Elevated Expression of PGR5 and NDH-H in Bundle Sheath Chloroplasts in C₄ Flaveria Species

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Cyclic electron transport around PSI has been proposed to supply the additional ATP required for C₄ photosynthesis. To investigate the nature of cyclic electron pathways involved in C₄ photosynthesis, we analyzed tissue-specific expression of PGR5 (PROTON GRADIENT REGULATION 5), which is involved in the antimycin A-sensitive pathway, and NDH-H, a subunit of the plastidial NAD(P)H dehydrogenase complex, in four Flaveria species comprising NADP-malic enzyme (ME)-type C₄, C₃–C₄ intermediate and C₃ species. PGR5 was highly expressed in the C₄ species and enriched in bundle sheath chloroplasts together with NDH-H, suggesting that electron transport of both PGR5-dependent and NDH-dependent cyclic pathways is promoted to drive C₄ photosynthesis.

Keywords: C₄ photosynthesis • Cyclic electron transport • Flaveria.

Abbreviations: FITC, fluorescein isothiocyanate; LHCII, light-harvesting complex II; NADP-ME, NADP-malic enzyme; NDH, NAD(P)H dehydrogenase; PGR5, PROTON GRADIENT REGULATION 5; RACE, rapid amplification of cDNA ends; TBST, Tris-buffered saline with Tween.

C₄ photosynthesis requires the coordinated functioning of two cell types, namely mesophyll and bundle sheath cells, with individual functions (Hatch 1987, Sage 1999). Although C₄ photosynthesis suppresses photorespiration by a CO₂-concentrating mechanism, it increases the energetic cost of CO₂ assimilation in comparison with C₃ photosynthesis. Consequently, two extra ATP molecules are required for each CO₂ molecule fixed to drive C₄ photosynthesis (Kanai and Edwards 1999). The extra ATP needed for C₄ photosynthesis was suggested to be produced by PSI cyclic electron transport activity, which contributes to generation of ΔpH across the thylakoid membrane (Kanai and Edwards 1999). Increased PSI cyclic electron transport compared with C₃ plants has been reported in a number of C₄ plants, including Sorghum bicolor and Zea mays (Herbert et al. 1990, Asada et al. 1993).

Two cyclic pathways around PSI have been identified in C₄ plants; the first pathway involves a plastidial NDH [NAD(P)H dehydrogenase] complex that is able to reduce plastoquinones from stromal NAD(P)H donors (Horvath et al. 2000) and the second pathway is an antimycin A-sensitive pathway involving PROTON GRADIENT REGULATION 5 (PGR5), which is localized in the chloroplast and considered to be a factor for major cyclic electron transport activity in C₃ plants (Munekage et al. 2002, Munekage et al. 2004, Munekage et al. 2008). The contribution of NDH-dependent electron transport to C₄ photosynthesis was suggested by its expression profile correlating with predicted ATP requirement in different cell types (Kubicki et al. 1996, Takabayashi et al. 2005). The contribution of PGR5-related electron transport to C₄ photosynthesis is unclear. However, Ivanov et al. (2007) reported that the oxidation level of P700 in bundle sheath strands isolated from Z. mays was dependent on antimycin A.

With the aim of investigating the nature of cyclic electron pathways involved in C₄ photosynthesis, we analyzed expression of PGR5 and NDH-H, a subunit of the NDH complex, in the dicot genus Flaveria (Asteraceae), which contains closely related C₄, C₃–C₄ intermediate and NADP-malic enzyme (ME)-type C₃ species and is a widely used model system for studying the C₄ photosynthesis evolutionary process (Sage 2004).

