Expression of linear and cyclic electron flow components in the bundle sheath chloroplasts along the developmental leaf gradient of maize (*Zea. mays*)

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Abstract During plant photosynthesis, ATP can be produced in the chloroplast by linear electron flow (LEF), as well as cyclic electron flow (CEF). As a C4 plant, maize (*Zea mays*) utilizes two distinct cell types, known as bundle sheath (BS) and mesophyll (M) cells, to facilitate the photosynthetic activity. BS cells function to fix the carbon dioxide in the form of reduced carbohydrates and carry out mostly CEF for ATP production. In contrast, M cells act as a carbon pump and provide carbon dioxide in the form of the C4 acid malate to BS cells and use LEF for ATP production. BS and M chloroplasts develop and differentiate from base to tip. In this honors thesis, I tested the hypothesis that with progressive leaf development, levels of Photosystem II proteins (OEC23, OEC33, D1, CP47) involved in LEF decrease in BS chloroplasts, while CEF proteins (NDH-L and PGR5) in the BS chloroplasts increase. This serves to switch from linear to cyclic electron flow in the BS chloroplasts along the leaf developmental gradient. Additionally, I investigated relationships between mRNA and protein levels of the selected CEF and LEF components to understand transcriptional and translational controls. Proteins and RNA were extracted from BS strands isolated from four different leaf sections of 9 day old plants and analyzed by western blot and reverse transcript – polymerase chain reactions (RT-PCR). My analysis showed increased levels of mRNA and proteins of all measured components from base to the middle of the leaf, followed by additional increase or decrease towards the leaf tip. These results suggest both transcriptional and post-transcriptional control mechanisms in maize leaf development. My data also suggest that BS cells isolated near the tip were not fully differentiated at the sampling stage.

Abbreviations

LEF	linear electron flow
CEF	cyclic electron flow
BS	bundle sheath
M	mesophyll
RT-PCR	reverse transcript – polymerase chain reactions
PSII	photosystem II
OEC	oxygen evolving complex
PGR5	proton gradient regulation5
PEP	phosphoenolpyruvate
PEPC	PEP carboxylase
NADPH	nicotinamide adenine dinucleotide phosphate hydride
NDH	NAD(P)H dehydrogenase complex

Introduction

In C3 plants, light-driven photosynthesis occurs in one cell type where activated electrons are fixed in nicotinamide adenine dinucleotide phosphate hydride (NADPH) by passing through photosystem II (PSII), cytochrome b6f (Cytb6f), photosystem I (PSI) and ferredoxin NADP reductase (FNR) with the assistance of plastoquinone (PQ), plastocyanin (PC) and ferredoxin (Fdx). Consequently, a proton gradient across the thylokaid membrane is created which subsequently leads to the synthesis of ATP by

ATP-synthase. In addition, two photosynthetic cyclic transport pathways, which generate ATP but not NADPH, have been identified in C3 plants: a pathway dependent upon ferredoxin:plastoquinone oxidoreductase (FQR) activity and the NAD(P)H dehydrogenase complex (NDH)-dependent pathway. In the FQR pathway, electrons activated in PSI are transferred to Fdx and instead of transferring electrons to $NADP^+$, reduced Fdx interacts with the FQR which allows the transfer of electrons into the quinone pool. Proton gradient can be created as a result of plastoquinol oxidation by the Cytb6f complex, driving ATP production by the ATP synthase. Recent studies have shown that a novel protein named proton gradient regulation5 (PGR5) is essential for the FQR-dependent CET (Munekage et al., 2004, 2002) and that overexpression of PGR5 increases the FQR-dependent CET (Okegawa et al., 2007, 2008). In the NDH dependent pathway, the NDH complex derives electrons from NAD(P)H or Fdx and subsequently transfer them into the PQ pool. Like the FQR dependent pathway, a proton gradient is generated as a consequence of plastoquinol oxidation, generating ATP (Fig 1). In Arabidopsis, Shimizu et al. (2008) proved that NDH-L is essential for stabilizing the NDH complex (Shimizu et al., 2008).

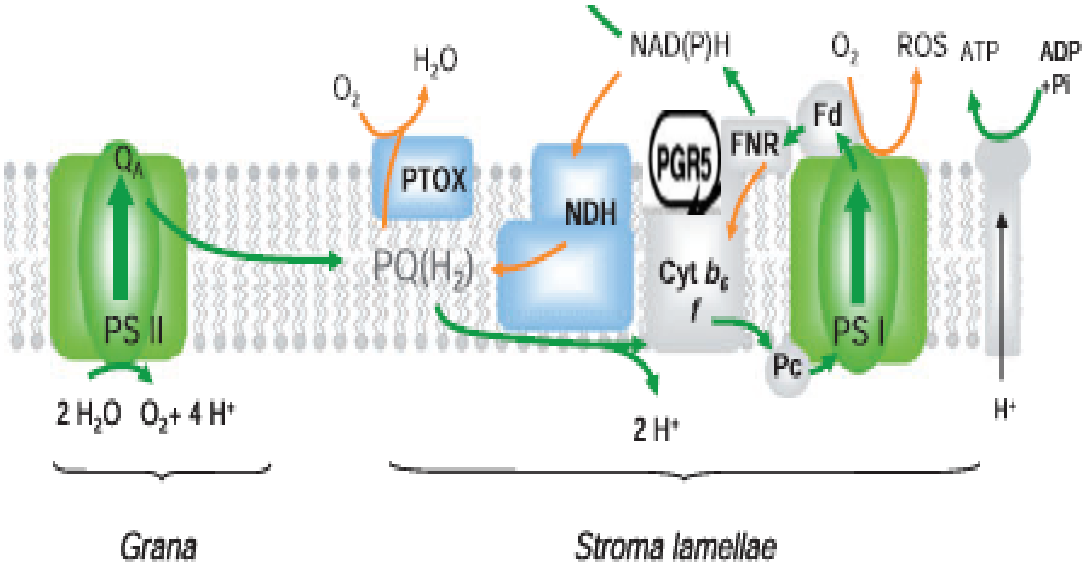

Figure 1: Photosynthetic electron flow in the chloroplast. In the chloroplast, the linear electron flow is PSII dependent whereas the cyclic electron flow is PSII independent. PGR5 and NDH regulate two separate cyclic electron flow pathways.

Certain taxa have developed a more efficient process of photosynthetic carbon fixation, known as C4 photosynthesis. In leaves of the C4 species maize (*Zea mays*), photosynthetic activities are partitioned between morphologically and biochemically distinct bundle sheath (BS) and mesophyll (M) cells. A single ring of BS cells surrounds the vascular bundle. Surrounding the BS are one or two concentric rings of specialized M cells, forming the classical Kranz anatomy (for review see Edwards and Walker, 1983) (Fig 2).

