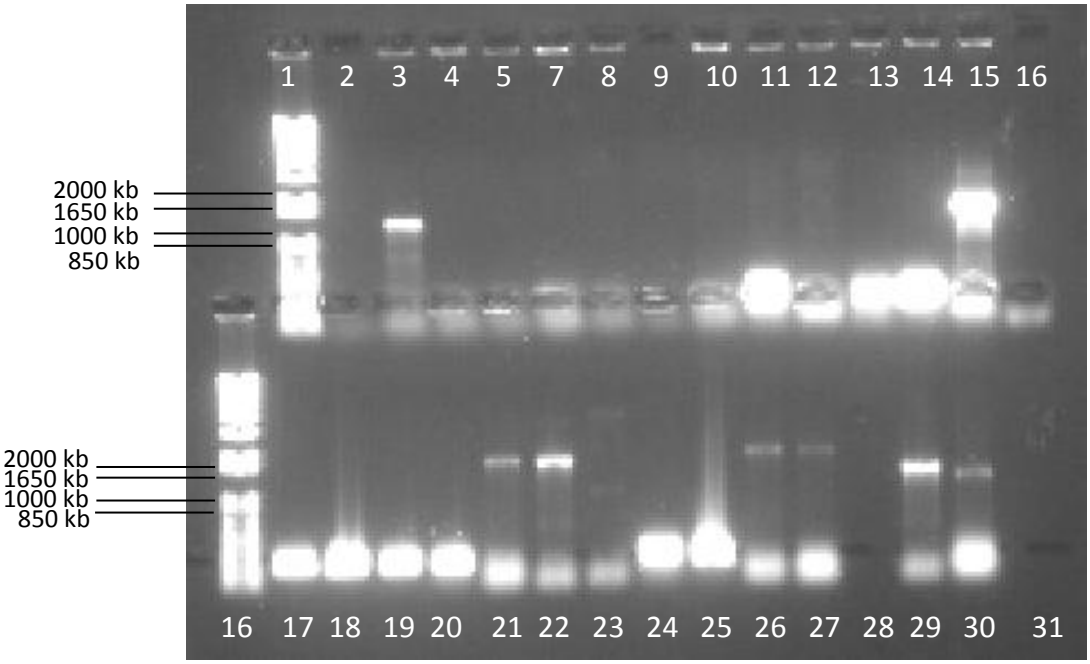
The PCR products were purified by running them on an agarose gel to separate the DNA fragments followed by DNA extraction using the QIAquick Gel extraction kit from Qiagen. The concentration was measured with NanoDrop. PCR products were cloned into a pET151 vector followed by transformation into a TOP10 host and the transformed TOP10 cells were plated on a LB plate with 100µg/ml ampicillin and incubated at 37^{o}C overnight. Cloning and transformation were performed according to the method described in subchapter 3.2.6. PCR.

PCR amplification was done 7 times over a period of 4 months. The first product from the first PCR amplification resulted in a cloned *PORA* product that was correct when sequenced. What differed from the protocol that time is that VENT polymerase was used, and the transformed TOP10 cells was incubated at 4^{o}C overnight before spread on a plate. The PCR amplifications performed afterwards was done with Taq polymerase which does not produce blunt end products. After that was discovered VENT polymerase was used again. The last times PCR products was produced they showed abnormal sizes when run on an agarose gel, or no bands at all. The reason for this was not detected.

4.4. Checking the transformed TOP10 cells with PCR screening

To check that the transformed TOP10 cells contained a pET151 vector with insert, the colonies of transformed cells were PCR screened. Each colony was streaked on a new LB plate with 100µg/ml ampicillin and amplified using GoTaq polymerase according to the method in sub chapter 3.2.7. Products from the PCR screened colonies were run on an agarose gel to see which of the colonies were positive transformed. If they were negative they would have a low band about 160bp. If they were positive they would have a band corresponding to their cloned product size plus 160 bp.

133 colonies were PCR screened. 12 of these were positive.



Well content:

1 – DNA ladder	16 – DNA ladder
2 – AU1	17 – BU1
3 – AU2	18 – BU2
4 – AU3	19 – BU3
5 – AU4	20 – BU4
6 – AU5	21 – BU5
7 – AU6	22 – BU7
8 – AU7	23 – BM1
9 – AU8	24 – BM2
10 – AU9	25 – BM3
11 – AM1	26 – BM4
12 – AM2	27 – BM5
13 – AM3	28 – BM6
14 – AM4	29 – BM7
15 – Control	

Figure 4-4. PCR transformation of AU, AM, BU and BM.

4.5. Sequencing the positive transformants

To confirm that the cloned pET151 vector contained an insert with a correct sequence, it was grown in LB medium containing 100µg/ml ampicillin and incubated overnight with shaking at 250rpm and 37^{o}C. The vectors were retrieved with QIAprep® spin miniprep kit. The retrieved vectors had their inserts checked by using the T7-promotor specific primers; T7, T7-rev, T7-50, T7-rev-50, and reagent list from

Table 3-6. PCR sequencing temperature cycle from Table 3-7 was followed. The method was performed as described in subchapter 3.2.9. The sequenced vectors were received as chromatograms. They were as analyzed described in subchapter 3.2.10 and the results dislplayed in a table.

Table 4-2 Sequence results from of the cloned POR A mature gene. The boxed content from forward and reverse primer is the part that is 100% identical to the sequence of PORA. Together they cover the whole sequence of the desired mature PORA product. The figure at the bottom of the table shows where and how much the two sequence products cover the whole sequence.

