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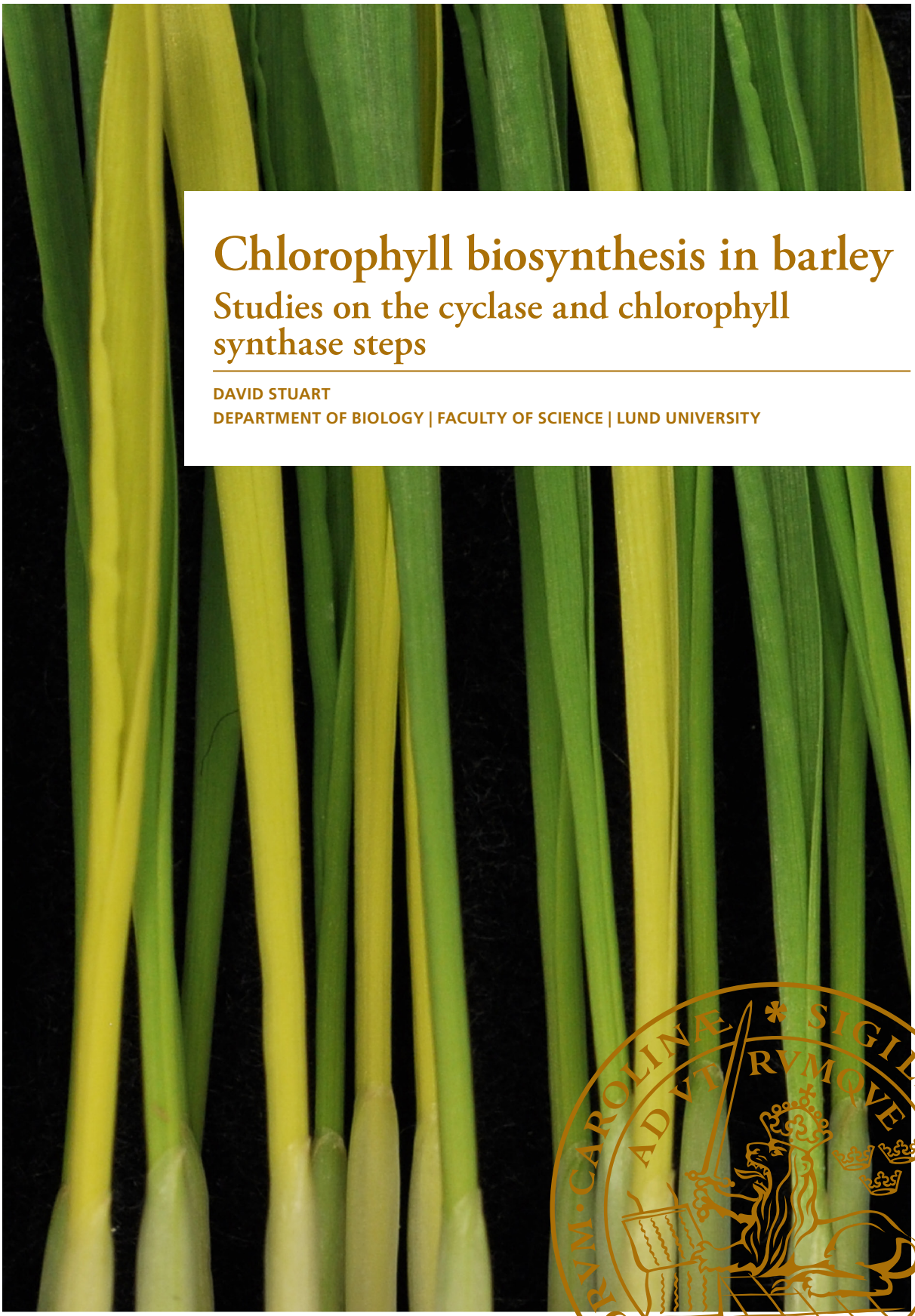

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David Stuart

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on 13th of June 2024 at 09.00 in the Lecture Hall A, Department of Biology, Sölvegatan 35B, Lund.

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The well-known green color of plants is due to the chlorophyll molecules used for light harvesting during photosynthesis, which takes place in chloroplasts. Biosynthesis of chlorophyll *a* is performed by 15 enzymatic steps and chlorophyll *a* can be interconverted to chlorophyll *b* via the chlorophyll cycle. Barley is a valuable model organism for studying chloroplast development and chlorophyll biosynthesis thanks to the availability of many chlorophyll mutants with abnormal pigment accumulation. Many of these mutants are deficient in enzymes of chlorophyll biosynthesis. Using barley as a model system, I have performed studies of two enzymatic steps in the chlorophyll biosynthetic pathway; the aerobic magnesium-protoporphyrin IX monomethyl ester cyclase and the chlorophyll synthase. I have identified the cyclase as a ferredoxin dependent enzyme and established the first *in vitro* assay using recombinant cyclase enzyme. I have also utilized the barley *viridis-k* chlorophyll mutants, which have impaired chlorophyll synthesis due to deficiency at the cyclase step. This revealed that a particular ferredoxin isoform, FdC2, is likely the main *in vivo* electron donor to the cyclase. By developing a pipeline for identification of genes deficient in barley mutants and applying this on barley *xantha-j* mutants, I connected this locus to the chlorophyll synthase. An AlphaFold generated structural model of the chlorophyll synthase suggested the active site to be subdivided into a prenyl pyrophosphate tunnel, the catalytic cavity, and a tetrapyrrole-binding pocket. A deep multiple sequence alignment contributed to the understanding of amino-acid residues. I suggest that binding of the isoprenoid substrate is a prerequisite for stable maintenance of chlorophyll synthase in the plastid. I further suggest that chlorophyll synthase is a sensor for coordinating chlorophyll and isoprenoid biosynthesis.

Key words: barley, Chlorina, chlorophyll synthase, *Hordeum vulgare*, Mg-protoporphyrin IX monomethyl ester cyclase, mutant, Viridis, *vir-k*, Xantha, *xan-j, xan-l, ycf54*

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Table of Contents

Abstract

The well-known green color of plants is due to the chlorophyll molecules used for light harvesting during photosynthesis, which takes place in chloroplasts. Biosynthesis of chlorophyll *a* is performed by 15 enzymatic steps and chlorophyll *a* can be interconverted to chlorophyll *b* via the chlorophyll cycle. Barley is a valuable model organism for studying chloroplast development and chlorophyll biosynthesis thanks to the availability of many chlorophyll mutants with abnormal pigment accumulation. Many of these mutants are deficient in enzymes of chlorophyll biosynthesis. Using barley as a model system, I have performed studies of two enzymatic steps in the chlorophyll biosynthetic pathway; the aerobic magnesium-protoporphyrin IX monomethyl ester cyclase and the chlorophyll synthase. I have identified the cyclase as a ferredoxin dependent enzyme and established the first *in vitro* assay using recombinant cyclase enzyme. I have also utilized the barley *viridis-k* chlorophyll mutants, which have impaired chlorophyll synthesis due to deficiency at the cyclase step. This revealed that a particular ferredoxin isoform, FdC2, is likely the main *in vivo* electron donor to the cyclase. By developing a pipeline for identification of genes deficient in barley mutants and applying this on barley *xantha-j* mutants, I connected this locus to the chlorophyll synthase. An AlphaFold generated structural model of the chlorophyll synthase suggested the active site to be subdivided into a prenyl pyrophosphate tunnel, the catalytic cavity, and a tetrapyrrole-binding pocket. A deep multiple sequence alignment contributed to the understanding of amino-acid residues. I suggest that binding of the isoprenoid substrate is a prerequisite for stable maintenance of chlorophyll synthase in the plastid. I further suggest that chlorophyll synthase is a sensor for coordinating chlorophyll and isoprenoid biosynthesis.

Populärvetenskaplig sammanfattning

Klorofyll är antagligen den mest synliga molekylen på jorden – dess gröna färg syns överallt där det finns tillräckligt med vatten för att hålla växter vid liv. Det är på klorofyllmolekylen som alla större näringskedjor är baserade, då växterna är basproducenterna. Också jordbruket är helt beroende av klorofyll och därmed också vår egen existens. Allt detta har skött sig själv under många miljoner år utan att vi haft en aning om hur klorofyll tillverkas i växtcellerna. Men ska vi i framtiden kunna effektivisera våra grödor så är de kan utnyttja ännu mer av solens energi så måste vi först lära oss hur klorofyll tillverkas och hur detta hänger ihop med bildandet av växternas kloroplaster och själva fotosyntesen.

Jag har arbetat med en fantastisk samling av kornmutanter som är defekta i klorofyllsyntes och kloroplastens utveckling. Dessa togs fram genom strålnings- och kemikaliebehandling av svenska och danska växtförädlare och forskare mellan 1928-1983. Jag har utnyttjat mutanterna för att identifiera de gener som de är defekta i. Mycket av genens funktion avslöjas genom att jag lyckats knyta genen till syntes av klorofyll men jag har också gjort molekylära och biokemiska analyser av mutanterna och de inblandade proteinerna. På detta sätt har jag lyckats förstå genen och mutanten i ett större molekylärt sammanhang. Mer specifikt har jag identifierat den gen vars protein ger elektroner till cyclasenzymet i klorofyllbiosyntesen. Jag har också identifierat den gen som kodar för klorofyllsyntaset, vilket katalyserar det sista steget i syntesen av klorofyll.

Projektet är av grundvetenskaplig karaktär men kunskap om generna är en förutsättning för att veta vilka gener man ska använda sig av för att i framtiden modifiera de odlade växterna så att de kan öka sin fotosyntes. Att studien baseras på korn som är den mest odlade grödan i Sverige gör steget till tillämpningar kortare även om dessa antagligen ligger långt fram i tiden.