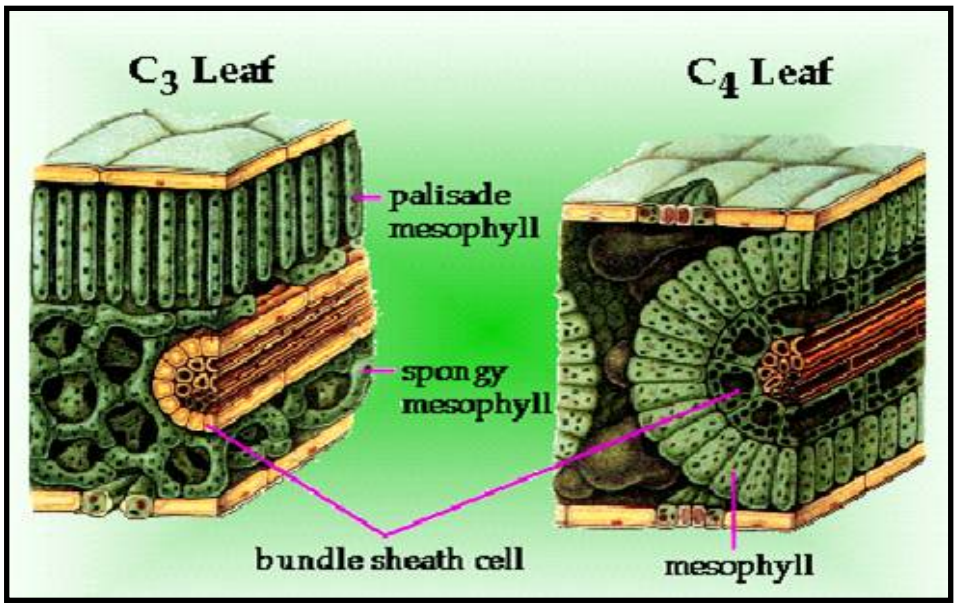

Figure 2: Kranz anatomy in C4 leaves. A fully developed maize leaf contains a single ring of BS cells surrounding the vascular bundle. Surrounding the BS cells are one or more concentric rings of M cells.

In addition to their difference in distribution within the leaf, these two types of cells are distinct in terms of their chloroplast features. The BS cells containing centrifugally arranged chloroplasts with large starch granules and unstacked thylakoids, while the M cells contain randomly arranged chloroplasts with stacked thylakoids and little to no starch (Edwards and Walker, 1983) (Fig 3). The oxygen-producing complex Photosystem II (PSII) predominantly accumulates in fully differentiated M chloroplasts and participates in linear electron flow (LEF). In contrast, BS chloroplasts contain only low amounts of active PSII complexes and therefore have low rates of LEF and low rates oxygen evolution (Majeran et al., 2005).

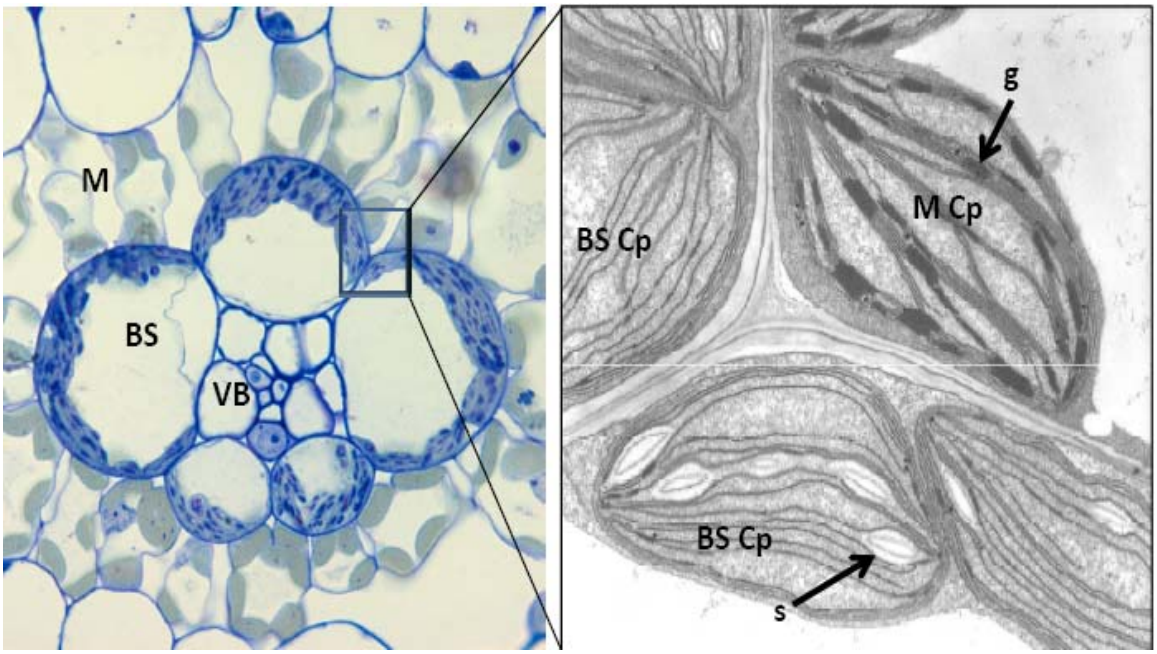

Figure 3: Different chloroplast features of BS and M cells. In the fully developed maize leaf, the BS cells centrifugally arrange chloroplasts with large starch granules and unstacked thylakoids, while the M cells randomly arrange chloroplasts with stacked thylakoids and little to no starch.

M and BS cells each accumulate a distinct set of C4 photosynthetic enzymes that enables them to cooperate in photosynthesis. The specific metabolic functions of differentiated BS and M chloroplasts were studied extensively by Majeran et al. (2005). As expected, there is a preferential BS chloroplast localization of proteins involved in the Calvin cycle such as Rubisco, phosphoribulokinase, and sedoheptulose-1,7-bisphosphatase, as well as enzymes involved in the oxidative pentose phosphate pathway (OPPP) such as transketolase. The malate-pyruvate shuttle involves enzymes PEP carboxylase (PEPC) and pyruvate phosphate dikinase (PPDK), which preferentially accumulate in M cells (Fig 4).

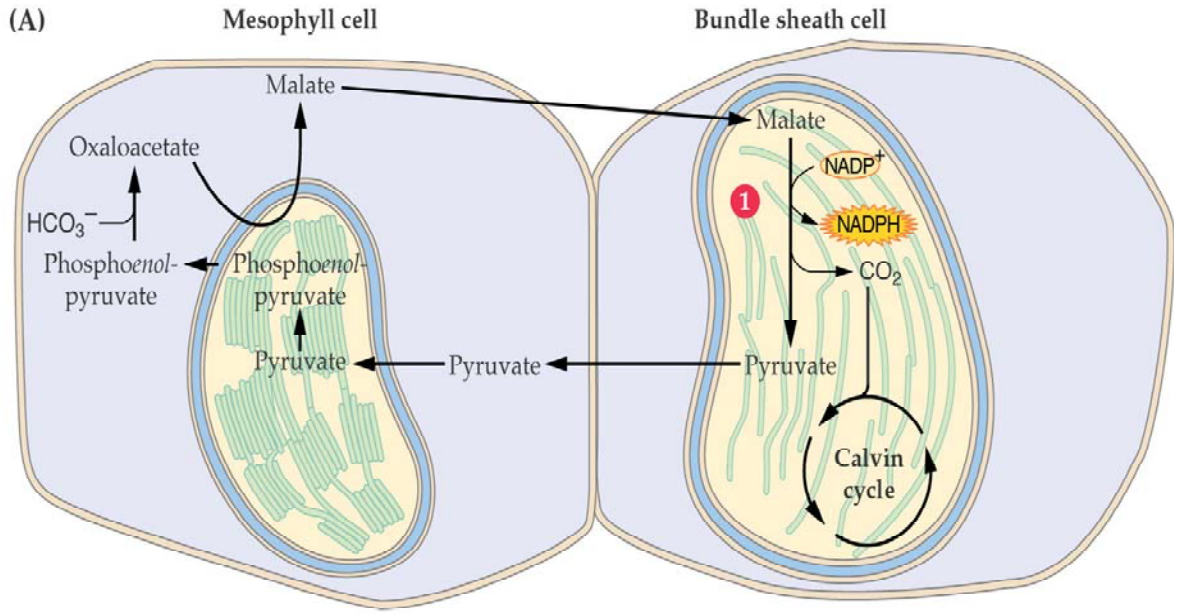

Figure 4: The malate-pyruvate shuttle and localization of the Calvin cycle in C4 leaves of maize. In the fully developed maize leaf, the BS cells specifically accumulate Rubisco and other Calvin cycle enzymes, while the M cells specifically express PEPC involved in the carboxylation of phosphoenolpyruvate, resulting in oxaloacetate production. Reduction of oxaloacetate in the M chloroplasts leads to the C4 acid malate. Decarboxylation of malate in the BS chloroplast results in increase of CO2 in the BS chloroplasts.