Forward primer. 17.11.11, using the T7 primer, 780 correct nucleotides
TGGGGGGTCTTTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCATCATCACCATCACCATGGTAAGCCTATCCCTAAC CCTCTCCTCGGTCTCGATTCTACGGAAAACCTGTATTTTCAGGGAATTGATCCCTTCACCTGCAAGAGGGAACAGAGCTTGAGGAATAATAAA GCGATTATTCGAGCTCAAGCAATCGCGACTTCAACTCCATCAGTCACAAAATCTTCCTTAGACCGCAAGAAAACACTTAGAAAAGGAAACGTG GTTGTCACGGGAGCTTCTTCAGGGCTAGGTTTAGCAACGGCAAAGGCATTAGCCGAGACAGGTAAATGGCACGTGATAATGGCGTGCAGAG ACTTCCTCAAGGCTGAGAGAGCCGCTCAATCTGCAGGGATGCCTAAGGACAGCTACACTGTGATGCATTTGGACTTGGCGTCTTTGGACAGC GTGAGGCAGTTTGTTGATAACTTCAGGCGAGCTGAGATGCCTCTCGATGTGTTGGTCTGCAATGCCGCAGTCTATCAGCCAACGGCTAATCAA CCTACTTTCACTGCTGAAGGGTTTGAGCTTAGCGTTGGGATAAACCATTTGGGCCACTTTCTTCTTTCAAGATTGTTGATTGATGACTTGAAGA ACTCCGATTATCCATCAAAACGTCTCATCATTGTTGGATCCATAACCGGAAACACTAATACATTGGCTGGTAATGTACCTCCAAAGGCTAATCT TGGTGATTTGAGGGGACTAGCAGGCGGGTTGAACGGGCTAAACAGCTCGGCGATGATAGATGGAGGAGATTTTGTTGGCGCAAAGGCGTAT AAAGATAGCAAAGTCTGCAACATGTTGACAATGCAGGAGTTTCATAGGCGTTTCCATGAAGACACCGGCATAACTTTTGCTTCCCTTTACCCCG GGTTGTATTGCAACGACTGGTTTGTTCAGAGAGCATATTCCTCTTTTCCGTACCTCTTCCCTCCTTTCCAGAAGTACATCAACAAAAGGTTACGT CTCGAGTCAGAGGCTGGGAAAAGACTGCTCAGTGGTTGGCTGAATCCAAGGCTTGACGAGTCGGGAGTGTTATTTGGGAAGCTTGGACCAG GACTTCGCCTCCATTTGAGAATCCAGCTTGCTTCTTCAGAGCCTTAGCGGAT
T7-50 reverse primer, 727 correct nucleotides
CGGGGGGAATTTGATTCCCCTTTCACCCTTGCCAAAAGAGGAAACAGGAGCTTGAGGGAAATAATTAAAGGCGAATTATTCCGAGTTTCAAA GGCAATTCGCCGACCTTCAACTCCCATTCAGTTCACAAAATTTCTTTCCTTAAGACCGCCAAGAAAAACACTTAGAAAAGGAAACGTGGTTTTTC ACCGGGAGCTTCTTCAGGGCTAGGGTTTAGCAACGGCAAAGGCATTAGCCCGAGACAGGTAAATGGCACGTGATAATGGCGTGCAGAGACT TCCTCAAGGCTGAGAGAGCCGCTCAATCTGCAGGGGATGCCTAAGGACAGCTACACTGTGATGCATTTGGACTTGGCGTCTTTGGACAGCGT GAGGCAGTTTGTTGATAACTTCAGGCGAGCTGAGATGCCTCTCGATGTGTTGGTCTGCAATGCCGCAGTCTATCAGCCAACGGCTAATCAACC TACTTTCACTGCTGAAGGGTTTGAGCTTAGCGTTGGGATAAACCATTTGGGCCACTTTCTTCTTTCAAGATTGTTGATTGATGACTTGAAGAAC TCCGATTATCCATCAAAACGTCTCATCATTGTTGGATCCATAACCGGAAACACTAATACATTGGCTGGTAATGTACCTCCAAAGGCTAATCTTG GTGATTTGAGGGGACTAGCAGGCGGGTTGAACGGGCTAAACAGCTCGGCGATGATAGATGGAGGAGATTTTGTTGGCGCAAAGGCGTATAA AGATAGCAAAGTCTGCAACATGTTGACAATGCAGGAGTTTCATAGGCGTTTCCATGAAGACACCGGCATAACTTTTGCTTCCCTTTACCCCGGT TGTATTGCAACGACTGGTTTGTTCAGAGAGCATATTCCTCTTTTCCGTACCCTCTTCCCTCCTTTCCAGAAGTACATCACAAAAGGTTACGTCTCC GAGTCAGAGGCTGGGAAAAGACTTGCTCAGGTGGTGGCTGATCCAAGCTTGACGAAGTCGGGAGTGTATTGGAGCTGGAACAAGACCTCGG CTTCATTTGAGAATCAGCTGTCTCAAGAAGCTAGCGATGTCGAGAAGGCTCGTAGAGTTTGGGAAGTCAGCGAGAAGCTCGTAGGCTTGGCC TAAAAGGGCGAGCTCAGATCCGGCTGCTAACTTACCCCAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTT GGGGCCTCTAAAACCTCA

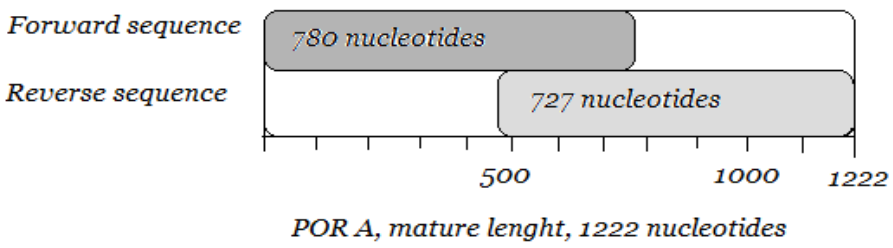


Figure 4-5. The coverage of PORA from the sequencing results in Table 4-2.

Only one clone had the correct sequence, which was mature PORA, AU (in colony nr 2 of transformed TOP10 cells (AU2)). The sequence results from AU2 are given in Table 4-2. The sequences have a length of 780 and 727 correct bases. For AU2 these two sequences overlap each other with a good margin (Figure 4-5).

Full length PORA (AM) had the whole sequence right except for one base. This base substitution would have resulted in a different amino acid when expressed as a protein and could not be used further. PORB did results in some positive transformants in the PCR screening process. However, when they were sequenced and the sequences run through the BLAST database (http://blast.ncbi.nlm.nih.gov/) they did not contain anything from the PORB sequence.