List of Papers

Paper I

Stuart, D., M. Sandström, Helmy M. Youssef, S. Zakhrabekova, P. E. Jensen, D. W. Bollivar and M. Hansson. 2020. Aerobic barley Mg-protoporphyrin IX monomethyl ester cyclase is powered by electrons from ferredoxin. Plants 9: 1157. https://doi.org/10.3390/plants9091157

Paper II

Stuart, D., M. Sandström, Helmy M. Youssef, S. Zakhrabekova, P. E. Jensen, D. Bollivar and M. Hansson. 2021. Barley *Viridis-k* links an evolutionary conserved C-type ferredoxin to chlorophyll biosynthesis. Plant Cell 33: 2834-2849. https://doi.org/10.1093/plcell/koab150

Paper III

Stuart, D., S. Zakhrabekova, M. Egevang Jørgensen, C. Dockter and M. Hansson. 2024. A pipeline for identification of causal mutations in barley identifies Xantha-j as the chlorophyll synthase gene. Plant Physiol. Published as an accepted manuscript. https://doi.org/10.1093/plphys/kiae218

Author's contribution to the papers

Paper I

Conceptualization, D.S., P.E.J., D.W.B. and M.H.; methodology, D.S., P.E.J., D.W.B. and M.H.; validation, D.S., P.E.J., D.W.B. and M.H.; formal analysis, D.S. and H.M.Y.; investigation, D.S., M.S., H.M.Y., S.Z., D.W.B. and M.H.; resources, M.H.; data curation, D.S. and M.H.; writing—original draft preparation, D.S., D.W.B. and M.H.; writing—review and editing, D.S., H.M.Y., S.Z., P.E.J., D.W.B. and M.H.; visualization, D.S., H.M.Y., S.Z. and M.H.; supervision, D.S., P.E.J., D.W.B. and M.H.; project administration, M.H.; funding acquisition, D.S., H.M.Y., S.Z., P.E.J., D.W.B. and M.H. All authors have read and agreed to the published version of the manuscript.

Paper II

D.S., D.B., M.H., and P.E.J. designed the experiments; D.S., H.M.Y., M.S., and S.Z. performed the experiments; D.S., D.B., and M.H. analyzed the data; D.S., D.B., M.H., and P.E.J. wrote the article. All authors read and approved the final article.

Paper III

DS and MH designed the research. DS, SZ, MEJ, CD and MH performed research. DS, SZ, MEJ, CD and MH analyzed data. DS and MH wrote the paper. All authors read and approved the final version of the manuscript.

Abbreviations

ALA	5-Aminolevulinic acid
ATP	Adenosine triphosphate
C5	Carbon 5
CAO	Chlorophyllide *a* oxygenase
CBR	Chlorophyll(ide) *b* reductase
CoA	Coenzyme A
CPOX	Coproporphyrinogen III oxidase
DPOR	Dark operative protochlorophyllide oxidoreductase
DVR	Divinyl reductase
FAD	Flavin adenine dinucleotide
Fd	Ferredoxin
FNR	Ferredoxin $NADP^+$ oxidoreductase
GluTR	Glutamyl-tRNA reductase
GSA-AT	Glutamate-1-semialdehyde aminotransferase
HCAR	7-Hydroxymethyl chlorophyll(ide) *a* reductase
kbp	Kilo base pairs
kDa	Kilo Daltons
LPOR	Light dependent protochlorophyllide oxidoreductase
Mbp	Mega base pairs
NADP	Nicotinamide-adenine-dinucleotide phosphate
NOL1	NYC1-like
NYC1	NON-YELLOW COLORING1
PBG	Porphobilinogen
PPOX	Protoporphyrinogen IX oxidase
PSI	Photosystem I
PSII	Photosystem II
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine

Introduction

Chlorophyll and photosynthesis

Chlorophyll *a* and *b* are the light harvesting pigments used by plants for photosynthesis, a process that harvests and converts light energy into chemical energy. This process takes place in the endosymbiont derived organelle known as the chloroplast (Schwartz and Dayhoff, 1978, Sagan, 1967). These organelles have an envelope composed of an inner and outer membrane surrounding the stroma (Figure 1). Within the stroma is the thylakoid membrane system, which separates the stroma from the thylakoid lumen (Staehelin, 2003). The thylakoids are further organized as stacks known as grana and un-stacked stromal thylakoids. The chlorophyll-protein complexes responsible for the harvest and conversion of light energy are located in the thylakoid membranes (Figure 2).

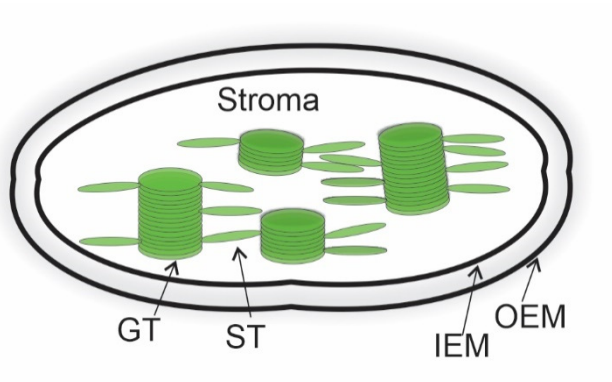

Figure 1 Schematic representation of a chloroplast. The organelle is enclosed by a double membrane consisting of the outer envelope membrane (OEM) and the inner envelope membrane (IEM) which surrounds the stroma. The thylakoid membrane system separates the stroma from the thylakoid lumen. Thylakoid membranes are organized into grana thylakoids (GT) which form stacks called grana and un-stacked stromal thylakoids (ST).

Chlorophylls are mainly found in the two photosystems, which perform charge separation (electron transfer) as well as in antenna complexes which help collect and funnel energy to the reaction centers in the photosystems (Caspy et al., 2020, Su et al., 2017). Electron transport begins at photosystem II where water is the electron donor at the lumen side of the membrane and the electrons are used to reduce plastoquinone to plastoquinol located within the thylakoid membrane (Hohmann-Marriott and Blankenship, 2011). Photosystem II contains a type II reaction center, a defining feature of which is using a quinone as the final electron acceptor (Hohmann-Marriott and Blankenship, 2011). The oxidation of water produces O_2 and H^+ at the lumen side and the protons required for the conversion of plastoquinone to plastoquinol are taken up from the stomal side. This results in a net decrease in pH within the lumen and this electrochemical gradient is used to drive ATP synthesis by the F_1F_0 ATP synthase also located in the thylakoid membranes (Hahn et al., 2018). Plastoquinol is oxidized back to plastoquinone by the cytochrome *b*$_6$*f* complex via the Q-cycle which releases protons into the lumen and takes up protons from the stroma, thus contributing to the electrochemical proton gradient across the thylakoid membrane (Stroebel et al., 2003). At the

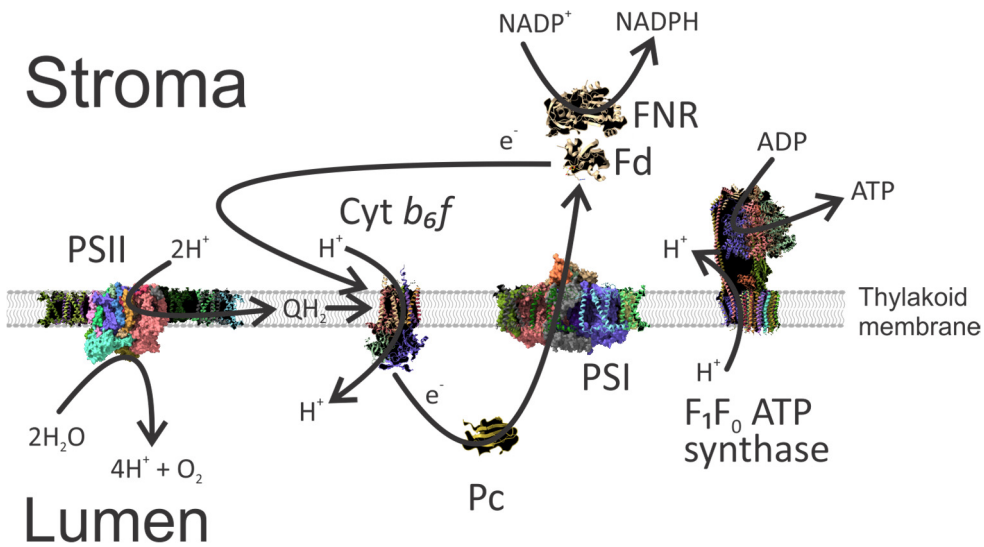

Figure 2 The main photosynthetic complexes located in the thylakoid membrane with the main routes of proton and electron transfer. PSII; photosystem II, image rendered from PDB 5XNL (Su et al., 2017). QH_2; plastoquinol. Cyt *b*$_6$*f*; cytochrome *b*$_6$*f* complex, image rendered from PDB 1Q90 (Stroebel et al., 2003). Pc; plastocyanin, image rendered from PDB 6YEZ (Caspy et al., 2020). PSI; photosystem I, image rendered from PDB 6YEZ. Fd; ferredoxin, image rendered from PDB 5H5J (Shinohara et al., 2017). FNR; Ferredoxin $NADP^+$ oxidoreductase, image rendered from PDB 5H5J. F_1F_0 ATP synthase rendered from PDB 6FKF (Hahn et al., 2018).

cytochrome b_6f complex, electrons are passed to the soluble electron carrier plastocyanin which is located in the thylakoid lumen. Plastocyanin is the electron donor to photosystem I (PSI) which transfers the electrons to the soluble 2Fe-2S cluster containing ferredoxin protein. PSI has a type I reaction center, a defining feature of which is having an iron sulfur cluster as the terminal electron acceptor (Hohmann-Marriott and Blankenship, 2011). Ferredoxin can in turn donate electrons to ferredoxin $NADP^+$ oxidoreductase (FNR) which reduces $NADP^+$ to NADPH. Alternatively, ferredoxin can donate electrons directly to ferredoxin dependent enzymes. The generation of low potential electrons originating from water that are channeled into other metabolic processes is generally referred to as linear electron transport. However, electrons from ferredoxin can be channeled back into the plastoquinone pool by reducing plastoquinone in a process dependent on PGR5 and PGRL1 (Hertle et al., 2013). This is known as cyclic electron transport since the electrons cycle around the cytochrome b_6f complex and PSI. Since electron transfer through the cytochrome b_6f complex contributes to the proton gradient across the thylakoid membrane, this is a mechanism to balance the cellular need for ATP versus NADPH, as well as providing a mechanism to maintain the proper redox balance of the plastoquinone pool.