In addition to being a carboxylase, Rubisco also functions as an oxygenase to react with oxygen, a process called photorespiration. What is more, these two substrates, carbon dioxide and oxygen compete for the same active site on the enzyme and the activity of Rubisco towards these substrates is determined by the relative amount of oxygen and carbon dioxide surrounding Rubisco, as well as temperature. Thus, the localization of Rubisco in the BS cells, which combines the low BS oxygen evolution rates and the high CO_2 concentration through import of inorganic carbon, improves

carbon fixation efficiency and suppresses photorespiration in BS cells. It was demonstrated that seven chloroplast-encoded and three nucleus-encoded maize NDH subunits show preferential BS expression with an average BS/M ratio of 3.0 (Majeran et al., 2008).

In maize (and other grasses), leaf differentiation progresses along a developmental gradient from base to tip, with undifferentiated cells that contain proplastids at the base, and fully differentiated cells containing chloroplasts at the leaf tip. The differentiation of BS and M cells into the formation of Kranz anatomy has been studied, but its regulation is still poorly understood. It is suggested that light has a significant role in C4 photosynthetic differentiation since maize leaves grown in the dark contain low levels of Rubisco in both M and BS cells while the presence of other C4 enzymes is barely detectable (Sheen and Bogorad, 1986). Mutagenesis studies resulted in the identification of genes that play a role in BS differentiation including *bundle sheath defective1 (bsd1)* (Langdale and Kidner, 1994) and *bundle sheath defective2 (bsd2)* (Roth et al., 1996). Lastly, it has been found through the *tangled1* mutant that the specification of BS cells is derived from procambial strand precursors and dependent on vein proximity (Jankovsky et al., 2001). These studies and others collectively suggest that there is a developmental gradient along the leaf with the youngest, incompletely differentiated cells at the etiolated base, followed by a C3-like region at the beginning of the greening section, and then fully differentiated C4 chloroplasts at the tip (Fig 5). Along the C4 developmental gradient, there are other developmental and metabolic gradients such as the source-sink gradient.

Although the differentiation of BS and M cells and the specific metabolic functions of mature BS and M chloroplasts have been studied, the specific details of how the cells develop and differentiate metabolically remain to be elucidated.

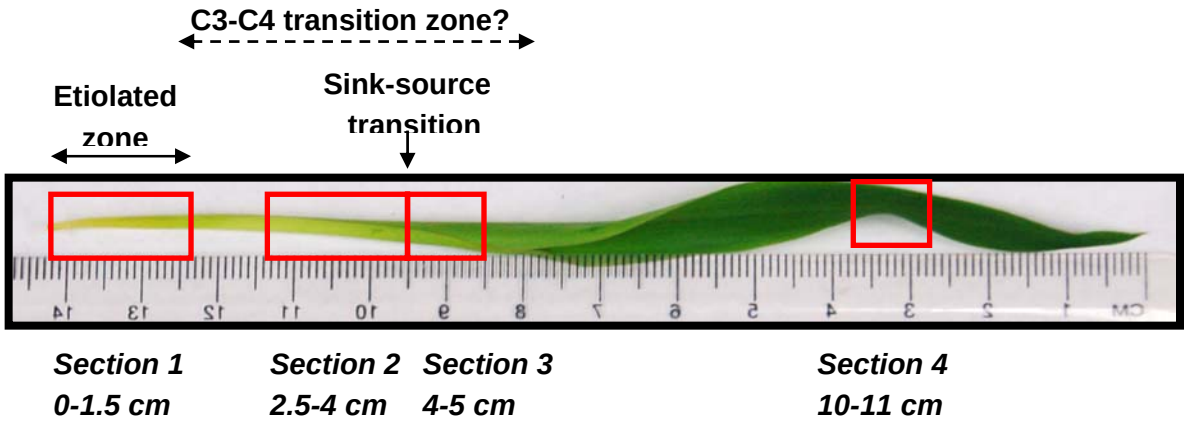

Figure 5: Example of the 3rd leaf of 9 day old maize seedlings and selection of leaf sections for analysis. BS strands from the four indicated leaf sections, representing four different maize developmental stages, were purified.

The regulation of photosynthetic gene expression has been studied intensively. Over the past decade, experimental evidence has accumulated to support intercompartmental control which involves signals from both the nucleus and the chloroplast (for review, see Eberhard et al., 2008). Further, it is reported that this intercompartmental regulation functions at both the translational and transcriptional levels (Beck, 2008; Eberhard et al., 2008; Wobbea et al., 2008).

The goal of this thesis project was to test the hypothesis that with progressive leaf development, levels of Photosystem II proteins (OEC23, OEC33, D1, CP47) involved in LEF decrease in BS chloroplasts, while CEF proteins (NDH-L and PGR5) in the BS chloroplasts increase. Furthermore, I investigated relationships between mRNA and

protein levels of the selected CEF and LEF components to understand transcriptional and translational controls. PEPC was used as marker for M cells. Proteins and RNA were extracted from BS strands isolated from four different leaf sections of 9 day old plants and analyzed by western blot and reverse transcript – polymerase chain reactions (RT-PCR). My analysis showed increased levels of mRNA and proteins of all measured components from base to the middle of the leaf, followed by additional increase or decrease towards the leaf tip. These results suggest both transcriptional and post-transcriptional control mechanisms in maize leaf development. My data also suggest that BS cells isolated near the tip were not fully differentiated at the sampling stage.

Results and Discussion

Isolation of BS strands and assessment of quality by light microscopy examination and western blots

BS strands were purified mechanically from four sections of the 3rd leaf of 9 days B73 plants. Sections of about 20-30 leaves were pooled for each replicate. These four sections correspond to the undifferentiated etiolated section (section 1), sections before (section 2) and after the sink-source transition point (section 3), and the differentiated and fully greened section of the source leaf zone (Fig 5). The C3-C4 transition point of BS and M cells is not clear but is likely to precede the sink-source transition zone (between section 2 and 3) and involves induction of enzymatic activities, as well as formation of cellular

structures, such as plasmodesmata and a thickened cell wall for BS cells. Comparing the samples isolated from the four leaf sections clearly shows increased amount of chlorophyll-containing chloroplasts from base to tip, consistent with greening (Fig 6).