4.6. Expression of mature PORA studied by small scale culture followed by coomassive staining and western blot

AU2 is a positive transformant with the correct sequence. It was expressed in a small scale to see how much is expressed. Controls used were plasmid 1 (protein Lil3-2 - full length), 4 (protein Lil3-2 - mature), empty pET15. These were transformed into BL21 cells and grown on plates. A colony from each plate was cultured in LB-medium with 100µg/ml ampicillin at 37^{o}C overnight with shaking at 250rpm. The plasmids were retrieved and purified by using the QIAprep® spin miniprep kit (Qiagen). The small scale expression was performed as described in sub chapters 3.3.1.

The cultures were harvested by lysozyme treatment, freeze- thawing and sonication (sub chapter 3.3.2). Supernatant and pellet were run on SDS PAGE (sub chapter 3.3.3). The gels were stained by commassive staining and western blotted (sub chapter 3.3.4 and 3.3.5). Western blot was performed with an anti-his

antibody because the expressed proteins from pET151 contain a his-tag. The blot showed that the cloned protein was produced in rich amounts. The coomassive staining showed the presence of the protein which has the expected size of 40kDa (including the his-tag).

4.7. Production of mature PORA by large scale culture and protein purification

Small scale culture showed good expression of mature PORA. A large scale culture was produced in order to produce a larger amount of protein and purify it. The pET151 plasmid containing cloned mature PORA was transformed into a culture of BL21 competent cells. A preculture was made by adding the transformed BL21 cells into LB-medium with 100µg/ml ampicillin and incubated overnight at 37^{o}C and shaking at 250rpm. The large scale culture was made by transferring the preculture to 400 ml of LB medium with 100µg/ml ampicillin. The large scale was incubated at 37^{o}C with shaking at 250 rpm until a OD_{600} value of 0.4 was reached. Then 1mM IPTG was added and the large scale culture incubated overnight at 16^{o}C with shaking at 250rpm. The cell membranes were destroyed by lyzosyme treatment, freeze-thawing and sonication.

Proteins from the cell extract were purified by filtering through a 0.45µm membrane and purified with metal chelate affinity chromatography using a his-tag column. The his-tagged protein was eluted with a high concentration of imidazole. Extract, flow through and collected samples were run on SDS PAGE and stained with coomasive staining (Figure 4-6). The top band of samples B1-B10 and C1-C10 is the expressed protein, mature PORA, which is expected to have a size of 40 kDa. This band is missing in the flow through which means that the protein is bound to the column.

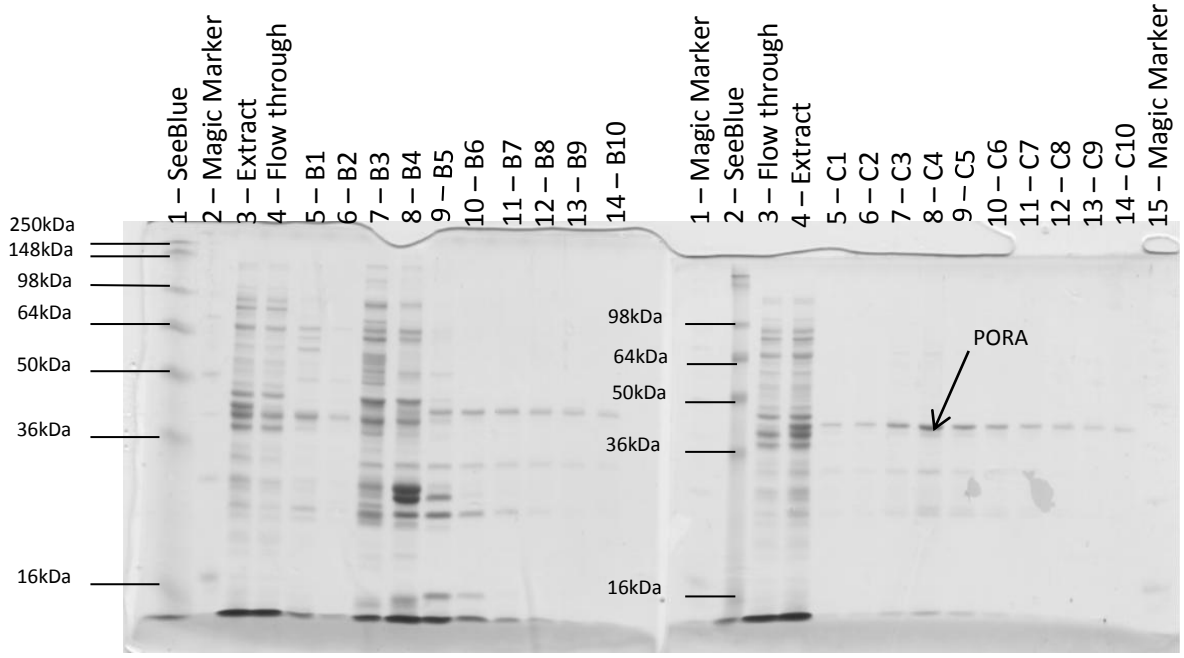


Figure 4-6. Coomassive staining of purified PORA fractions after reverse phase chromatography. SeeBlue and Magic Marker are used as protein markers.

Samples C3, C4, C5 and C6 which had the highest amount of mature PORA protein was collected in a dialysis tube. The tube was put in solution of 25mM Hepes and 300mM NaCl and desalted overnight at 4^{o}C with stirring.

4.8. Removal off the histidine tag (his-tag)

After being desalted the proteins were supposed to be cleaved by TEV protease as described in sub chapter 3.3.8. However this was not successful in this experiment. After TEV treatment the sample protein purified using the his-tag column followed by SDS PAGE of the sample and coomassive staining. This showed that the histidine tag was still in place because the protein was eluted in the buffer with high imidazole concentration, instead of the one with low concentration. Other members of the lab reported the same problem with TEV and it was decided to continue without cutting off the histidine tag. Out of the 4 ml from C3, C4, C5 and C6 that were desalted, 2ml was concentrated down to 100 µl by spinning it in two 10K concentration tubes for 25 minutes at 10 000rpm. The other 2 ml were kept unconcentrated. The stock of PORA were stored at 4^{o}C.