The microbial route to chlorophyll biosynthesis

While plants contain only chlorophyll *a* and *b*, there are a number of other chlorophylls and bacteriochlorophylls found in other photosynthetic organisms spread throughout the tree of life. Only cyanobacteria and eukaryotes with plastids perform oxygenic photosynthesis and contain both type I and type II reaction centers. Numerous bacterial groups such as *Acidobacteria*, *Chloroflexi*, *Chlorobi*, *Firmicutes*, *Gemmatinomadetes*, and *Proteobacteria* contain photosynthetic members that perform anoxygenic photosynthesis and contain only a single photosynthetic reaction center of either type I or type II (Hamilton, 2019). These bacteria generally produce bacteriochlorophylls but many of the biosynthetic steps use enzymes which are homologous to those found in cyanobacteria, algae and plants. Most of the genes involved in (bacterio)chlorophyll biosynthesis were thus first identified in bacterial species such as *Rhodobacter capsulatus* which contains all the genes required for photosynthesis in a 46 kbp cluster termed the photosynthesis gene cluster (Bollivar et al., 1994b). Many genes were thus named "*bch**" where the * is a letter designation for the gene. This naming convention was then transferred to chlorophyll synthesizing organisms as "*chl**". Many genes, however, have alternative names that differ between organisms mainly for historical reasons since the genes have kept the names from the mutants, which were used for the discovery of the genes.

Chlorophyll mutants in barley

A rich variety of chlorophyll mutants are available in barley (*Hordeum vulgare* L.). The many mutants were induced by physical and chemical mutagenesis in breeding programs aiming for high-yielding barley cultivars. Mutants with low or no chlorophyll are not associated with high yield, but the chlorophyll mutants were easily observed in the M_2 generation already at the seedling stage and thus served as indicators for the success of the mutagenic treatment (Lundqvist, 1992). When the barley chlorophyll mutants were generated, they were classified based on their phenotype as *albina* (white), *xantha* (yellow), *viridis* (light green lethal), *chlorina* (light green viable), and *tigrina* (transverse striped) (Hansson et al., 2024). Mutants with the same phenotype were given an identifying number and then crossed to each other to identify allelic complementation groups which were designated by a letter. An example name would then be *xantha-l.35*. When it was first discovered in the field after mutagenesis with ethyleneimine in 1957 (Henningsen et al., 1993), it was given the name *xantha-35* because it was the 35th yellow mutant isolated. It was found that crosses to for example *xantha-81*, but not *xantha-27*, could generate yellow plants, which demonstrated that *xantha-35* and *xantha-81*, but not *xantha-27*, are deficient in the same gene. Their names were then extended to *xantha-l.35* and *xantha-l.81*, respectively, to indicate that they are allelic. For many years, very few molecular analyses could be performed with the barley mutants. The large 5.4 Mbp diploid genome was a major obstacle until a reference genome sequence was published (Mascher et al., 2017). The availability of a reference genome sequence has created a renaissance for the many available mutants since map-based cloning and marker analyses can now take a short-cut when transferring information between genetic and physical maps. Still, there is a challenge to study chlorophyll mutants since the recessive mutations, except for the light green *chlorina* mutants, are lethal in homozygous form. Therefore, the mutations must be kept in heterozygous stocks and each propagation has to be followed by segregation analyses in order to sort heterozygous green mutants from homozygous green wild-type plants. There are also advantages using barley as a model organism for chlorophyll biosynthesis. This is because the large barley seed can support also a lethal mutant with energy to form a reasonably large mutant seedling that can be used, not only for molecular studies, but also for biochemical analyses.

Chlorophyll biosynthesis

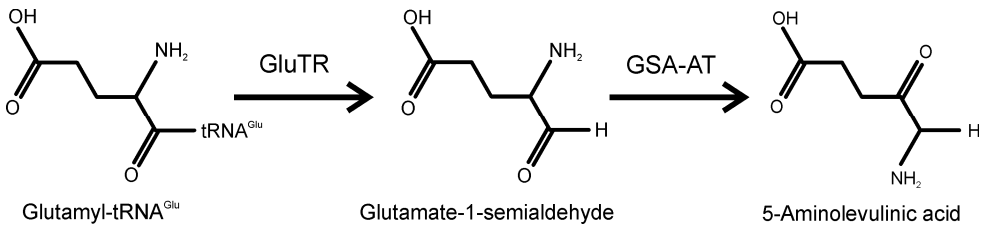

Figure 3 The C5 pathway for 5-aminolevulinic acid biosynthesis utilized by plants. Activated glutamate in the form of glutamyl-tRNA^{Glu} is first converted to glutamate-1-semialdehyde by glutamyl-tRNA reductase (GluTR) followed by conversion to 5-aminolevulinic acid by glutamate-1-semialdehyde aminotransferase (GSA-AT.

Chlorophylls are cyclic tetrapyrrole molecules and the biosynthetic pathway in plants involves numerous enzymatic steps. The first part of the pathway is shared with heme biosynthesis and the enzymatic steps are often called the "common" or "shared" steps since they are shared in common for both heme and chlorophyll biosynthesis until the red colored intermediate protoporphyrin IX. The first step specific for chlorophyll biosynthesis is insertion of a Mg^{2+} ion into protoporphyrin IX and the chlorophylls specific steps are therefore generally referred to as the "chlorophyll branch" or the "Mg branch" and ends with the synthesis of the characteristically green chlorophylls *a* and *b* in plants. Chlorophyll *a* and *b* can be interconverted via the chlorophyll cycle.

Shared steps of chlorophyll and heme biosynthesis

The common steps start with the synthesis of 5-aminolevulinic acid (ALA), the first dedicated intermediate of tetrapyrrole biosynthesis. There are two known pathways for ALA synthesis. One is the Shemin pathway that produces ALA by condensation of glycine and succinyl-CoA (Shemin et al., 1955). The second pathway is the carbon 5 (C5) pathway which produces ALA from glutamate (Beale and Castelfranco, 1973, Beale et al., 1975). Plants utilize the C5 pathway for ALA synthesis by the action of two enzymes (Figure 3), glutamyl-tRNA reductase

(GluTR) followed by glutamate-1-semialdehyde aminotransferase (GSA-AT). Activated glutamate in the form of glutamyl-tRNA^{Glu} (Schön et al., 1986, Huang et al., 1984) is first converted by GluTR into glutamate-1-semialdehyde by conversion of the carboxyl group to an aldehyde. GSA-AT next produces ALA by swapping the positions of the amino group and the carbonyl group (Kannangara and Gough, 1978, Grimm, 1990).

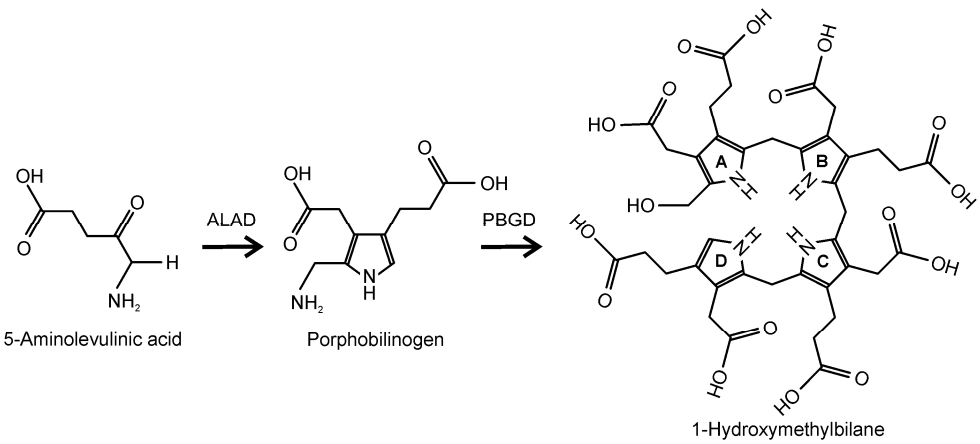

Figure 4 Biosynthesis of 1-hydroxymethlbilane (HMB) from 5-aminolevulinic acid (ALA). The enzyme ALA dehydratase (ALAD) forms the first pyrrole, porphobilinogen (PBG), by condensing two molecules of ALA. Four molecules of PBG are then combined by PGB deaminase (PBGD) to form the linear tetrapyrrole HMB. The four pyrrole rings are denoted as ring A, B, C, and D.