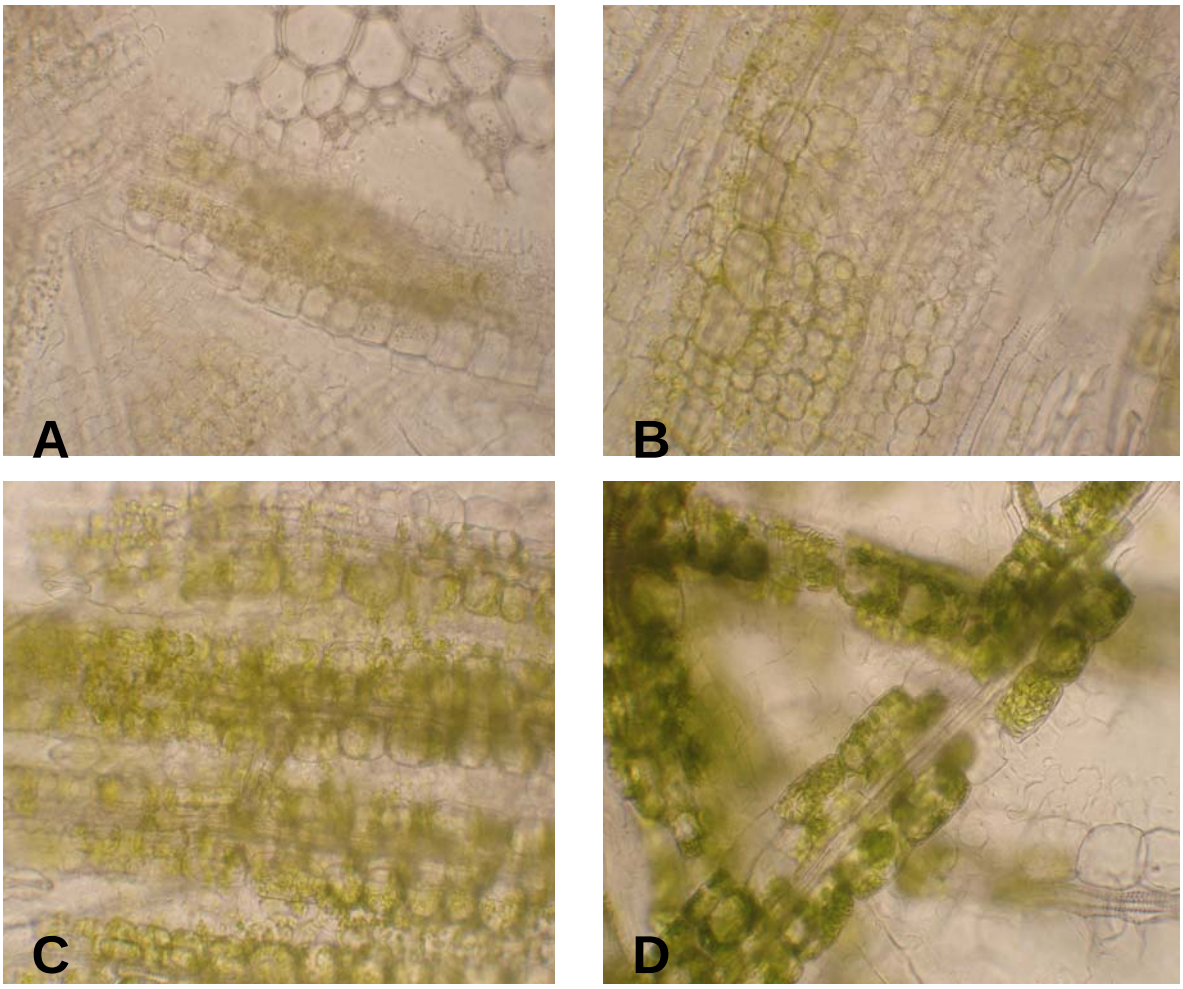

Figure 6: Microscopy examination of purified BS samples. A: section 1 (0-1.5cm); B: section 2 (2.5-4cm); C: section 3 (4-5cm); D: section 4 (10-11cm).

Microscopy examination further showed that the BS strands in sections 1 and 2 were not very distinct and not well developed, resulting in a mixture of different cell types (Fig 6A, B). Thus the enrichment of BS strands in samples of sections 1 and 2 was relatively low. In section 3, BS strands are clearly visible and appear to be enriched. The shape of the chloroplast containing cells in the bundle sheath was round and not yet the more rectangular shape observed in section 4 (Fig 6C). In section 4, a strong enrichment of mostly intact BS strands was observed, with a minor contamination of cells that appeared to have lost their cellular content (Fig 6D). Thus we conclude that the purity (or enrichment) of BS strands dramatically increased from base to tip sections and that chloroplast development was strongly induced from base to tip.

To further evaluate the induction of C4 pathways and the purity of the BS strands, total cellular proteins were extracted from each section using SDS and other denaturing components. An equal amount of proteins from each section was analyzed by 1-D SDS polyacrylamide gel electrophoresis (PAGE) followed by western blot using a specific antiserum against the M-specific protein marker PEPC. In addition, I also included a protein extract from the total leaf section 1 and 4; these samples represent the whole cellular population in each section. In total three independent experiments were carried out. Fig 7 shows the PEPC western blot data for each of these experiments.

The PEPC protein content of the BS section 4 is on average 47% of that of the corresponding whole leaf section, suggesting on average a two-fold enrichment of BS cells (Fig 7). We did observe some variation of purity between the independent replicates,

with the highest purity in the last preparation (prep 3) (Fig. 7). Had more time available, I would have made additional independent preparations and only used the best three preps.

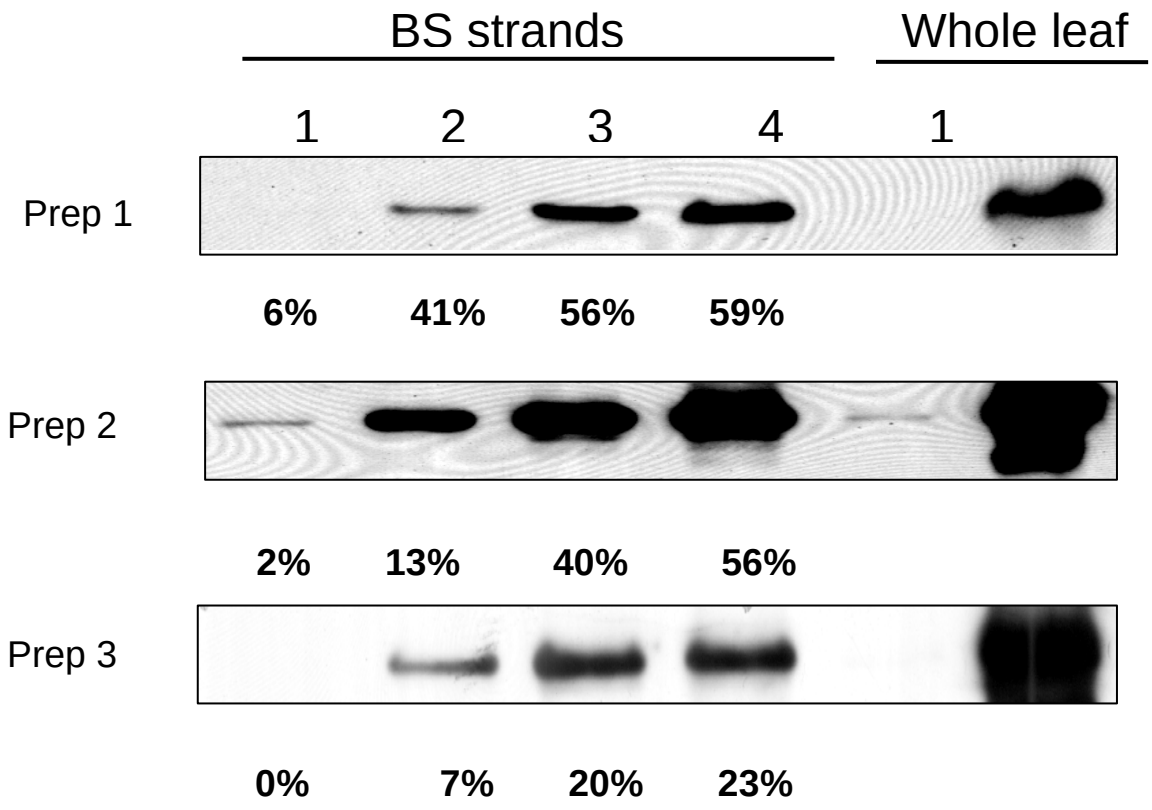

Figure 7: Presence of PEPC in BS strands as compared to whole leaf sections using a western blot in three biological replicates (prep 1, 2, 3). From left to right, the bands correspond to section 1 (0-1.5cm), section 2 (2.5-4cm), section 3 (4-5cm), section 4 (10-11cm), whole leaf base (0-1.5cm) and whole leaf tip (10-11cm) respectively. Equal amounts of protein were loaded in each lane. The relative accumulation levels (in %) of analyzed proteins in the BS samples as compared to the whole leaf section 4 are indicated.