4.9. Confirming correct amino acid sequence by Mass Spectrometry (MS) analysis

MS analysis was performed according to the described method in sub chapter 3.3.9. The bands were cut out of a SDS gel with samples from the protein purification (Figure 4-6). The upper band from C3, C4 and C5 were cut out and prepared for MS. The results from MS was inserted into a table (Table 4-3) and the sequence coverage calculated as the ratio of the number of amino acids in identified peptides divided by the number of amino acids in the complete protein sequence.

The sequence coverage for the three bands (C3, C4 and C5) were 13%, 11% and 9%. One sequenced peptide produced a score of 199%, which is very high.

Table 4-3. MS results of three samples of the expressed PORA product.

Identified proteins	Gene	Accession number (UniProt)	Detected signals (*m/z*, charge state)	*De novo* sequenced peptides	Score, %	Sequence coverage (%)
Protochlorophyllide reductase A CKREQSLRNNKAIIRAQAIATSTPSVTKSSLDRKKTLRKGNVVVTGASS GLGLATAKALAETGKWHVIMACRDFLKAERAAQSAGMPKDSYTVM HLDLASLDSVRQFVDNFRRAEMPLDVLVCNAAVYQPTANQPTFTAE GFELSVGINHLGHFLLSRLLIDDLKNSDYPSKRLIIVGSITGNTNTLAGN VPPKANLGDLRGLAGGLNGLNSSAMIDGGDFVGAKAYKDSKVCNM LTMQEFHRRFHEDTGITFASLYPGCIATTGLFREHIPLFRTLFPPFQKYIT KGYVSESEAGKRLAQVVADPSLTKSGVYWSWNKTSASFENQLSQEA SDVEKARRVWEVSEKLVGLA	*PORA*	Q42536	801.90 $[M+2H]^{2+}$ 985.40 $[M+2H]^{2+}$ 1118.90 $[M+2H]^{2+}$	VVTGASSGLGLATAK TSASFENQLSQEASDVE K GGLNGLNSSAMIDG	35 86 75	13%
Protochlorophyllide reductase A CKREQSLRNNKAIIRAQAIATSTPSVTKSSLDRKKTLRKGNVVVTGASS GLGLATAKALAETGKWHVIMACRDFLKAERAAQSAGMPKDSYTVM HLDLASLDSVRQFVDNFRRAEMPLDVLVCNAAVYQPTANQPTFTAE GFELSVGINHLGHFLLSRLLIDDLKNSDYPSKRLIIVGSITGNTNTLAGN VPPKANLGDLRGLAGGLNGLNSSAMIDGGDFVGAKAYKDSKVCNM LTMQEFHRRFHEDTGITFASLYPGCIATTGLFREHIPLFRTLFPPFQKYIT KGYVSESEAGKRLAQVVADPSLTKSGVYWSWNKTSASFENQLSQEAS DVEKARRVWEVSEKLVGLA	*PORA*	Q42536	969.49 $[M+2H]^{2+}$ 1119.07 $[M+2H]^{2+}$	DSYTVMHLDLASLDSVR GLAGGLNGLNSSAMIDG GDFVGAK	111 199	11%
Protochlorophyllide reductase A CKREQSLRNNKAIIRAQAIATSTPSVTKSSLDRKKTLRKGNVVVTGASS GLGLATAKALAETGKWHVIMACRDFLKAERAAQSAGMPKDSYTVM HLDLASLDSVRQFVDNFRRAEMPLDVLVCNAAVYQPTANQPTFTAE GFELSVGINHLGHFLLSRLLIDDLKNSDYPSKRLIIVGSITGNTNTLAGN VPPKANLGDLRGLAGGLNGLNSSAMIDGGDFVGAKAYKDSKVCNML TMQEFHRRFHEDTGITFASLYPGCIATTGLFREHIPLFRTLFPPFQKYIT KGYVSESEAGKRLAQVVADPSLTKSGVYWSWNKTSASFENQLSQEAS DVEKARRVWEVSEKLVGLA	*PORA*	Q42536	637.30 $[M+2H]^{2+}$ 801.50 $[M+2H]^{2+}$	AQAIATSTPSVTK GNVVVTGASSGLGLATAK	33 66	9 %

4.10. Plastid extraction

Etioplasts were isolated from 4.5 days dark grown barleys (*Hordeum vulgare)* according to the method in sub chapter 3.4.2. The isolation took place in darkness except for a green light source. 400µl of isolated etioplasts to a concentration of 5.4 x 10^5 etioplasts/µl were extracted. The etioplasts were treated with acetone according to the method (sub chapter 3.4.3) to separate the proteins from the pigments. Proteins sediments were left in the fume hood with the lid open to evaporate the acetone. The pigments were divided into aliquots of 1x10^7 plastids.

4.11. BSA standard curve to determine POR content in etioplasts and in the expressed mature PORA protein

1, 2, 5 and 10 ng of BSA together were run on a SDS gel with SDS PAGE to make a standard. 1, 2, 5 and 10 µl of expressed mature PORA protein was run on the same gel. 1, 2, 5, 10 and 20 µl of protein from the etioplast extract was run on separate SDS gel with SDS PAGE (Figure 4-7).