In the next step, two ALA molecules are condensed by ALA dehydratase to form porphobilinogen (PBG) which is a pyrrole (Boese et al., 1991, Schaumburg et al., 1992) (Figure 4). Further, PBG deaminase combines four PBG molecules to produce the linear tetrapyrrole 1-hydroxymethylbilane (Witty et al., 1993, Jones and Jordan, 1994). It thus takes a total of eight ALA molecules to produce one tetrapyrrole molecule. The next step is inversion of ring D and a ring closure catalyzed by uroporphyrinogen III synthase to form the first cyclic tetrapyrrole intermediate (Figure 5) (Tan et al., 2008). The enzyme uroporphyrinogen III decarboxylase next produces coproporphyrinogen III by decarboxylating the acetate groups on each of the four pyrrole rings (Mock et al., 1995). This is followed by conversion of the propionate side chains on rings A and B to vinyl groups by the oxygen-dependent coproporphyrinogen III oxidase (CPOX) to produce protoporphyrinogen IX (Madsen et al., 1993, Kruse et al., 1995). The last of the common steps is a six electron oxidation performed by protoporphyrinogen IX oxidase (PPOX) which converts the methylene bridges to methine bridges between the pyrrole rings to produce the first porphyrin of the pathway (Figure 6) (Jacobs

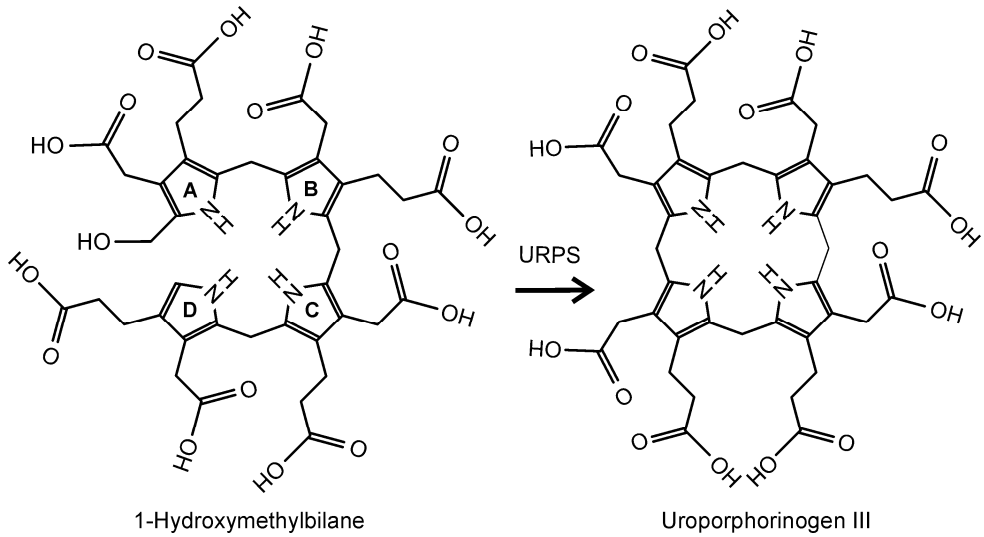

Figure 5 From a linear to a ring-closed tetrapyrrole. HMB is converted by uroporphyrinogen III synthase (URPS) to the first cyclic tetrapyrrole intermediate, uroporphyrinogen III. In the process, the D ring is turned leaving an isomer with two propionyl groups pointing down from rings C and D.

and Jacobs, 1987, Lermontova et al., 1997). At this point the pathway forks and metabolites are diverted to either heme biosynthesis after insertion of Fe^{2+} into the macrocycle by ferrochelatase or to chlorophyll biosynthesis after insertion of Mg^{2+} by the magnesium chelatase.

Magnesium chelatase

The insertion of Mg^{2+} into protoporphyrin IX (Figure 7) is performed by the magnesium chelatase which is a multi-subunit enzyme made up of three subunits. These are encoded by the *bchI*/*chlI*, *bchD*/*chlD*, and *bchH*/*chlH* genes (Bollivar et al., 1994b, Jensen et al., 1996a), which correspond to the *Xantha-h*, *Xantha-g*, and *Xantha-f* genes in barley (Jensen et al., 1996b) and have a mass of approximately 40 kDa, 70 kDa, and 140 kDa, respectively. The enzyme is located in the stroma but XanF can associate to thylakoid and envelope membranes depending on the buffer conditions, especially the Mg^{2+} concentration (Walker and Weinstein, 1995).

The reaction is ATP and Mg^{2+} dependent (Fuesler et al., 1981) and requires a Mg^{2+} concentration greater than that of ATP (Jensen et al., 1998). Early studies established that the reaction mechanism proceeds through a slow ATP dependent activation step followed by a faster, also ATP dependent metal chelation step (Walker and Weinstein, 1991). The activation step was shown to require the XanH/BchI and XanG/BchD subunits as well as ATP (Willows et al., 1996) and

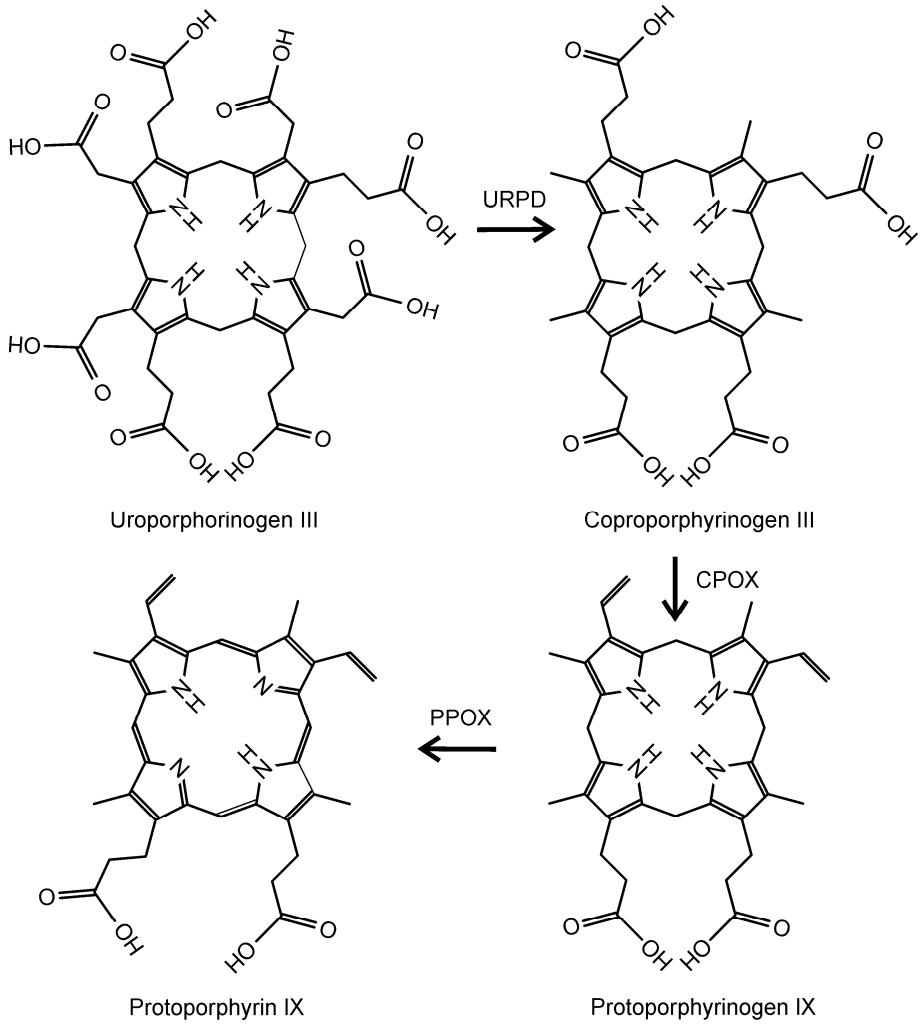

Figure 6 The three final enzymatic steps shared between chlorophyll and heme biosynthesis. Uroporphyrinogen III is converted to coproporphyrinogen III by uroporphyrinogen III decarboxylase (URPD). Next, protoporphyrinogen IX is produced by the action of the enzyme coproporphyrinogen III oxidase (CPOX). Finally, protoporphyrin IX is produced by the enzyme protoporphyrinogen IX oxidase (PPOX).

require high protein concentrations which is suggestive of multi-subunit complex assembly. With the cyanobacterial enzyme however, it was shown that there are two separate activation steps. One part is the ATP dependent activation of ChlI and ChlD and the other is an ATP dependent activation of ChlH with protoporphyrin IX (Jensen et al., 1998). Both the activation steps as well as catalysis require ATP.

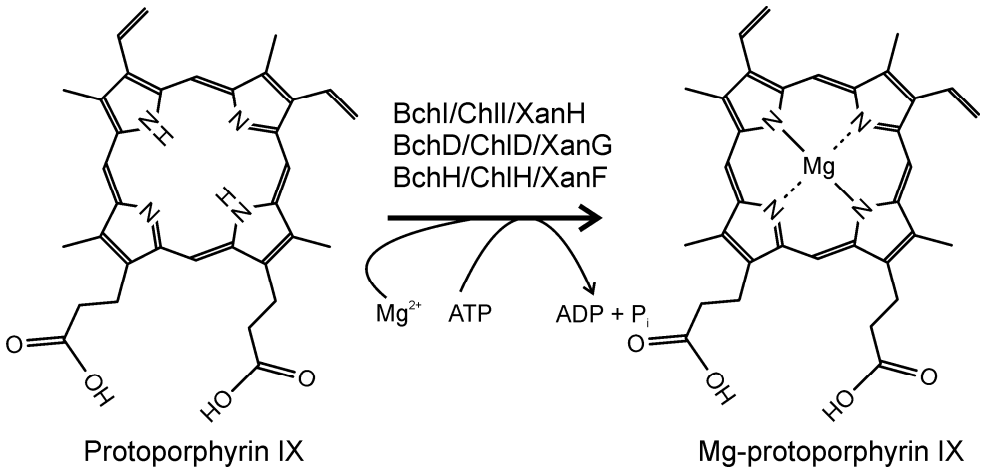

Figure 7 The magnesium chelatase reaction inserts Mg^{2+} into protoporphyrin IX to form Mg-protoporphyrin IX. The reaction is performed by a multi-subunit enzyme made up of the BchI/ChlI/XanH, BchD/ChlD/XanG, and BchH/ChlH/XanF proteins. ATP is required for complex assembly as well as catalysis.