Development of protocols for mRNA purification and linear amplification by RT-PCR

In order to study transcriptional regulation of gene expression, a protocol for purification of intact total RNA was developed. The purified RNA was treated by DNase to ensure that in particular chloroplast DNA did contribute to the quantification of transcript (this is

particularly important for chloroplast genes since many do not contain introns). Since chloroplast-encoded transcripts do not have a polyA tail, random primers (as opposed to polyA primers) were used for cDNA synthesis. Considerable time and effort was invested in optimization of cDNA synthesis and primer design for linear amplification of specific transcripts (cDNAs). Control experiments confirmed that chloroplast DNA did not contribute to the transcript quantification (data not shown).

Expression of the nuclear-encoded malate-pyruvate shuttle component PEPC

The results of the mRNA profiling show up-regulation of PEPC mRNA levels from section 1 to section 2, indicating that in the C3-like developmental stage (prior to formation of differentiated BS strands as shown by the microscopy in Fig 6), there is an increase in the PEPC transcription or stabilization. In subsequent developmental stages, the PEPC transcript level decreases at a constant rate, suggesting that the differentiation of the bundle sheath triggers the down-regulation of PEPC transcription and/or increases the PEPC mRNA degradation (Fig 8A). One mode of the transcriptional regulation depends on nuclear factors (Mitchell and Tjian, 1989). Three kinds of nuclear factors, MNF1, MNF2a and MNF2b, interact with the 5'-flanking promoter region of the maize PEPC and may regulate the gene expression (Yanagisawa and Izui 1990, 1992). Another mode for selective transcription of PEPC is DNA methylation (Ngernprasirtsiri et al., 1989). Methylation of PEPC occurred in the bundle sheath (particularly at the final stages of tissue differentiation), but not in the mesophyll (Yanagisawa and Izui, 1990).

It is predicted that PEPC protein level should be up regulated in both BS and M cells before entering the bundle sheath lineage, and down-regulated after the C3-C4 transition point. Surprisingly, the PEPC protein level is increasing along the gradient from the base to the tip with the maximum change per centimeter in the transition sections (Fig 8A). This likely indicates that the leaf section 4 does not represent fully differentiated cells. In addition the strong induction of PEPC in M cells in section 4, combined with the varying contamination of M cells in the purified samples also contributes to this increase.

The changes of PEPC mRNA and protein levels show a different dynamics and indicate the dominance of translational control of the PEPC gene during bundle sheath development. Sheen and Bogorad (1987) showed that in mature mesophyll cells the maximum accumulation of PEPC transcript precedes that of the corresponding protein, indicating that PEPC is translationally regulated. My data suggest that PEPC expression in the BS cells is regulated in a similar way.

Expression of the central enzyme of the Calvin cycle, chloroplast-encoded RBCL

The mRNA level of RBCL increased 5-fold along the developmental gradient (Fig 8B). From the base to section 2, when etioplasts begin to develop into chloroplasts, the RBCL mRNA level is slightly up regulated. From section 2 to section 3, the RBCL transcript level increases very strongly, coinciding with significant greening of the plastids. From section 3 to the tip, mRNA continued to increase.

In terms of protein level, RBCL shows up-regulation along the gradient, which is in agreement with the metabolic role of BS cells in carbon dioxide fixation (Fig 8B). In section 4, RBCL levels are 4-5 fold higher than in total leaf section (Fig 8B), consistent with near exclusive accumulation in BS cells. Also, the protein changes show a similar pattern as the mRNA changes in the sense that the transition sections (from 2 to 3) have the highest increase per centimeter, and the C3-like sections at the base have the lowest. The mRNA and protein results reveal a translational control of RBCL protein accumulation. For the first two sections, the protein accumulation is initially slower than mRNA accumulation and as it gets closer to the transition point, protein accumulation gradually catches up and exceeds the mRNA accumulation. These results together suggest different translational mechanisms held by proplastids/etioplasts and mature chloroplasts. Cheniclet and Carde (1987) showed that the number of ribosomes increases as proplastids differentiate into chloroplasts. Hence, in the base, the number of ribosomes might be a bottle-neck for translational activity. As proplastids develop into chloroplasts, more ribosomes become available, releasing this bottleneck. In addition, their roles in carbon metabolism might participate in deciding their regulation pattern. Although the C3–C4 transition in Rubisco gene expression in maize leaves is not necessarily correlated with sink–source transition (Patel and Berry, 2008), the overall pattern of sink to source transition from the tip to the base still exists. The sink-specific gene sucrose synthase (SUS), involved in breakdown of imported sucrose from the source areas, is abundant in the leaf base and decreased from base to tip (Hardin et al., 2003). Thus, accelerating

protein synthesis of RBCL is a good strategy to fulfill the carbon fixation requirement and formation of source leaf sections.

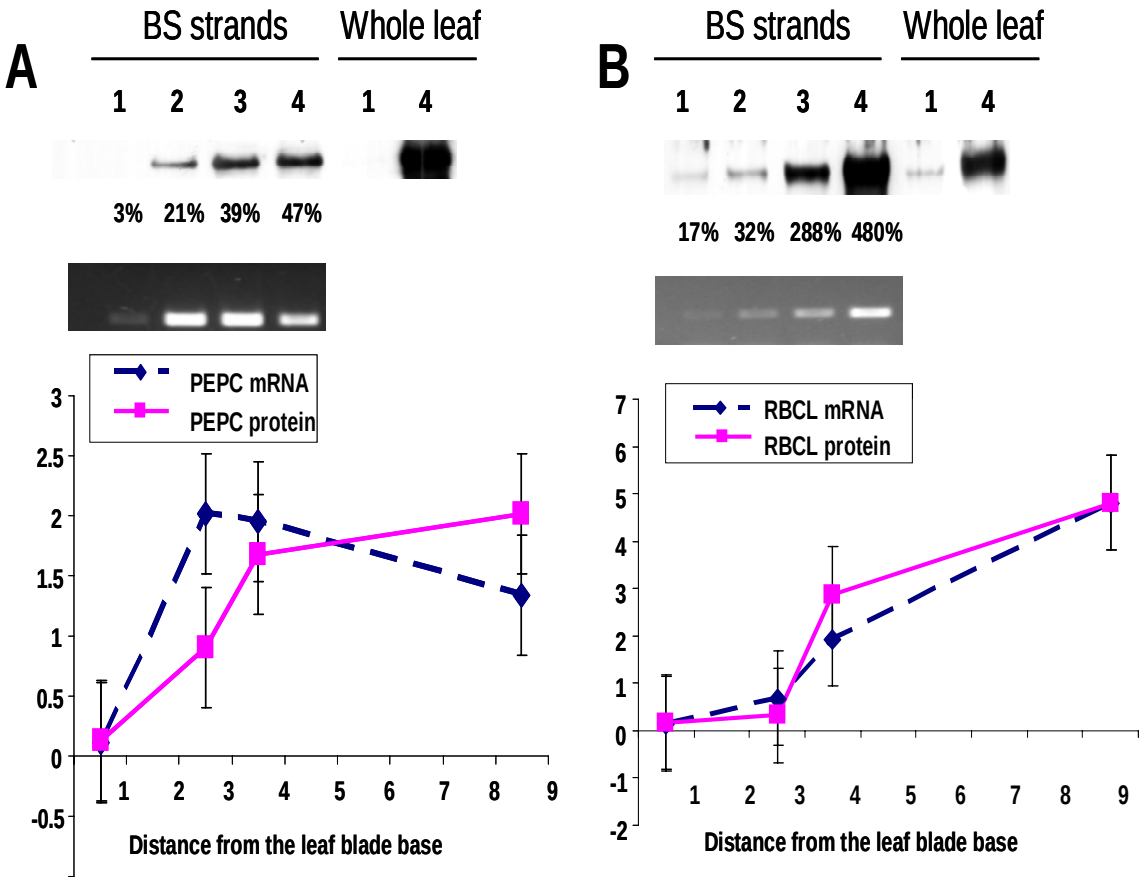

Figure 8: The expression pattern of PEPC (A) and RBCL (B) mRNA and protein: the top panel represents the western blot bands and the bottom panel represents the RT PCR bands. From left right, the bands correspond to section 1 (0-1.5cm), section 2 (2.5-4cm), section 3 (4-5cm), section 4 (10-11cm) respectively. For comparison, the PEPC and RBCL protein levels in the total cell extracts in