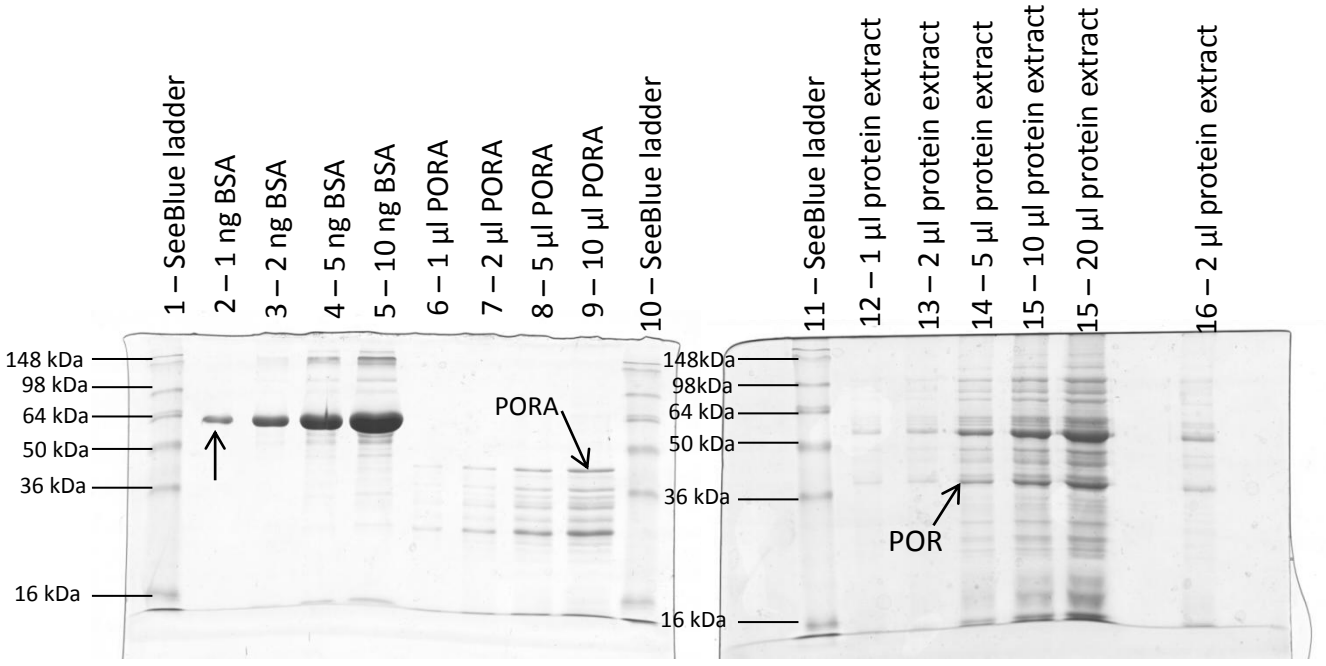


Figure 4-7. BSA standard run on SDS gels as together with the expressed PORA and extracted proteins from the plastid isolation. The purpose was to estimate the amount of expressed POR and POR in the protein extract in order to find the

relationship between POR and pigments in etioplasts, and use that relationship in the reconstitution part. The arrows point out the bands look the most alike, but also indicates the band that would be POR (from etioplast) and PORA that has a molecular weight corresponding to that protein. In lane 14 the 2 µl were not pipetted successfully, this was corrected for in lane 16.

Calculation of POR in barley etioplast and PORA:

- 1 ng BSA = 5 µl PORA (expressed) = 2.5 µl POR in etioplast extract
- POR in etioplast extract: 1ng / 5 µl = 0.2ng POR/µl
- PORA (expressed): 1ng /5µl = 0.2ng PORA/µl

POR:pigment ratio in etioplast extracted etioplasts:

- 0.2 ng POR/µl = $5.4x10^5$ pigments/ µl

Calculations of the amount PORA needed for $1x10^7$ plastids:

- $1x10^8$ plastids/ $5.4x10^5$ pigments/ µl x 0.2ng POR/ µl = 3.7 ng
- 37 ng POR / 0.2ng PORA/ µl = 18.5 µl of PORA

4.12. Reconstitution

The method from sub chapter 3.5 was followed when performing the reconstitution. With 25mM Hepes and 300mM NaCl as a reference, a spectra was made for 50µl of PORA of unknown concentration (Figure 4-8 A). The stock of mature PORA was vortexed well before being measured. A peak at 273nm had an absorbance of 0.022. This peak shows that the mature PORA is present in the sample. The 50 µl of the measured mature PORA was transferred into $1x10^7$ etioplasts, mixed well together before measuring the absorbance (Figure 4-8 B). The small peak at 634nm corresponds to pchlide with an absorbance of about 0.05. 3mM NADPH was added to the mature PORA with etioplasts and measured (Figure 4-8 C). The NADPH gave a clear peak between 300 and 400nm.

The mixture of mature PORA, pchlide and NADPH were illuminated for 5 seconds followed by a measurement. Then it was illuminated again and the absorbance measured fFigure 4-10). No change in the absorbance spectra after illumination was seen. A decrease of the peak at 634nm and an increase of a peak around 670 were expected.