The XanF/ChlH subunit binds the substrate, protoporphyrin IX (Willows et al., 1996). Structural studies have shown that the XanH/ChlI and XanG/ChlD proteins form two stacked hexameric rings and the current model for the reaction is that this complex formation is the activation step and that the ChlH protein which binds protoporphyrin IX then acts as a substrate for the ChlID complex (Lundqvist et al., 2010). It has been shown using recombinant magnesium chelatase subunits that inhibition of the reaction occurs when increasing the D subunit beyond a 1:2 ratio to the I subunit (Jensen et al., 1998). As an explanation for this observation, it has been suggested that the D hexamer may form first and act as a scaffold for assembly of the I hexamer (Axelsson et al., 2006). It is interesting to note that the *in vivo* accumulation of the D subunit appears dependent on accumulation of the I subunit (Lundqvist et al., 2010, Lake et al., 2004, Hansson et al., 1999). This apparent degradation of free D subunits may be a cellular control mechanism to maintain a suitable D:I ratio in the cell. In addition to the three subunits of the magnesium chelatase enzyme, plants and cyanobacteria appear dependent on an additional protein, GUN4, for efficient magnesium chelation *in vivo*. The GUN4 protein is porphyrin binding and may be involved in delivery of protoporphyrin IX and

removal of Mg-protoporphyrin IX from the enzyme (Larkin et al., 2003). GUN4 also lowers the required Mg^{2+} concentration (Davison et al., 2005).

Methyl transferase

After magnesium chelation, the enzyme Mg-protoporphyrin IX O-methyl transferase, encoded by *bchM*/*chlM* (Bollivar et al., 1994a, Smith et al., 1996), forms a methyl ester on the propionate side chain of ring C (Figure 8) using S-adenosylmethionine (SAM) as the methyl donor (Tait and Gibson, 1961). No mutants in the methyl transferase gene of barley have been described. In Arabidopsis the enzyme has been localized to both the thylakoid and envelope membranes (Block et al., 2002).

The enzymatic reaction requires S-adenosylmethionine and is competitively inhibited by S-adenosylhomocysteine and S-adenosylmethionine (Shieh et al., 1978). It appears that activity of the methyl transferase is stimulated by the large subunit of the magnesium chelatase in both bacterial and eukaryotic systems (Hinchigeri et al., 1997, Alawady et al., 2005, Shepherd et al., 2005) which suggests a conserved substrate channeling mechanism. The enzyme has also been shown to be stimulated by addition of phospholipids to assay mixtures (Sawicki and Willows, 2007).

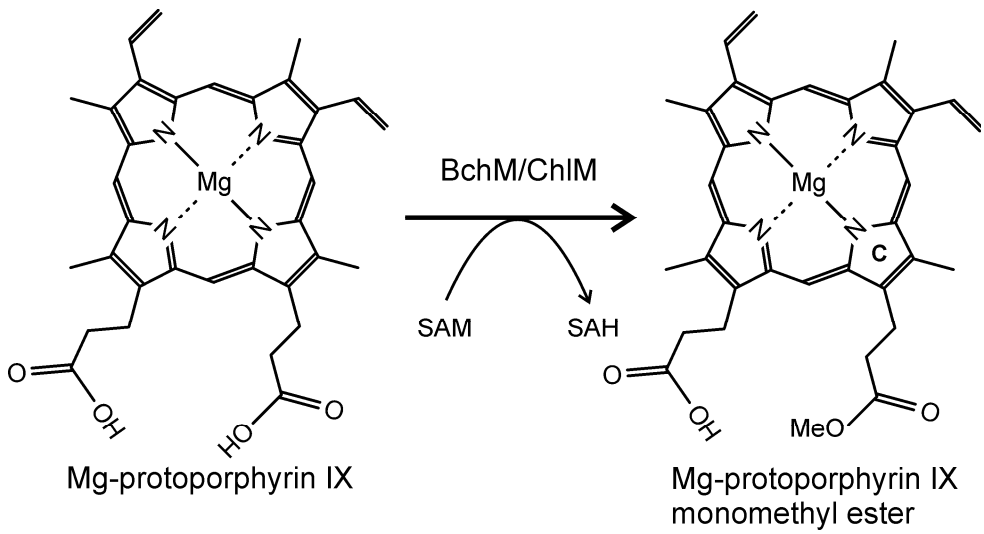

Figure 8 The methyl transferase reaction produces a methyl ester on propionate group on ring C. The reaction is performed by BchM/ChlM and uses S-adenosylmethionine (SAM) as the methyl donor. SAM is converted to S-adenosylhomocysteine (SAH).

Cyclase

After formation of the methyl ester the next enzyme produces the fifth ring E (Figure 9) which also results in the first green intermediate of the chlorophyll biosynthesis pathway (Bryant et al., 2020). This is a multi-step reaction that proceeds through addition of a carbonyl group to C13[1] followed by a carbon-carbon bond formation between C13[2] and the carbon bridging ring C and D which produces ring E. There are two unrelated enzymes performing this reaction. One enzyme is a hydratase encoded by *bchE* and is only found in photosynthetic bacteria. The other enzyme

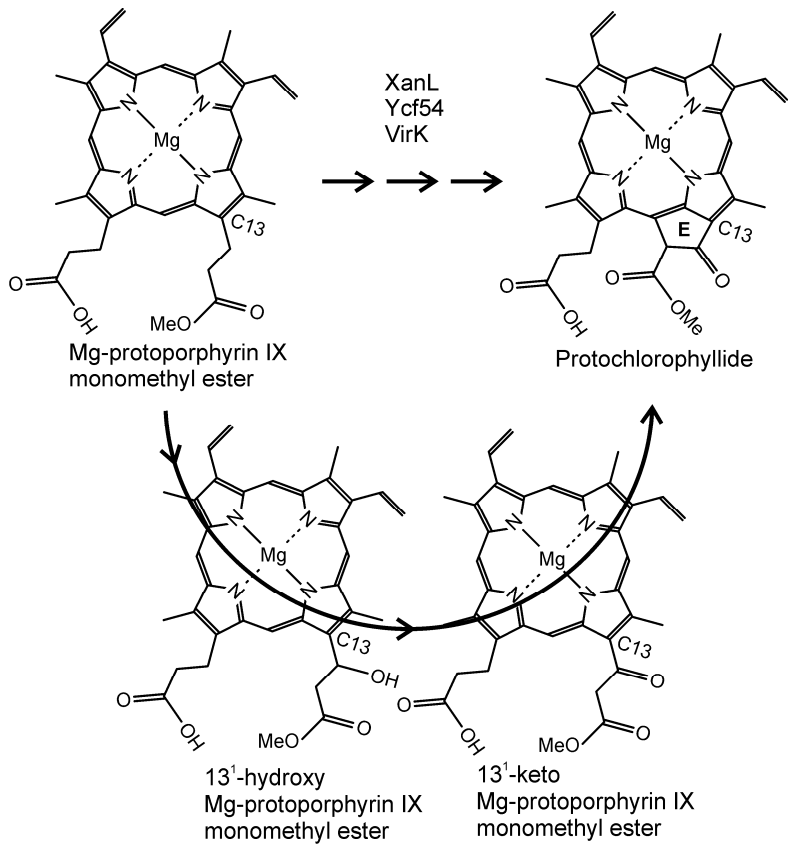

Figure 9 The cyclase reaction forms the fifth ring E of the chlorophyll molecule by converting Mg-protoporphyrin IX monomethyl ester to protochlorophyllide. The reaction proceeds through a C13[1] hydroxy followed by a C13[1] keto intermediate and all three transformations are catalyzed by XanL. An auxiliary protein, Ycf54, is important for the reaction *in vivo*. The enzyme requires electrons and these are provided by reduced ferredoxin. The VirK protein, the barley ortholog of Arabidopsis FdC2, is suggested to be the *in vivo* ferredoxin isoform responsible for donating electrons to XanL.

is a carboxylate bridged diiron monooxidase where the incorporated oxygen atom comes from molecular O_2. As this is the only enzyme found in plants all references to the cyclase from here on refer to the oxygenase.

In barley, the enzyme performing this reaction is encoded by the *Xantha-l* gene (Rzeznicka et al., 2005). In photosynthetic bacteria performing anoxygenic photosynthesis, the gene has been named *acsF* while in the cyanobacterium Synechocystis, the two homologs have been named $chlA_I$ and $chlA_{II}$ (Pinta et al., 2002, Minamizaki et al., 2008). In *Chlamydomonas reinhardtii* there are two homologs which have been named *CRD1* and *CTH1* while in Arabidopsis the gene is named *CHL27* (Tottey et al., 2003, Moseley et al., 2002, Moseley et al., 2000). In addition to the cyclase enzyme, plants contain an auxiliary protein, Ycf54, which is required for optimal function of the cyclase enzyme (Hollingshead et al., 2012). The role(s) of Ycf54 are poorly understood but it does not appear to be required for catalysis and there are cyclases in bacteria which are not dependent on Ycf54 (Chen et al., 2017). Studies of the cyclase have been hampered by the lack of catalytically active recombinant enzyme for *in vitro* activity assays – a hurdle which we recently overcame. When XanL is produced recombinantly in *Escherichia coli* it is not catalytically active unless co-expressed with Ycf54 (Stuart et al., 2020). Ycf54 did not co-purify with XanL, further supporting that it is not required for catalysis and suggesting a role in folding or maturation of XanL. The cyclase enzyme has been localized to both the inner envelope and thylakoid membranes in Arabidopsis (Tottey et al., 2003) and Ycf54 has been localized in soluble as well as membrane fractions from barley etioplasts (Bollivar et al., 2014). *In vitro* enzymatic assays have showed that the cyclase requires a reductant supplied as NADPH as well as both soluble and membrane fractions from etioplasts which suggested at least one more unidentified component required for cyclase activity (Rzeznicka et al., 2005). Other diiron monooxygenases generally do not utilize NAD(P)H directly so it was likely that the unidentified component is involved in electron transfer from NADPH to the cyclase. In developing a recombinant *in vitro* enzyme activity assay, we recently identified the soluble component as ferredoxin and that FNR is also required to reduce ferredoxin using NADPH (Stuart et al., 2020). This clearly demonstrated that the cyclase is a ferredoxin dependent enzyme. Plants contain a number of ferredoxin isoforms. Based on evidence from barley mutants at the *Viridis-k* and *Xantha-l* loci, the FdC2 ferredoxin isoform encoded by the *Viridis-k* gene in barley is the *in vivo* electron donor to the cyclase (Stuart et al., 2021). The FdC2 isoform is conserved in all plants and cyanobacteria and must thus have an indispensable function. The involvement of FdC2 in chlorophyll biosynthesis is the first clear role for the protein. Previous biochemical characterizations of FdC2 from rice and *Thermosynechococcus elongatus* indicate that FdC2 is most likely reduced by PSI and not via FNR *in vivo* (He et al., 2020, Schorsch et al., 2018). This would imply that most electrons for the cyclase step of chlorophyll biosynthesis are provided by PSI via FdC2 and would explain the cyclase deficient phenotype of the barley *vir-zb.63* mutant which lacks photosystem I activity (Steccanella et al., 2015).