Expression of nuclear-encoded and plastid-encoded PSII components

Chloroplast-encoded PS II D1 (psbA) mRNA shows up-regulation from the base to the tip (Fig 9A). The change per centimeter is relatively small from the base to the section 2 and afterwards it increases to a constant value. It is known that psbA transcripts are remarkably stable with a half-life ranging between 10 and 40 hours (Mullet & Klein, 1987; Klaff and Gruissem, 1991). Therefore, the increase of PS II D1 mRNA is very likely to be a consequence of increased transcription as BS cells and their chloroplasts develop. Surprisingly, PS II D1 protein levels increased from the base to the tip. Whereas I expected a decrease towards the tip, mRNA and protein show similar dynamics from base to tip.

CP47 mRNA shows up-regulation from the base to the section 2 and down regulation from section 3 to the tip (Fig 9B). Furthermore, in the first three sections, the increase per centimeter decreases as it moves towards the tip. CP47 protein is slightly up-regulated from the base to the transition section and is considerably up regulated from there (Fig 9B).

In particular for CP47, the protein synthesis efficiency (protein/mRNA) is relatively low at the early stage. This might be related to chlorophyll synthesis. As chlorophyll binding proteins, it is has been well documented that the synthesis of D1 and CP47 can only take place if there is sufficient supply of chlorophyll a (Eichacker et al., 1990; Kim et al., 1994). At the early stage when proplastids/etioplasts are still developing into chloroplasts, chlorophyll synthesis is insufficient, therefore restricting the synthesis of D1

and CP47 proteins. Another limiting factor of their early translation is the insufficient ribosome number mentioned before.

Nuclear-encoded OEC23 and OEC33 are two co-regulated genes (Fig 9C, D) that have similar transcript accumulation patterns as PEPC (Fig 8A), which suggest these three nuclear-encoded genes might have a similar transcriptional regulation mechanism. Their protein levels increase towards the tip, which is in contrast to the hypothesis that these two subunits should become less abundant after the sink-source and C3-C4 transition zones (Fig 9C, D). However, protein levels in section 4 of the BS strand were 31-46% lower than in the total cell extract from section 4, indicating that OEC23 and OEC33 preferentially accumulate in M cells. The discrepancy between my expectations and the obtained results might be attributable to their relative stability in the thylakoid lumen. Indeed, a pool of free, unassembled OEC proteins capable for assembly has been found in the thylakoid lumen (Akiko Hashimoto, et al., 1997) and it was suggested that these unassembled subunits function as a store to ensure rapid reassembly of functional, oxygen-evolving PSII complexes during the PSII repair cycle. Because protein assay is carried out on proteins from the entire bundle sheath cells, hence, it is possible that the amount of OEC subunits incorporated into thylakoids PS II complex is decreasing and this trend is overwhelmed by the increase in unassembled OEC subunits. OEC subunits exhibit transcriptional control from the base to the section 2 and translational control from section 2 to the tip. It reveals that there might be some unidentified BS specific lineage factor that promotes protein turnover of OEC subunits.

The four PS II subunits all increased in the BS strands along the leaf gradient. Since the harvested leaf 3 is relatively young and since photosynthetic activity increases from the leaf base towards the tip, PS II subunits are generally upregulated from the base to the tip at this age. However, compared to the total leaf extract in section 4, they were clearly down-regulated in BS section 4 (between 49 and 12%), indicating that they were down-regulated in the strands as compared to M cells. Nevertheless, we did expect that these proteins levels for these subunits would decrease from section 3 to 4 in the BS strands, as PSII activity is down-regulated in the context of C4 cell specialization. This suggests that BS cells and chloroplasts had not reached there fully differentiated state. Furthermore, a recent study by the Van Wijk lab (Majeran et al., 2008) showed that accumulated PSII subunits in BS cells do not appear to be assembled in a complete PSII complex. Therefore, the actual activity of the PS II complex might be decreasing towards the BS tip, even if the measured subunits increase their accumulation level. Recent researches discovered that PsbT, a low molecular weight chloroplast-encoded protein, is required for the efficient repair of photodamaged PSII and the absence of PsbT caused the loss of PS II activity and PS II protein subunits (Monod et al., 1994; Ohnishi and Takahashi, 2001).