The relative abundances of PGR5 and NDH-H were analyzed by immunoblotting in a C₄-type species, F. pringlei, a C₃–C₄ intermediate species, F. anomalae, and two NADP-ME C₃-type species, F. trinervia and F. bidentis (Fig. 1). The abundance of major electron transport complexes was analyzed using specific antibodies against PsbO for PSI, the Rieske protein for the cytochrome b₆f complex, and PsAC for PSI (Fig. 1). The C₄ F. pringlei and the C₃–C₄ intermediate F. anomalae showed a similar relative abundance of PGR5, NDH-H and subunits of the major electron transport chain. In contrast, relative amounts of PGR5 and NDH-H in both C₄ species were four and eight times higher than in C₃–C₄ intermediate and C₃ species, respectively. While PsbO was less abundant in C₄ species than in C₃ and C₃–C₄ intermediate species, PsAC was...
Fig. 1 Immuno blot analysis of PGR5, NDH-H, PsbO, Rieske protein and PsAC in C₃ F. trinervia, C₄ F. bidentis, C₃–C₄ F. anomala and C₄ F. pringlei. Total membrane proteins were extracted from leaves of each Flaveria species. Lanes were loaded with 20 µg of proteins for detection of PGR5 and NDH-H, 5 µg of protein for detection of PsbO, 10 µg of proteins for detection of Rieske protein and PsAC, and a dilution series of F. bidentis as indicated.

slightly more abundant in C₄ species. The abundance of the Rieske protein was similar among all the species compared.

To compare the amino acid sequence of PGR5 between C₃ and C₄ species, the full-length PGR5 gene was cloned from cDNA libraries of F. trinervia, F. bidentis and F. pringlei. Alignment of the deduced protein sequences using the ClustalW algorithm (Supplementary Fig. S1) shows that the sequence is highly conserved among C₃ and C₄ Flaveria. The program TARGETP (www.cbs.dtu.dk/services/TargetP) predicted that the N-terminal sequence of these PGR5 homologs contains a chloroplast-targeted transit peptide. The C-terminal sequence used to raise the PGR5 antibody (from Ala102 to Leu121) was 100% identical among the Flaveria species, indicating that the variation in immunodetection signal intensity reflects actual differences in PGR5 protein levels.

To investigate the localization of PGR5 and the NDH complex in C₃ and C₄ Flaveria, in situ immunolabelling was performed using both anti-PGR5 and anti-NDH-H antibodies (Fig. 2). Transverse sections of the leaf lamina of each Flaveria species were stained with toluidine blue. Numerous chloroplasts were colored blue in the mesophyll and bundle sheath cells (Fig. 2A–C). Transverse sections, prepared from the same leaf samples, were labeled with either the pre-immune serum or the immune serum and subsequently labeled with secondary antibodies conjugated to fluorescein isothiocyanate (FITC). Overlay images of the FITC fluorescence in green and autofluorescence in red were visualized by confocal microscopy. The background labeling with pre-immune serum was very low in all cases (Fig. 2G–H, M, N) compared with the control section (labeling without primary antibody, Fig. 2D–F). There was very little immunolabelling for PGR5 in the C₃ F. pringlei (Fig. 2J). Although FITC fluorescence was observed in the vascular bundle, which did not contain chloroplasts (Fig. 2J), similar FITC fluorescence patterns were observed in leaf transverse sections of the Arabidopsis pgr5 mutant lacking PGR5 protein (data not shown), indicating that the FITC fluorescence observed in the vascular bundle was not caused by the PGR5 protein. In F. bidentis, more intense immunolabelling for PGR5 was observed in bundle sheath cells compared with mesophyll cells (Fig. 2K). The strong FITC fluorescence superimposed onto chloroplasts resulted in yellow fluorescence and indicated specific immunolabelling for PGR5. A similar result was obtained for F. trinervia (Fig. 2L). These results showed that PGR5 was enriched in bundle sheath chloroplasts of C₄ Flaveria. Immunolabelling for NDH-H showed exclusive localization of NDH-H in bundle sheath chloroplasts of the C₄ F. bidentis but only very faint staining of NDH-H in mesophyll cells of the C₄ F. bidentis and C₃ F. pringlei (Fig. 2O, P).