Although a single enzyme is responsible for catalysis, the cyclase reaction proceeds through three steps, each of which is a two-electron oxidation. The enzyme requires a reductant in order to reduce the second oxygen atom at each step. The cyclase proceeds through two intermediates (Wong et al., 1985, Walker et al., 1988). First, the C13[1] carbon is hydroxylated. The hydroxyl group is then oxidized to a carboxy group. Finally, a carbon-carbon bond is formed to produce the E ring which completes the conversion of the red Mg-protoporphyrin IX monomethyl ester to the first green intermediate protochlorophyllide (Figure 9).

Protochlorophyllide oxidoreductase

The next step is the hydrogenation of the double bond between C17 and C18 in ring D to convert protochlorophyllide to divinyl chlorophyllide a (Figure 10). Two enzymes exist that perform this reaction. The first is the dark operative protochlorophyllide oxidoreductase (DPOR) which is related to nitrogenase and consists of three components BchL, BchN, and BchB (Burke et al., 1993). The second is the light dependent protochlorophyllide oxidoreductase (LPOR). Angiosperms only contain the LPOR enzyme and chlorophyll biosynthesis is thus halted at protochlorophyllide when plants are grown in the dark.

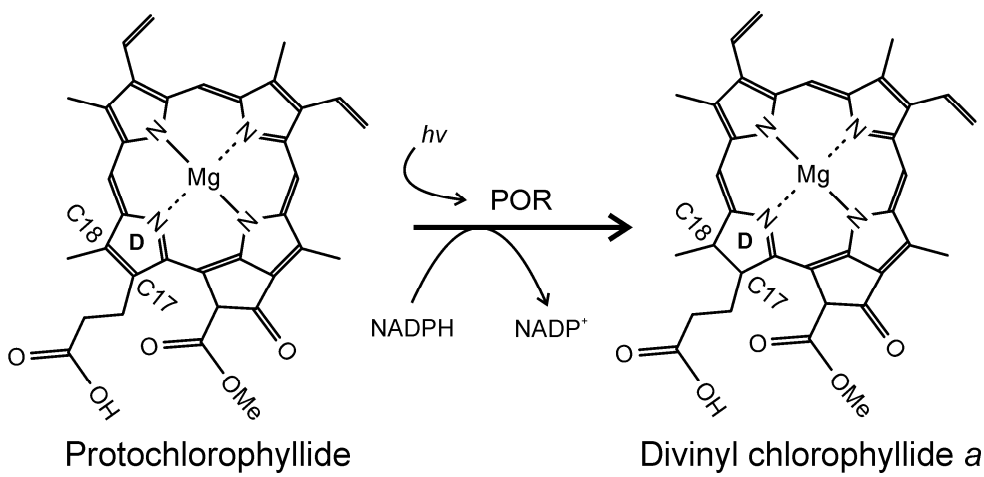

Figure 10 The conversion of protochlorophyllide to divinyl chlorophyllide *a* in angiosperms is performed by the light dependent protochlorophyllide oxidoreductase (LPOR). In addition to light (*hv*), the enzyme also requires NADPH to reduce the double bond between C18 and C17.

Dark grown angiosperms thus undergo a process known as etiolation where the chloroplasts halt development as etioplasts (Solymosi and Schoefs, 2010). The LPOR reaction can thus be considered as the first of the late steps of the Mg branch as flux through the pathway after LPOR only occurs in the light. Etioplasts accumulate a crystalline matrix known as a prolamellar body that contains protochlorophyllide:LPOR complexes. After illumination, the protochlorophyllide is converted to chlorophyllide and the prolamellar body starts to disperse and forms the thylakoid membranes as the plastids develop (Solymosi and Schoefs, 2010).

Divinyl reductase

Plants and most oxygenic phototrophs produce monovinyl chlorophylls where the C8 vinyl group has been reduced to an ethyl group (Figure 11). The enzyme performing this reaction is the divinyl reductase (DVR) and the plant enzyme was first identified by characterization of Arabidopsis mutants that accumulated divinyl chlorophylls (Nakanishi et al., 2005, Nagata et al., 2005) and later a rice mutant which also accumulated only divinyl chlorophylls (Wang et al., 2010). The enzyme has a putative C-terminal transmembrane domain (Nakanishi et al., 2005) and enzymatic activity has been isolated from chloroplast membrane fractions (Parham and Rebeiz, 1995).

Substrate specificity of DVR has been investigated using recombinant DVR from the monocots rice and maize, as well as dicots Arabidopsis and cucumber (Wang et

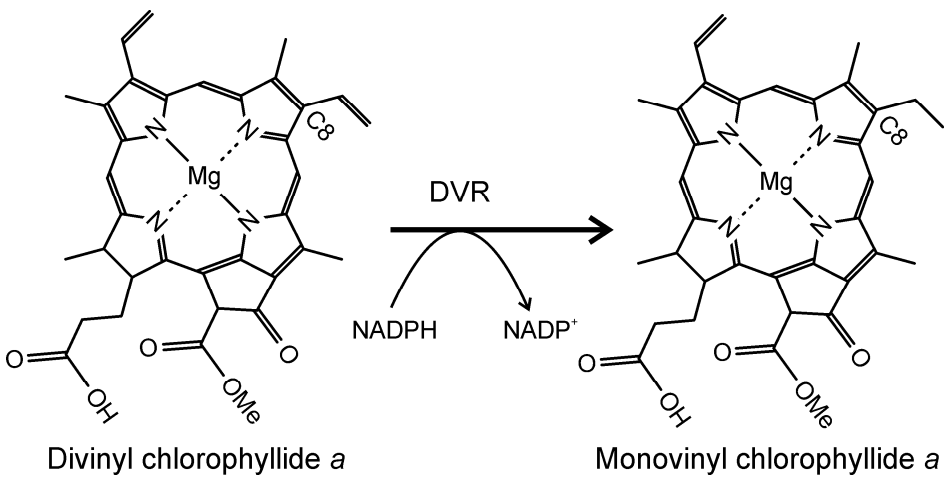

Figure 11 The reduction of the C8 vinyl group to an ethyl group is performed by the divinyl reductase (DVR) enzyme. The reaction requires NADPH.

al., 2013). Both monocots and dicots had highest activity with divinyl chlorophyllide *a* and activity decreased with earlier intermediates in the pathway but was detectable all the way back to divinyl Mg-protoporphyrin IX for monocot DVR and divinyl Mg-protoporphyrin IX monomethyl ester for dicot enzymes. The monocot enzymes had higher activity for a given substrate and also efficiently converted divinyl chlorophyll *a* (Wang et al., 2013) but not divinyl chlorophyll *b* or divinyl chlorophyllide *b* (Wang et al., 2010). In general, chlorophyllide is used synonymously with the monovinyl species if nothing is specified.

Chlorophyll synthase and geranylgeranyl reductase

The final conversion of chlorophyllide to chlorophyll is the formation of a phytol ester on the C17 propionate group (Figure 12). This is performed by the thylakoid bound enzyme chlorophyll synthase (ChlG) which can utilize either phytol pyrophosphate or geranylgeranyl pyrophosphate as a substrate (Oster et al., 1997). The geranylgeranyl moiety is reduced by geranylgeranyl reductase (ChlP) which can occur either before or after esterification to chlorophyllide (Bollivar et al., 1994c). Both oxygens of the ester originate from the C17 propionate group and the reaction is believed to occur by nucleophilic attack by the carboxylate anion on the

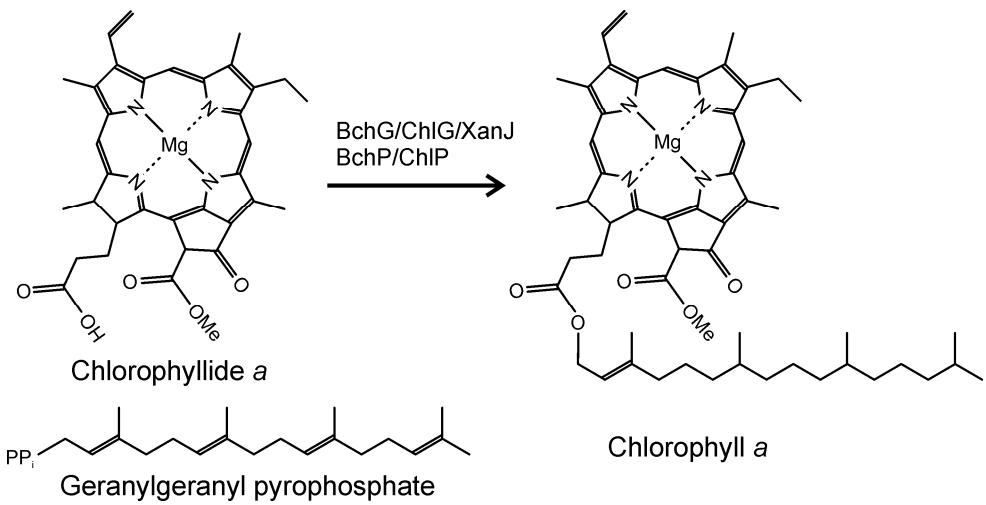

Figure 12 The conversion of chlorophyllide to chlorophyll involves esterification of a phytol "tail" on the C17 propionate group. Ester formation is performed by the chlorophyll synthase enzyme (BchG/ChlG/XanJ) which can use either geranylgeranyl pyrophosphate or phytyl pyrophosphate. The enzyme geranylgeranyl reductase (BchP/ChlP) converts the geranylgeranyl chain to a phytyl chain either before or after esterification.

pyrophosphoryl activated alcohol (Emery and Akhtar, 1987). Chlorophyll synthase has been shown to interact with the Alb3 membrane insertase and it is suggested that this is part of a mechanism to coordinate chlorophyll biosynthesis with the insertion of chlorophyll into chlorophyll binding proteins in the thylakoid membrane (Proctor et al., 2018, Chidgey et al., 2014).