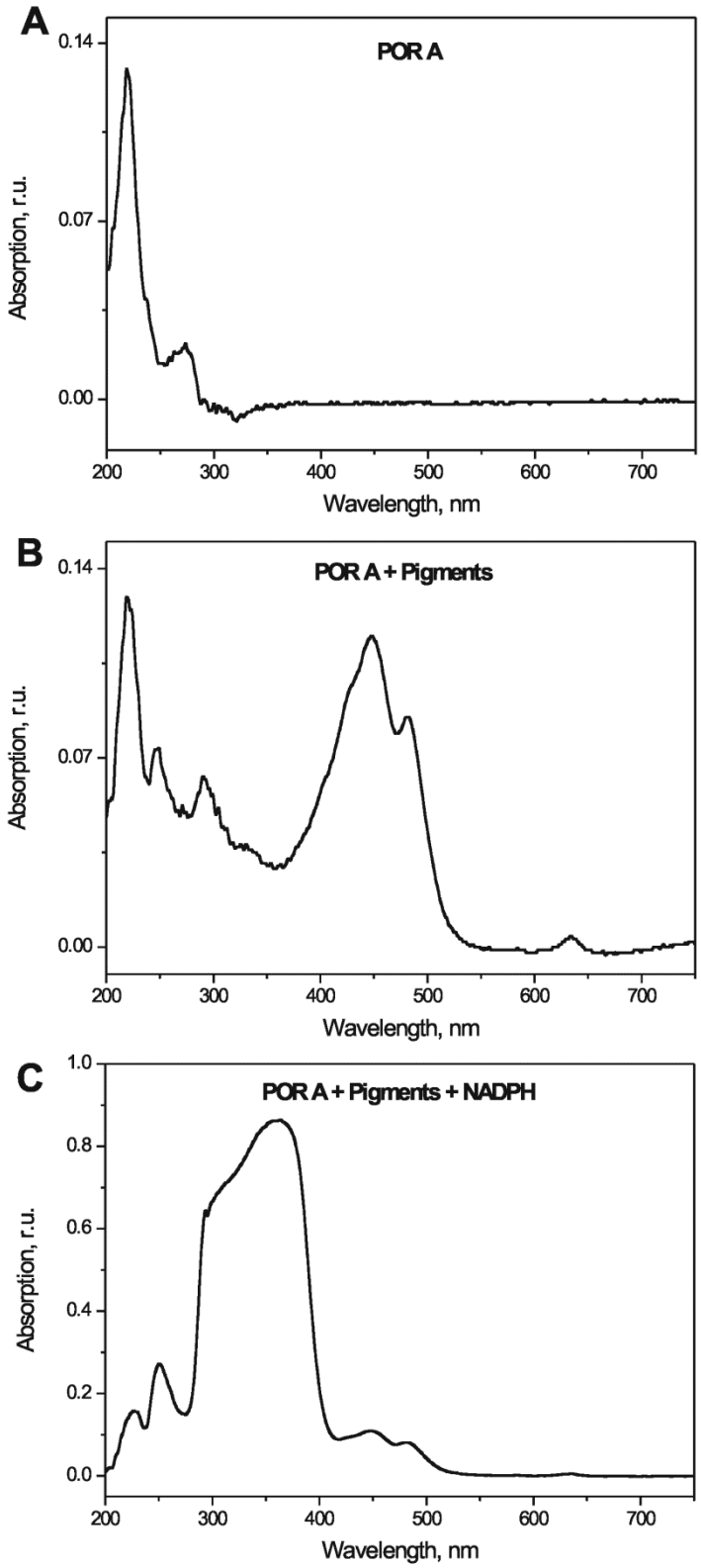


Figure 4-8. Absorption spectra of PORA, POR together with pigments, and PORA, pigments NADPH.

A) The peak at 273 nm with an absorption of 0.022 corresponds to the distinctive peak of PORA. This shows that the PORA is present in the protein extract.

B) The peak at 634 indicates Pchlide. The amount corresponds to 1x10^{8} plastids solubilized in 50 µl of PORA extract of an unknown concentration

C) Adding NADPH to gives the characteristic peak between 300 and 400. The peak is very high because of the high concentration 3mM.

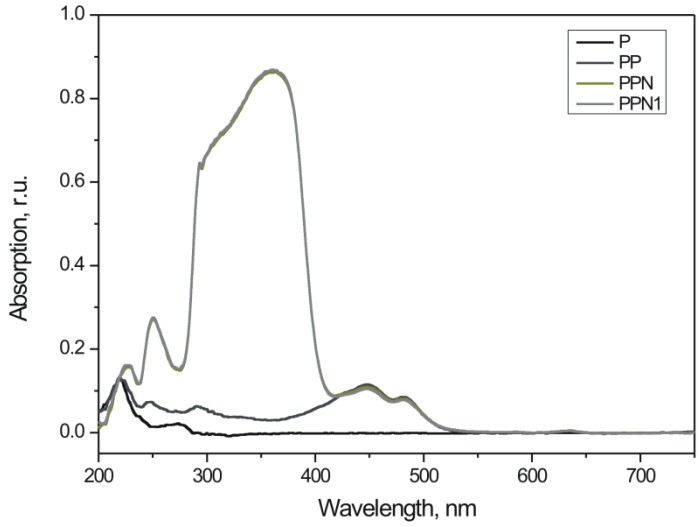


Figure 4-9. Adding the three individual spectra from figure 4-8 produces this spectra.

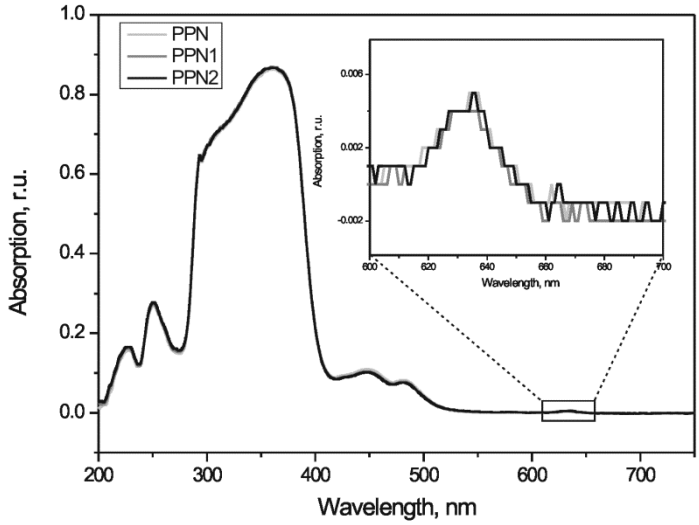


Figure 4-10. After PORA, pigments and NADPH was mixed together the sample was illuminated twice. The illumination did not change any part of the spectra, nor the band at 634 nm which indicates the substrate, pchlide. It was excpected that a band around 670-680 would arise as well as the decrease of the 634 peak, which would have meant that pchlide was converted to chlide. PPN stands for PORA, pigment and NADPH. The following number says how many illuminations the sample has been given.

A solution of 25mM Hepes, 300mM NaCl and 10% glycerol was used as a reference. 8.8ng of mature PORA, $1x10^7$ etioplasts, 3mM NADPH and 10% glycerol was mixed well together and the absorbance

measured between 600 to 700nm. The peak at 634nm had an absorbance of 0.04 showing the presence of pchlide. The mixture of mature PORA, pigments and NADPH was illuminated for 5 seconds and measured again. This was repeated 10 times before it was pipetted out and into a eppendorf tube and put on ice overnight. Incubation took place in the dark. The next the day mixture was pipetted out and measured again. It was illuminated for 5 seconds and measured. This was repeated 3 times (Figure 4-11). It was expected that the peak at 634 would decrease and a peak around 670 would increase. This would indicate that pchlide was transformed into chlide. This did not happen.