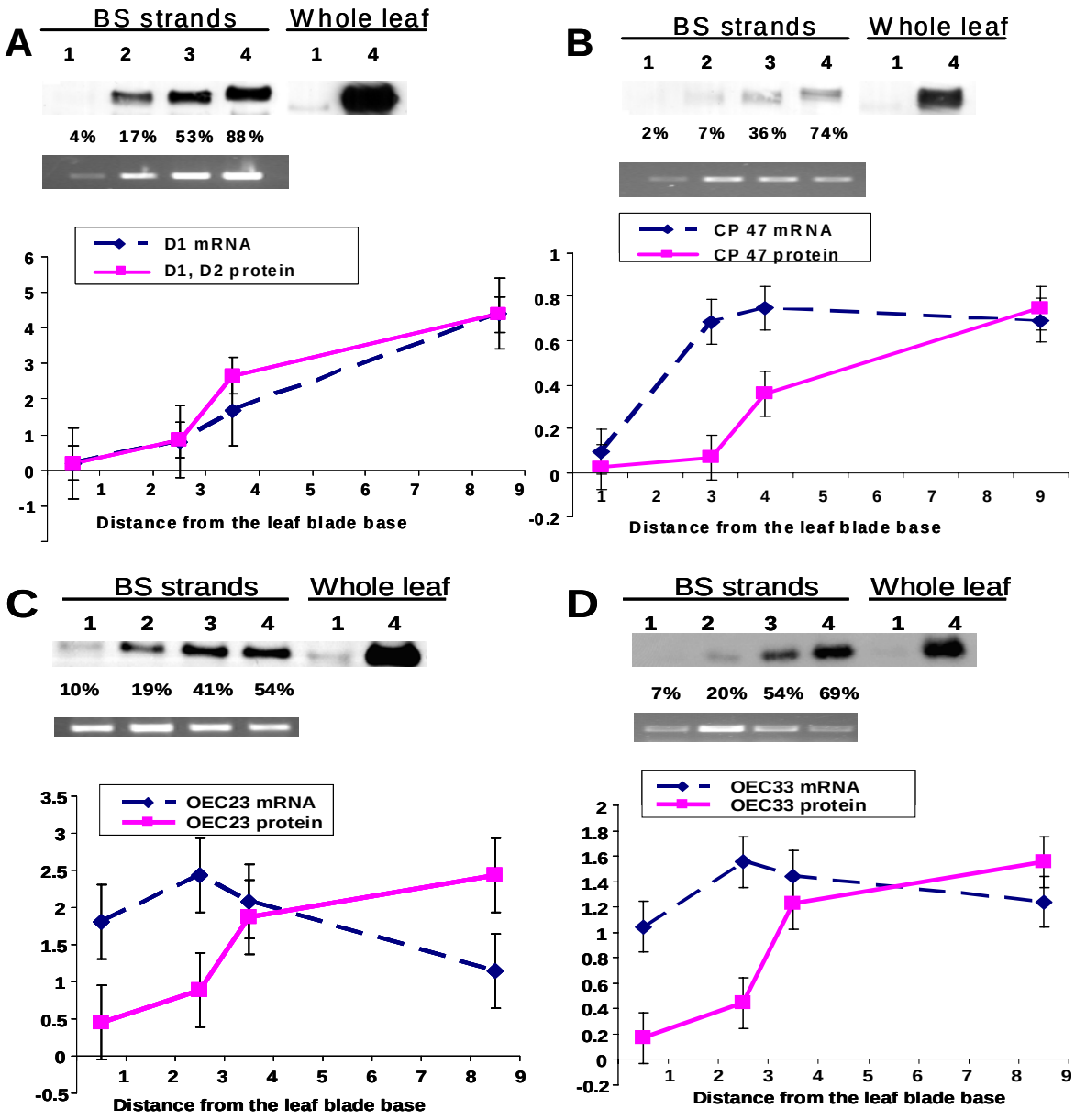

Figure 9: The expression pattern of selected PSII subunits, D1 (A), CP47 (B), OEC23 (C) and OEC33 (D): the top panel represents the western blot bands and the bottom panel represents the RT PCR bands. From left right, the bands correspond to section 1 (0-1.5cm), section 2 (2.5-4cm), section 3 (4-5cm), section 4 (10-11cm) respectively. For comparison, the D1, CP47, OEC23 and OEC33 protein levels in the total cell extracts in sections 1 and 4 are shown. The graph is the combination of the mRNA and protein intensities after normalization. The relative accumulation levels (in %) of analyzed proteins in the BS samples as compared to the whole leaf section 4 are indicated.

Expression of nuclear-encoded PGR5 and NDH-L involved in NDH-independent and NDH-dependent cyclic electron flow

Overall, PGR 5 transcript showed early up-regulation along the developmental gradient (Fig 10B). This suggests that the PGR 5 transcript might be subject to lineage dependent transcription regulation or stabilization. Similar to the mRNA, PGR 5 protein is up-regulated from the base to the tip, ultimately resulting in 85% higher accumulation levels in BS strands than in total leaf extract in section 4 (Fig 10B). This indicates a strong preferential accumulation in BS strands as compared to M cells. However, the increase occurred mostly from section 3 to 4, indicating a strong post-transcriptional control (Fig 10B). Because PGR 5 dependent cyclic electron flow produces ATP, this pattern might correspond to the energy demand of bundle sheath cells for carbon fixation. In addition, Munekage et al. (2002) proposed that in Arabidopsis, PGR5 is essential to prevent photosystem I photoinhibition. As maize PGR5 protein showed high homology to Arabidopsis PGR 5 protein, it is highly likely that maize PGR5 is the functional homologue. In the base, undifferentiated proplastids barely contain chlorophyll and therefore, the base section can hardly absorb light and activate electrons for photosynthesis. Hence, PGR5 is unnecessary for energy production and photoprotection there. As cells become more differentiated, more PGR5 is produced to satisfy the energy demand and photoprotection. Compared with the tip section, the transition sections are closer to the sink as well as contain less chlorophyll. Thus, less PGR 5 is required for them compared with the tip section.

The results suggest that PGR5 is regulated on the translational level along the entire gradient. The translational signals are likely to be retrograde signals from the chloroplast or controlled proteolysis in the chloroplast. These retrograde signals have only been shown to regulate the transcription activity, the translation events might be a secondary effect. For example, one signal is the reactive oxygen species (ROS) produced in the course of photosynthetic electron flow. Gadje et al. (2006) identified the up-regulation of transcription factors stimulated by ROS in Arabidopsis via microarray. With the increase in photosynthetic activity from the base to the tip, ROS production increases as well.

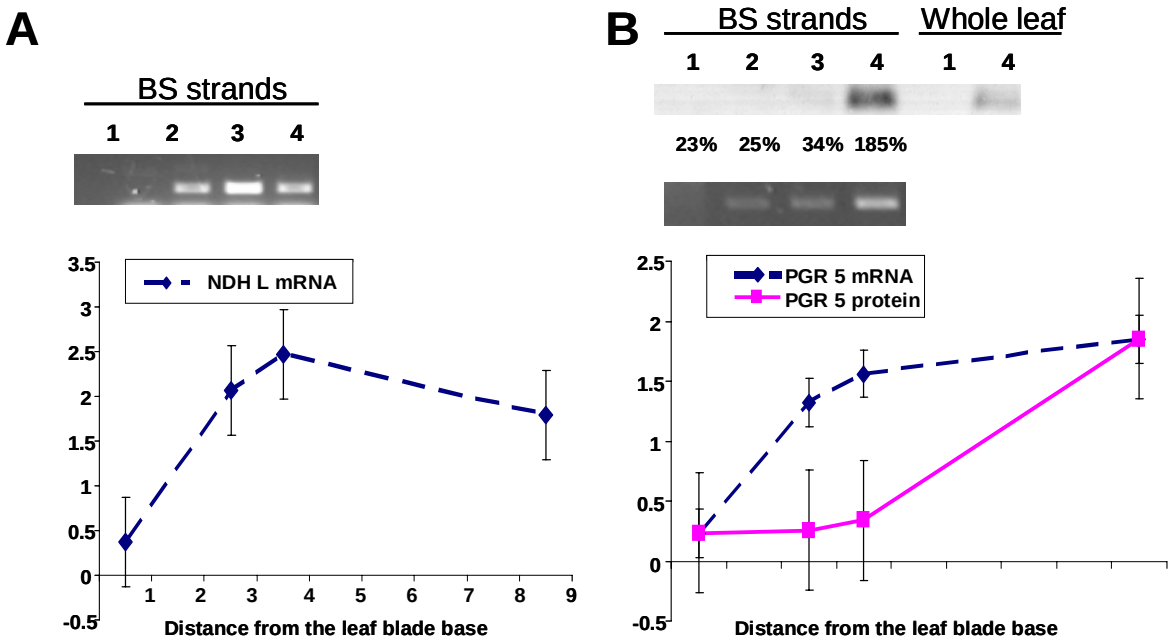

Figure 10: The expression pattern of photosynthetic cyclic electron components NDH-L (A) and PGR5 (B): the top panel represents the western blot bands and the bottom panel represents the RT PCR bands. From left right, the bands correspond to section 1 (0-1.5cm), section 2 (2.5-4cm), section 3 (4-5cm), section 4 (10-11cm) respectively. For comparison, the PGR5 protein levels in the total cell extracts in sections 1 and 4 are shown The graph is the combination of the mRNA and protein intensities after normalization. No signal was detected on the western blot for the NDH L protein. The relative accumulation levels (in %) of analyzed proteins in the BS samples as compared to the whole leaf section 4 are indicated.

The NDH-L transcript shows a similar pattern as CP 47 (Fig 10A). The protein profiling of NDH-L was unsuccessful due to the inactivity of the antiserum (originally developed against Arabidopsis protein), which was verified by adding Arabidopsis chloroplast as the positive control (not shown). NDH-L is expected to be up regulated along the developmental gradient because the NDH complex not only helps ATP production, but also provides carbon dioxide as mentioned in the introduction. This dual functionality is conducive for carbon fixation in BS cells.

Conclusions and Suggestions for Future Research

The results have revealed that BS strands can only be isolated after the sink-source transition point, and the great variability in BS strand purity can confound the quantitative data. Therefore, more samples should be prepared and their purity should be carefully assessed before quantification. In addition, a good protocol for mRNA extraction and RT PCR was developed which can be applied for large scale quantitative analysis in future experiments. Moreover, selected photosynthetic genes that have different regulation mechanisms are grouped. For the post transcriptionally regulated genes, future studies can be carried out to clarify the mechanism.