Chlorophyll synthase from plants can utilize chlorophyllide *a* and chlorophyllide *b* (see chlorophyll cycle below) equally well (Benz and Rüdiger, 1981). Interestingly, ChlG is highly specific for chlorophyllide while BchG is highly specific for bacteriochlorophyllide, although the specificity can be altered with a single amino acid substitution (Kim et al., 2016). In barley, chlorophyll synthase is encoded by the *Xantha-j* gene (Stuart et al., 2024). As part of this thesis, we recently identified this gene using bulk segregant analysis by whole genome sequencing of an F_2-mapping population made by crossing the mutant *xan-j.59* with the cultivar Quench. The mutant *xan-j.64* has an amino-acid substitution that is likely to prohibit binding of the geranylgeranyl/phytol pyrophosphate substrate and fails to accumulate the XanJ enzyme, suggesting that substrate binding stabilizes the enzyme *in vivo*. Treatment of plants with clomazone, which inhibits isoprenoid biosynthesis, resulted in up-regulation of chlorophyll synthase transcript levels but no accumulation of the protein. This further supports that chlorophyll synthase is degraded *in vivo* when the isoprenoid substrate is lacking. This may be a mechanism to balance the supply of isoprenoids between chlorophyll biosynthesis and carotenoid biosynthesis.

The chlorophyll cycle

The two chlorophylls present in plants, chlorophyll *a* and *b*, differ by having either a methyl group or a formyl group on C7 and can be interconverted via the chlorophyll cycle (Figure 13). The enzyme responsible for synthesis of chlorophyll *b* was first identified in Chlamydomonas and named chlorophyll *a* oxygenase (CAO) and contains a Rieske type iron sulfur cluster and a mononuclear iron cluster and is a monooxygenase enzyme (Tanaka et al., 1998). This is consistent with earlier isotope labeling experiments showing that the 7-formyl oxygen atom comes from molecular oxygen (Schneegurt and Beale, 1992, Porra et al., 1994). The enzyme was later renamed to chlorophyllide *a* oxygenase (still CAO) when it was shown that enzymatic activity of the recombinant enzyme was only obtained when chlorophyllide *a* was used as a substrate (Oster et al., 2000). This study also established that CAO is a ferredoxin dependent enzyme and identified 7-hydroxymethyl chlorophyllide *a* as an intermediate. In barley, 15 mutants in the

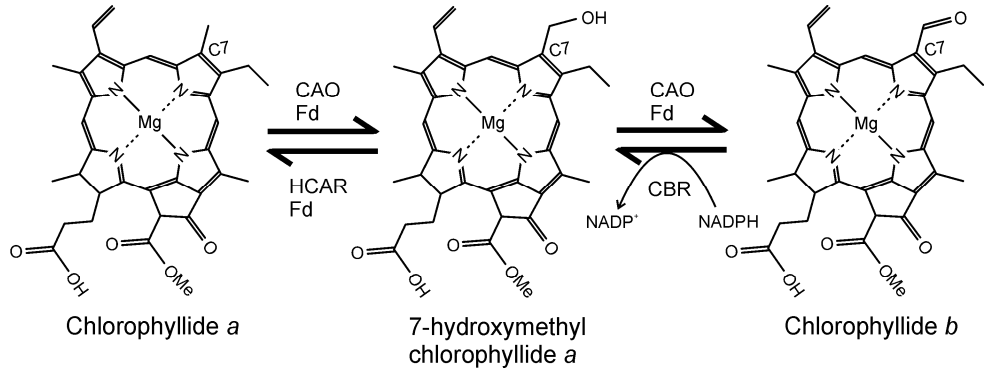

Figure 13 Chlorophyll *b* is synthesized from and converted back to chlorophyll *a* via the chlorophyll cycle. The enzyme chlorophyllide *a* oxygenase (CAO) converts chlorophyllide *a* to chlorophyllide *b* via the C7 hydroxymethyl intermediate and is not able to convert esterified pigments. Chlorophyll *b* is converted back to chlorophyll *a* by the sequential action of chlorophyll(ide) *b* reductase (CBR) followed by 7-hydroxymethyl chlorophyll(ide) *a* reductase HCAR. CBR uses NADPH and HCAR is ferredoxin dependent.

HvCAO gene have been characterized and the gene has been named *fch2* and *chlorina-f2* in different mutant collections (Mueller et al., 2012). Depending on the severity of the mutations the phenotype ranges from increased chlorophyll *a*/*b* ratios to accumulation of only chlorophyll *a*. Chlorophyll(ide) *b* can be converted back to Chlorophyll(ide) *a* by two sequential reactions catalyzed by chlorophyll(ide) *b* reductase (CBR) followed by 7-hydroxymethyl chlorophyll(ide) *a* reductase (HCAR) (Kusaba et al., 2007, Meguro et al., 2011).

Two genes implicated in the chlorophyll(ide) *b* reductase reaction were first identified in rice by analysis of stay-green mutants which are mutants that do not degrade chlorophyll during senescence. The first gene was named *NON-YELLOW COLORING1* (*NYC1*) and the second was identified by homology and named *NYC1-like* (*NOL*). Both encode short-chain dehydrogenase/reductase enzymes (Kusaba et al., 2007). The NYC1 protein has three trans-membrane domains. NOL only contains one catalytic domain and enzymatic activity has only been obtained with recombinant NOL which requires NADPH as a reductant and is able to convert chlorophyll *b*, chlorophyllide *b*, pheophorbide *b*, pheophytin *b*, as well as light harvesting complex II bound chlorophyll *b* to the corresponding 7-hydroxymethyl species. NOL has been localized to the stromal surface of thylakoid membranes and while NYC1 has not been localized it is presumably found in the thylakoid membrane based on the presence of the three transmembrane domains (Sato et al., 2009).

The final conversion of the 7-hydroxymethyl group to a methyl group is performed by HCAR which was first identified in the Arabidopsis genome based on its

homology to a *Synechocystis* DVR, which is unrelated to the DVR used by plants (Meguro et al., 2011). The enzyme is ferredoxin dependent and contains two 4Fe-4S clusters as well as binding FAD (Wang and Liu, 2016, Meguro et al., 2011). This inter-conversion between chlorophyll *a* and *b* is thought to be important for adapting to changing light conditions because chlorophyll *b* is only found in the light harvesting complexes, more of which are needed under low light conditions. In addition, the conversion of chlorophyll(ide) *b* to chlorophyll(ide) *a* is required for chlorophyll degradation which proceeds though pheophorbide *a* and is inhibited by pheophorbide *b* (Hörtensteiner et al., 1995). This also explains the stay-green phenotype of CBR and HCAR mutants.

Research results

My thesis work had two main tracks that were partly overlapping. The first track was the study of the cyclase step of chlorophyll biosynthesis. This step has been the least understood step of chlorophyll biosynthesis and all components required for catalysis were not known. Barley as a model organism for studying the cyclase has the advantage of a well-developed *in vitro* enzyme assay using fractionated barley plastids as well as chlorophyll mutants at two loci known to be involved in the reaction. The *Xantha-l* locus was known before the start of the project. *Xantha-l* encodes the main catalytic component of the cyclase. The *Ycf54* component was known but the function was not understood. The *Viridis-k* locus had not yet been identified but shown to be essential for the cyclase reaction. During my thesis work, I identified and analyzed the function of *Viridis-k* (Paper 2) and explored *Ycf54* to develop an *in vitro* cyclase enzyme assay based on recombinant proteins (Paper 1).

The second track of my thesis work was to develop a pipeline for the identification of the causal mutations behind the pigment deficient phenotypes in barley chlorophyll mutants. For many of these mutants little is known and once the causal mutation is identified the mutants can be used to study the biological role of the identified gene. The work on the identification of the *Viridis-k* gene (Paper 2) was important for the development of the pipeline, which was fine-tuned through the identification of the *Xantha-j* gene (Paper 3).

Paper 1

Paper 1 concerns the magnesium protoporphyrin IX monomethyl ester cyclase step of chlorophyll biosynthesis. We utilized *in vitro* enzymatic assays using fractionated barley etioplasts to establish that ferredoxin and FNR are likely involved in transferring electrons from NADPH to the cyclase enzyme XanL. This was first shown by using antibodies against ferredoxin and FNR as inhibitors of enzymatic activity. Using immunoblots, ferredoxin was localized to the soluble plastid fraction while FNR was found in both soluble and membrane fractions. Commercially available spinach ferredoxin was able to substitute for the soluble plastid fraction in enzymatic assays when combined with the membrane fraction. This showed that the cyclase is a ferredoxin dependent enzyme and that the previously unidentified soluble component is ferredoxin. Having identified an

electron donor for the cyclase, we sought to produce the catalytic component, XanL, recombinantly. When XanL was produced in *E. coli* it was not enzymatically active in assays. However, when it was co-expressed with Ycf54 the enzyme was active. Ycf54 did not co-purify with XanL suggesting a role other than being directly involved in catalysis. Although other studies also indicate that the cyclase reaction can occur without Ycf54 and that the auxiliary protein is likely to have regulatory roles (Chen et al., 2021b, Hollingshead et al., 2016), one cannot rule out that it can also have a direct although non-essential role in the reaction. Recombinant XanL and Ycf54 that were produced separately did not result in enzymatic activity when combined *in vitro*. This suggests that Ycf54 may have a role in folding or maturation of XanL *in vivo* although it may also be that the activation of XanL by Ycf54 is dependent on some factor not present in our enzymatic assays.