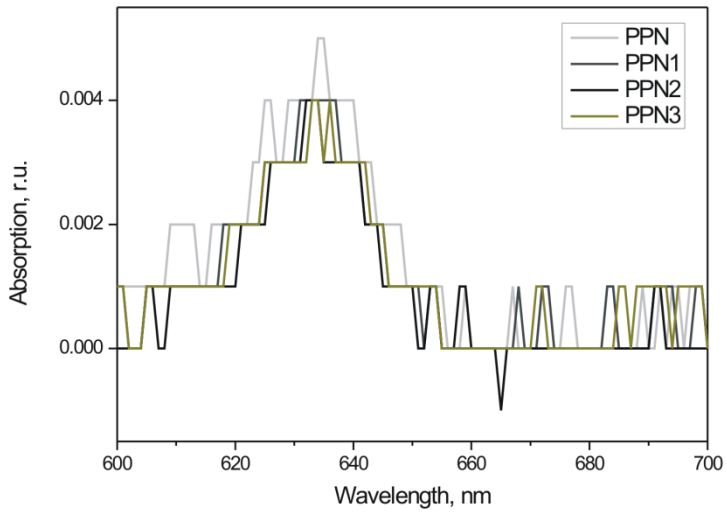


Figure 4-11. *A sample with PORA, pigments and NADPH were mixed together, illuminated 5 times and then incubated on ice overnight. The day after absorption spectra was taken as well as illuminations to see if the reaction would occur after the incubation. PPN is pigments, PORA and NADPH. PPN1 is when PPN has been illuminated 1 time after the incubation, PPN2 when the sample has been illuminated two times after the incubation, and PPN3 three illuminations.*

5. Discussion

5.1. Cloning

The initial goal was to clone both *PORA* and *PORB*. The desired constructs were to be of mature length (without signal sequence) and full length (with signal sequence). Proteins synthesized in the cell sometimes contain a signal sequence to make sure it is transported to the right destination. When the protein arrives to its destination the signal sequence is cleaved off, sometimes this is necessary to make the protein active. PORA and POR contain a signal sequence for the chloroplast, often called a transit peptide. However the length of this is unknown and it has to be predicted somehow. This was done in two ways in this experiment. A commonly used prediction server, ChloroP, was used to predict a theoretical cleavage site. The other mature sequences were predicted according to the chemical predicted cleavage site for POR A and POR B in barley (1). These two methods gave different lengths of signal sequence. A wrongly predicted cleavage site can in the worst case scenario lead to loss of important structure and functionality. Therefore the two different mature lengths were produced in order to see if they would give different results in the reconstitution experiment.

The PCR amplification went well to start with. In the first round of PCR amplification VENT polymerase was used. The stock of VENT polymerase ran out so Taq polymerase was used instead. But Taq polymerase produces a PCR product with a 3' overhang and this was a problem because blunt end products are needed in order to clone into pET151. VENT polymerase was used again for the rest of the PCR amplification experiments. Even after all the PCR amplifications, clonings and transformations, in the end only one *PORA* was successfully cloned. Parameters regarding salt concentrations and temperature in the PCR temperature cycle was optimized, the volume of PCR products and vectors were changed. The first round of PCR amplification produced PCR products which had nice bands on the agarose gel, showing that DNA products of correct length were produced. This also confirms that the primers were working. In the last rounds of making PCR products there were produced unexpected bands when running the PCR products on agarose gels. It was suspected that it might be the PCR machine that did not work well, because of its troubles in the past.

Even when VENT polymerase was used, there was a problem with a low number of transformed colonies. Either it is something wrong with the pET151 vector batch that makes it difficult to clone into or there was a failure when transforming into TOP10 competent *E.coli* cells. Cloning in general is unpredictable and it can be very time consuming.

The only successful cloned product was the mature *PORA* (ChloroP predicted signal sequence), named AU2 after its number when PCR screened. There was another clone that was almost correct (for full sequence of *PORA, AM4),* being PCR amplified and cloned at the same time as AU2. AU2 were sequenced to check if the sequence was correct. The sequence results (Figure 4-2) confirmed that it was 100% identical to the sequence of *PORA* from *Arabidopsis Thaliana*. AM4 had almost the whole sequence of *PORA* correct - except for one nucleotide difference. The base substitution would have resulted in a different amino acid when expressed, and therefore it could not be used.

There were none positive transformants of *PORB* detected. The template for *PORB* was checked by sequencing with its specific primers in case it was something wrong with the clone I had received. Template for *PORB* was confirmed to be present by sequencing.

5.2. Expression of AU2 protein

Eventually it was decided to keep working with the only positive clone that had successfully been produced, the mature *PORA* (AU2).

Small scale expression of AU2 showed that the protein was well expressed and present in the soluble part of the cell lysate. Its solubility corresponds with the fact that it is an enzyme with a polar outer surface (8). Large scale culture of protein production was induced with IPTG. The protein was purified from the cell lysate by centrifugation, filtration and metal chelate affinity chromatography. The elution AU2 in the 500mM imidazole produced two clear bands and several small bands in between them (Figure 4-6). The highest band indicated by an arrow is the protein that corresponds with the molecular weight of mature PORA which is 40kDa (38 kDa protein + 2kDa histidine tag). The lower band on the gel is roughly 27 kDa. This lower band could be product of incomplete length or maybe degraded product.

A band from a 12% SDS gel that were thought to be the AU2 protein was analyzed with mass spectrometry (MS). The band was cut out from a 12% SDS gel where the purified proteins had been run (Figure 4-7). Three samples were analyzed. One of the sequenced peptides had a score of 199, which is very good (Table 4-3). The sequenced peptides are equally divided from the start to the end of the amino acid sequence. The first 16 amino acids and the last 16 amino acids from the sequence were not covered. The complete protein is not fully sequenced. But it is enough to say that the protein AU2 is PORA from *Arabidopsis thaliana* and the chances for it being correctly cloned and expressed is high.

The treatment with TEV protease did not work and the histidine tag were not cut off from the mature PORA protein. Ideally it would be better to have it cleaved off in case it influences the folding of PORA or

the way it binds to its substrate. A PORA protein with maltose binding tag (MBP-tag) have been used for reconstitution with good results (35).

5.3. Etioplast extraction

A standard curve was produced to determine the ratio between POR and etioplasts in dark grown barley seedlings. The relationship was found to be $2.7x10^6$ etioplasts per 1ng of POR.