The experiments presented in this thesis can be refined and extended in the following ways. First, as mentioned before, the assembly state of PS II should be considered. Thus, in future experiments, instead of extracting the whole protein, only membrane proteins should be purified to exclude the influence of unassembled proteins.

Besides, more subunits should be investigated and this might reveal the assembly mechanism of PS II. Second, in order to investigate the expression level of NDH complex, NDH antisera need to be generated. Considering the role of NDH-L in Arabidopsis, investigating NDH-L first is a good starting point. However, like PS II, the NDH complex is an assembly of multiple subunits and there might be differences between different species. Therefore, antisera against subunits other than NDH-L should be included as well. Finally, to acquire a complete and accurate picture, the most ideal approach is doing large scale transcript analysis and protein profiling via high throughput technologies such as DNA microarrays and mass spectrometry. This will allow us to have more insights into the developmental process of BS strand formation and differentiation in maize.

Materials and Methods

Maize genotype, growth and isolation of bundle sheath strands.

Approximately 300 *Zea mays* plants (inbred B73) were grown in a growth chamber for 9 days under 16 h light/8 h dark, 400 µmol photons·m^{-2}·s^{-1} until the third leaf was emerging. The third leaf was separated from the plant at the ligule. Four sections corresponding to main leaf developmental stages were cut from the leaf: a section containing the bottom 1.5 centimeter; followed by a 1 cm gap, the next 1.5 cm section and its adjacent 1 cm section; the last section is a 1 cm section which is 10cm away from

the bottom. Each section was stored in an extraction buffer of 350 mM sorbitol, 50 mM Hepes-KOH, pH 8, 2 mM EDTA, 5 mM ascorbic acid, and 5 mM L-cystein. Bundle sheath strands were isolated by blending in a Warring blender in the buffer at ¼ power for 3 intervals of 10 minutes with an inverted (blunt) blade. The purity of the BS strands was assessed by light microscopy and additional blending was applied as needed. The isolated strands were kept at -80^{o}C for long term storage.

Total BS protein extraction and 1D SDS-PAGE.

BS proteins were extracted by grinding in liquid nitrogen and an extraction buffer (75mM, Tris, 2% SDS, PH 8) supplemented with a cocktail of protease inhibitors. Protein concentrations for the soluble fraction were determined by the bicinchoninic acid (BCA) assay. For 1-DE SDS PAGE, proteins were equilibrated with SDS (0.2%), Na_2CO_3 (100 mM), DTT (100 mM), and sucrose (10%) and separated on 12% Tricine gels (Schägger and von Jagow, 1987).

Western Blot followed by SDS-PAGE of BS proteins

1D SDS-PAGE gels were transferred to nitrocellulose membranes in transfer buffer (150 mM glycine, 2 mM Tris and 20% MeOH). Membranes were then blocked in 5% milk 1X TBST (40mM Nacl, 10mM Tris, 0.01% Tween 20, PH 7.5) solution for one hour. Antisera generated in rabbits against Rubisco large subunit, phosphoenopyruvate carboxylase, PSII subunits D1, D2, CP47, OEC23, OEC33, PGR5 and NDH subunit L

were used for overnight primary binding. Afterwards, a secondary antibody, which was generated from goat and conjugated to the enzyme horse radish peroxidase, was used for detection. Transfer membranes were added with the 1:1 mixture of ECL1 (2.5mM luminol, 400µM p-coumaric acid, 100mM Tris-HCl) and ECL2 (5.4mM H202, 199mM Tris-HCl) solution followed by 4 minutes exposure with light sensitive films in a sealed case. Film development was carried out by an automatic developer.

10µg, 20 µg, 30 µg of Arabidopsis chloroplast proteins were loaded to examine the activity of NDH-L antibody (According to Munekage et al. (2002), 4µg of Arabidopsis chloroplast is enough for signal detection.).

Image acquisition and quantification of Western Blot bands

Western Blot films were scanned into the computer by using scanner Personal Densitometer SI (Molecular Dynamics) with digital resolution 12Bits and pixel size 50 micron. Quantification of the bands on the films was performed after background correction. For each biological replicate, protein accumulation level was expressed as the relative density after normalizing to the corresponding density of 10-11cm whole leaf. The average values of three biological replicates were used for plotting and analysis.

RNA extraction

Purified BS strands were ground in liquid nitrogen followed by reacting with TRIzol reagent. Chloroform was then added to homogenized samples. The aqueous phase was transferred and reacted with precipitation solution (0.8 M Na-citrate, 1.2 M NaCl) after centrifugation. Dry RNA pellets were resuspended in 50μL RNase-free water. RNA concentrations were determined using NanoDrop ND-1000 (Thermo Scientific).

DNase treatment and reverse transcript reaction

RNA samples were treated with Promega RQ1 RNase-free DNase prior to reverse transcript reaction (RT). RT reactions were carried out using SuperScript III First-Strand Synthesis System (Invitrogen). Since plastid genes do not contain polyA tail, random hexamers were used as the primer for cDNA synthesis.

cDNA sequence acquisition primer design

Expressed sequence tag (EST) sequences were acquired from the database Plant Proteome DataBase for *Arabidopsis thaliana* and maize (*Zea mays*) (PPDB) with accessions from the publication of Majeran et al., (2008). The validity and accuracy of these sequences were verified with Basic Local Alignment Search Tool (BLAST) using translated EST query. Conservative regions of sequences whose similarity values are greater than 190 were used to design primers. The software FAST-PCR was used for the primary primer designing. Parameter of output primers were examined with the software Vector-NTI. Only primer pairs with high compatibility were used. The following primer

pairs are used in the reactions: 18s ribosomal RNA forward: tcctgacacggggaggtagt; reverse: gcgatggcttgctttgagca; RBCL: forward: atgcgctggagagaccgtttcgtc; reverse: gcatgcattgctcggtgaatgt; PEPC: forward: agccacttgagctgtgctaca; reverse: agctccgacagcagccactc; PSII D1: forward: aagcctgtggggtcgcttctgca; reverse: tccaagggcgcatacccagacgga; CP 47: forward: tctggttgggctggttcgatgg; reverse: aacaagccacccctgcaagaa; OEC 23: forward: ccgtccaagtggaaccccagca; reverse: cggtgatgagctggtgcttgc; OEC 33: forward: cgaaccagtgcccgaccatcga; reverse: aagctctcgggcttgccggt; PGR 5: forward: agaccactcgcttccccgaag; reverse: gcagaactcgttgatgacctgcga; NDH-L: forward: tctacgtgcgcctcaagtcc; reverse: acggctgcattcgtgcatgg.

PCR reaction and agarose electrophoresis

Because different genes have different abundance, PCR reactions were carried out under different cycles for quantification. The following cycle numbers were used: 18s ribosomal RNA: 16; RBCL: 20; PEPC: 30; PSII D1:16; CP 47: 18; OEC 23: 30; OEC 33: 30; PGR 5: 30; NDH-L: 30. For each cycle, denaturation is under 94°C for 2 minutes, annealing is under 58°C for 55 seconds and extension is under 72°C for 1 minute. PCR products were loaded into 1% agarose gel with 0.001μg/mL ethidium bromide for electrophoresis.

Image acquisition and quantification of DNA in Agarose gel

The software Alphamager 2200 was used for agarose gel analysis. For each biological replicate, band quantification was performed after background subtraction by comparing the normalized band intensities. Within each biological replicate, normalization is based on the intensities of loading control. In order to combine the results of three biological replicate, a second normalization was carried out based on the maximum values of each biological group. RNA accumulation levels are expressed as the average intensity of three biological replicates after normalization.

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