Paper 2

In paper 2 we identified the gene disrupted in barley *Viridis-k* mutants, which are deficient in the cyclase reaction. These mutants, although not completely blocked at the cyclase step, are able to produce only trace amounts of chlorophyll. The mutants were previously identified as deficient in the cyclase reaction because they accumulate substrate of the cyclase when fed with ALA. We identified the gene encoding the ortholog of the Arabidopsis FdC2 ferredoxin as a candidate gene and showed that the two allelic *vir-k.23* and *vir-k.170* mutants both contain mutations in the gene. That the correct gene had been identified was further confirmed by genetic complementation of the *vir-k.23* mutant by agroinfiltration, which restored chlorophyll synthesis around the infiltration site. Given that the *vir-k* mutants are deficient in the cyclase reaction, as well as the specific ortholog of FdC2, suggests that this may be the main *in vivo* electron donor. A pre-requisite for this is that FdC2/VirK must be able to transfer electrons to the cyclase. We therefore tested and confirmed that VirK can donate electrons to the cyclase in our recombinant *in vitro* assays. In addition, immunoblot analysis of the three available mutants of the cyclase enzyme, *xan-l.35*, *xan-l.81*, and *xan-l.82*, provided further evidence that VirK is associated with the cyclase enzyme in planta. The *xan-l.35* mutant accumulates less XanL protein and *xan-l.81* mutants accumulate more compared to the wild-type and both have missense mutations. The *xan-l.82* mutant has a nonsense mutation and thus accumulates no protein. In these mutants and the wild type, the protein levels of Ycf54 and VirK follow the levels of XanL which further supports that VirK is likely important for the cyclase since *in vivo* VirK levels depend on the cyclase levels. Furthermore, both *xan-l.81* and *xan-l.82* are completely blocked at the cyclase step so the changes in Ycf54 and VirK are likely to be an effect of altered cyclase enzyme levels rather than some general effect due to a block in chlorophyll biosynthesis at the cyclase step. No significant difference

in *Vir-k* mRNA levels were detected, further suggesting that the altered protein levels are not due to transcriptional changes but rather by post-transcriptional processes. VirK orthologs are conserved among oxygenic phototrophs which suggests that the protein has at least one function which is essential for oxygenic photosynthesis.

Paper 3

The use of genetic markers in plant breeding provides fast and efficient screening of breeding lines by genetic approaches compared to phenotypic methods. Preferably the genetic marker should be in the casual gene regulating a desired trait. In paper 3, we describe a relatively cheap and simple method for identification of mutated genes. We applied this on mutant *xan-j.59* but the method should be applicable for any locus where a mutant with a clear phenotype is available. The method is a variant of bulk-segregant analysis, which we apply on F_2-mapping populations. In short, a mapping population is produced and planted. In case of a recessive mutation, leaves of the homozygous mutant plants are collected and pooled. Genomic DNA is isolated from the pooled leaves and sent for genomic sequencing. SNPs linked to the mutation are searched for in the DNA sequence data, which reveals the chromosomal location of the mutation. The DNA sequence data is also likely to contain the DNA sequence information of the mutated gene. Therefore, the data is filtered for homozygous mutations in the identified linked region. In case of the *xan-j.59* sequence data, 4202 homozygous SNPs were found in the mapped region. Only two SNPs were located in high-confidence genes and caused changes of amino-acid residues in the corresponding proteins. One of the proteins was annotated as SKI family transcriptional corepressor 1 and the other as chlorophyll synthase. The later is a likely candidate gene of a yellow *xan-j.59* mutant deficient in chlorophyll biosynthesis since chlorophyll synthase catalyzes the last enzymatic step in the pathway of chlorophyll. The identification of *Xan-j* as the chlorophyll synthase gene was confirmed by sequencing of the other allelic mutants *xan-j.19* and *xan-j.64*. In *xan-j.19*, a one base pair deletion was identified in exon 13, which causes a frameshift in the gene and a truncated protein that contains 340 native amino-acid residues followed by 5 incorrect residues. Mutant *xan-j.59* has an early nonsense mutation that results in a peptide of 53 residues once the chloroplast transit peptide is removed. The missense mutation in *xan-j.64* causes an S212F substitution. An AlphaFold generated structure of the barley chlorophyll synthase showed the location of Ser-212 in relation to the binding site of the two substrates. Our model suggested that Ser-212 abolishes binding of the geranylgeranyl pyrophosphate/phytyl pyrophosphate substrate. By watering barley plants with clomazone, which is an inhibitor of chloroplastic isoprenoid biosynthesis, a white phenotype was obtained. By western blot analyses, no XanJ

protein could be detected in the white plants. We suggested that there might be a requirement of geranylgeranyl pyrophosphate/phytyl pyrophosphate binding to the active site of chlorophyll synthase to stabilize the enzyme. We further suggested that chlorophyll synthase might function as a sensor for coordinating chlorophyll and isoprenoid biosynthesis.

Future perspectives

Determining the mechanism behind how Ycf54 activates the cyclase enzyme is an important future direction of study that we made possible by the establishment of a recombinant production system. Although the system needs to be improved such that the components can be purified to homogeneity, it also opens up the possibility of further biochemical and structural studies. One interesting avenue to explore would be site-directed mutagenesis to produce variants of XanL that are unable to perform some of the reaction steps. Such mutants would be valuable for producing the reaction intermediates in usable quantities and studying the reaction mechanism of individual steps. The diiron cluster of XanL is coordinated by four glutamate residues and two histidine residues. A potential approach would be replacing the glutamates, individually and in all combinations, with aspartates in the hope that small disruptions in the diiron clusters localization may disrupt specific reaction steps. Chen et al. later published a recombinant assay system for the cyclase from the purple nonsulfur bacteria *Rubrivivax gelatinosus* (Chen et al., 2021a). Their system could also be used for studies of the cyclase reaction mechanism as it is likely to be conserved. However, as the *R. gelatinosus* cyclase is not dependent on Ycf54, their system is not suitable for studies on how Ycf54 regulates cyclase function in plants and cyanobacteria.

At first glance the results from paper 1 and paper 2 may seem contradictory since the first paper identifies canonical ferredoxin as the electron donor to the cyclase while paper 2 postulates that FdC2/VirK is the specific electron donor. The key difference is that in paper 1, ferredoxin was identified as the electron donor in *in vitro* assays with plastid extracts and also used in assays with the recombinant cyclase enzyme. These experiments are run under non-physiological conditions and tell us that regular ferredoxin can donate electrons to the cyclase enzyme. The assertion in paper 2 that VirK is likely the main electron donor comes from *in vivo* experiments utilizing barley mutants that are deficient in the cyclase enzyme or VirK. Mutations in either of these genes results in accumulation of cyclase substrate when plants are fed ALA (Steccanella et al., 2015). That combined with immunoblots showing that *in vivo* VirK accumulation is dependent on cyclase enzyme levels provides a strong indication that Virk is the main *in vivo* electron donor to the cyclase. It is likely that most, if not all, chloroplastic ferredoxins can donate electrons to the cyclase *in vitro*. We have tested and confirmed that this is possible with a number of different recombinant barley ferredoxins (Unpublished) and in assays with the recombinant *R. gelatinosus* cyclase reported by Chen et at., they used spinach or recombinant *Anabaena* ferredoxin. Future studies could thus use recombinant systems to test kinetics or binding energies for the different

chloroplastic ferredoxins which may provide some indication of the specificity of VirK as the donor to the cyclase enzyme. However, it is important to keep in mind that such experiments would not be representative of physiological conditions. *In planta*, there are likely to be many competing interaction partners so the efficiency with which a specific ferredoxin can donate electrons to the cyclase will be strongly influenced by the levels of competing electron acceptors. Therefore, such experiments would ideally be combined with *in vivo* experiments. For example, a systematic knockout of all ferredoxin genes could be done to confirm that the cyclase deficient phenotype is specific to FdC2/VirK mutants. Although beyond the scope of the present thesis work, such a collection of mutants would additionally be a valuable resource for future studies of specific roles of individual ferredoxins.

While VirK is likely the main *in vivo* electron donor to the cyclase enzyme it remains to be determined where those electrons originate from. One possibility is that the main electron donor to VirK *in vivo* is photosystem I. In the dark, chlorophyll biosynthesis halts, at least in angiosperms, at the step after the cyclase since POR is light dependent. Having a dedicated ferredoxin mainly reduced by PSI would thus decrease competition for reducing equivalents from NADPH in the dark when cyclase activity is not needed. Consistent with this idea, ALA feeding of the *vir-zb.63* mutant, that is deficient in PSI activity, indicates a deficiency at the cyclase reaction, particularly in the light (Steccanella et al., 2015). Further studies of this mutant could provide valuable insights into the involvement of photoreduced VirK in the cyclase reaction.

Compared to the cyclase enzyme, considerably more is known about the chlorophyll synthase. Several biochemical studies have been published in which an enzymatic assay was established based on recombinant enzymes (Oster et al. 1997). Substrate specificity (Oster et al. 1997), cofactor requirement and essential amino-acid residues were analyzed (Schmid et al. 2001). Also, the substrate binding order was determined (Schmid et al. 2002). It is striking that structural data of the chlorophyll synthase is not available, which is most likely because the protein is a membrane bound enzyme and probably challenging to crystalize. We used AlphaFold to predict a 3D model of the barley chlorophyll synthase, which can be regarded as a theoretical alternative to x-ray crystallography. In order to get more detailed information about the chlorophyll synthase and its reaction mechanism, future studies should focus on structural studies since that would reveal key information about substrate binding and important amino-acid residues in the active site and of structural importance.

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