5.4. Reconstitution

After the AU2 protein was purified and the pigments from etioplasts were extracted, they were reconstituted with the cosubstrate NADPH. Absorbance spectra were made adding one reagent at a time, identifying the presence of each reagent (Figure 4-8). The peak between 250 and 300nm corresponds to POR, the small peak at 634nm corresponds to protochlorophyllide and the big broad peak between 300 and 400nm corresponds to NADPH. It was certain that all of the reagents were there. The sample was illuminated several times but the spectra did not change (Figure 4-10). Meaning that protochlorphyllide was not converted into chlorophyllide by the enzyme AU2. Even after incubation there was no activity (Figure 4-11). The presence of glycerol did not make a difference, even though it is thought to stabilize the complex of AU2, pchlide and NADPH.

The presence of many bands of AU2 (Figure 4-6, lane C3-C10) makes it likely to assume that half of the produced proteins or more do not have the right molecular weight and would become inactive as an enzyme. So even with low amounts of active enzymes and pigments it should be enough to transform some of the pchlide to chlide, which would be detected on the absorbance spectra.

It is still not known if post translational modifications take place for the mature POR proteins. This does not happen when expressed in *E.coli* and that can prevent the protein from becoming active. However recombinant PORA has been used in reconstitution before. In one experiment, a MBP-PORA (organism not given) was produced by *E.coli*. The cell lysate was directly added to the pigments and NADPH. Absorption spectra before and after illumination showed that pchlide had been transferred to chlide and that the MBP-POR was a functional enzyme (35). Thus POR may not need post-translational modifications.

The third option is if the histidine-tag interferes and prevents correct folding of the protein, or prevents proper binding to its substrate and cosubstrate. A fourth option is that the length of the signal sequence predicted by ChloroP is not long enough compared to the findings of *Eichacker et al. (1).* When comparing to the determined cleavage site in *Barley* the signal sequence for PORA in *Arabidopsis*

thaliana, the signal sequence from ChloroP is too short. There is a difference of these two signal sequences of 20 amino acids. These 20 amino acids could be enough to prevent proper folding and function of the protein.

5.5 Future experiments

More experiments would have given answers to what went wrong in the constitution part. It could simply be that more optimization of the experiment was needed. Repeating the experiment of Vengadasalam S. (35) with cell lysate instead of purified protein would be useful. Because if it worked, it would mean that the purification process is damaging to the AU2.

Running AU2 with pigments on a native gel could tell us if they are able to bind to each other (Figure 3-1). This was the next step of the strategy of cloning POR. Also the use of fluorescence spectroscopy could be used to support the absorption spectroscopy results.

A last option would be to clone different forms of *PORA* and *PORB*. Maybe the cloned mature PORA, AU2, is not functional. Maybe the signal sequence is very important PORA and must be cleaved at the correct site. To clone more *PORA* with different lengths would be interesting in order to see if they would end up as active enzyme. Especially the AL sequence would be interesting to clone. If the AL sequence were found to be active and transforming pchlide into chlide, it would support the determined cleavage site for PORA and PORB in barley that was determined by *Plöscher et al. (1)*.

5.5. Other experiments to be done with POR

The reconstitution part is simply a way to check that the protein can work as an enzyme and capable of binding pigments. The intended purpose with expression of the mature PORA protein would also be protein-protein interaction experiments with the protein Lil3. The Lil3 protein is found in the stages from etioplasts to chloroplasts (36). The function of it is still not clear and it is of interest to find out which proteins it interacts with. Lil3 has been found in complexes in etioplasts right after illumination (36). Tanaka *et a*l. found that lil3 binds to geranyl geranyl reductase (GGR), and they propose that lil3 might have a stabilizing role for GGR (37). PORA and PORB could be candidates for interaction with Lil3.

6. Conclusion

A mature sequence of *PORA* from *Arabidopsis thaliana* has been cloned into a pET151 vector, the clone was called AU2 due to its number when PCR screened. The sequence has a length of 1065 nucleotides. The sequence was confirmed by PCR sequencing to be *PORA* from *Arabidopsis thaliana* with 100% correct bases.

AU2 was transformed into a BL21 strain of competent *E. coli* and expressed in a small scale. The protein was expressed with a histidine tag that can be detected by western blot. SDS PAGE followed by western blot and coomassive staining confirmed that the protein had been expressed. Expected size of AU2 is 40 kDa (38kDa AU2 + 2kDa histidine tag) which was confirmed on the stained SDS gels with a strong band in that region according to the protein marker (SeeBlue).

AU2 proteins were expressed in large quantities with a large scale culture. The cell lysate was purified by centrifugation, filtration and metal chelate affinity chromatography. The purified AU2 product was run on 12% SDS gel with SDS PAGE which showed not only one band but several bands. One strong band is according to the protein marker thought to be AU2, the other bands are of lower molecular weight. These lower bands are probably incomplete expressed AU2, or degraded protein.

The band in the 12%SDS gel was confirmed to be PORA by Mass Spectrometry (MS). Three samples were analyzed and several peptides were sequenced with a high score. This confirms that the protein AU2 is PORA.

Etioplasts were extracted from 4.5 day old dark grown *Hordeum vulgare*. The proteins and pigments were separated by acetone treatment and speed-vacuum centrifugation. The ratio between POR and etioplasts in the extraction were determined to be 2.7×10^6 etioplasts per 1ng of POR.

The pigments from the etioplasts were used for a reconstitution experiment with AU2 and NADPH. Absorbance spectra did not show a decrease pchlide at 634 nm and increase of chlide at ~670nm, even after overnight incubation. Therefore AU2 did not perform the enzyme activity under those circumstances. More experiments are needed to determine whether it is active or not. Fluorescence spectrophotometry (which is more sensitive) and reconstitution on native gel (shows if AU2 can bind to the pigment) are additional experiments that can be done to determine binding between AU2 and pigments.

More products of *PORA* and *PORB* would have to cloned and expressed if AU2 is not an active enzyme. PORA and PORB products would be used to study protein-protein interactions with itself and with the protein Lil3, in order to understand more about the roles they play in the transition from etioplasts to chloroplasts.

7. References

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