In NADP-ME-type C₄ photosynthesis, 3.3 ATP/2.1 NADPH molecules are estimated to be required per CO₂ molecule fixed in mesophyll cells, which is a similar ratio to that in C₃ photosynthesis, whereas only 2.3 ATP per CO₂ fixed is considered to be required in bundle sheath cells since NADPH is supplied by decarboxylation of C₄ acid (Kanai and Edward 1999). Increased expression of PGR5 and NDH-H in bundle sheath cells of NADP-ME-type C₄ Flaveria suggested promotion of both PGR5-dependent and NDH-dependent cyclic activities to fulfill the ATP requirement of C₄ photosynthesis. In a previous study, expression profiles of NDH-H were well correlated with the predicted ATP requirement in C₄ cell types, in contrast to PGR5. This was observed in both NAD-ME-type C₄ plants and NADP-type C₄ plants (Takabayashi et al. 2005). From these results, it was suggested that the NDH complex mainly energizes C₄ photosynthesis. However, if the expression of PGR5 was normalized to the cytochrome f level, whose expression is comparable between mesophyll and bundle sheath cells (Kubicki et al., 1996, Majeran et al., 2008), the PGR5 expression could be correlated with the predicted ATP requirement in C₄ cell types in those plants, with the exception of the case of NAD-ME-type Portulaca oleracea (Takabayashi et al. 2005). Here, we showed increased expression of PGR5 from the C₃ over the C₃–C₄ intermediate to the C₄ species of the genus Flaveria (Fig. 1B) and used an immunolabelling technique which showed enrichment of PGR5 protein in bundle sheath cells (Fig. 2). This result suggests that a PGR5-dependent pathway contributes to ATP production, which drives C₄ photosynthesis. However, the abundances of PGR5, NDH-H and major electron transport complexes were similar between the C₃–C₄ intermediate F. anomala and C₄ F. pringlei. The proportion of PSI/PSII is similar between C₃–C₄ intermediate species and C₄ species of Flaveria (Pfündel and Pfeffer 1997). Together, these findings suggest that the composition of the electron transport chain has remained unchanged during the evolution of C₃ to C₃–C₄ intermediate species in Flaveria.

In C₄ Flaveria, two morphologically distinct chloroplast types were observed, as in S. bicolor and Z. mays (Laetsch 1971, Hofer et al. 1992). While mesophyll chloroplasts contain numerous grana thylakoid membranes, bundle sheath chloroplasts...
In situ immunolocalization of PGR5 and NDH-H in leaf tissue of C₃ F. pringlei (A, D, G, J, M, O), C₄ F. bidentis (B, E, H, K, N, P) and C₄ F. trinervia (C, F, I, L). Transverse sections of the lamina for anatomical observation were stained with toluidine blue (A–C). Localization of PGR5 and NDH-H was visualized by the green fluorescence of the FITC-labeled antibody. Leaf sections were stained with primary anti-PGR5 serum (J–L), anti-NDH-H serum (O, P) or without primary antibody (D–F). Pre-immunization sera for PGR5 (G–I) and for NDH-H (M, N) were used to analyze background labeling. Leaf sections were subsequently stained with secondary antibody (anti-rabbit-IgG–FITC conjugate). Overlaid images of green FITC fluorescence and red auto fluorescence were visualized by confocal microscopy. Scale bars = 100 µm.
contain grana-free thylakoid membranes (Laetsch 1971). Enrichment of PGR5 on bundle sheath chloroplasts suggests that PGR5 is probably localized on non-appressed thylakoid membranes. To test this hypothesis, thylakoid membranes isolated from C₃ F. pringlei or C₄ F. bidentis leaves were fractionated into grana and stroma lamellae by digitonin treatment (Cuello and Quiles 2004). SDS–PAGE revealed protein patterns typical for grana and stroma lamellae in C₃ F. pringlei; light-harvesting complex II (LHCII) was enriched in the grana thylakoid fraction, and α and β subunits of ATP synthase were enriched in the stroma lamellae fraction (Fig. 3A). A similar protein pattern was observed in the C₄ species, with the exception of LHCII, which were also detected in the stroma lamellae fraction (Fig. 3A). Immunoblotting against Lhcb1 and the ε subunit of ATP synthase (AtpE) showed that Lhcb1 and AtpE were mainly localized in the grana thylakoid fraction and stroma lamellae fractions, respectively, in both F. pringlei and F. bidentis, but a small amount of Lhcb1 was also detected in stroma lamellae in F. bidentis (Fig. 3B). A higher amount of Lhcb1 was detected in the stroma lamellae fraction of F. bidentis than that of F. pringlei, which is probably due to localization of LHCII in agranal thylakoid membranes of bundle sheath chloroplasts in C₄ species (Hofer et al. 1992). Immunoblotting against PGR5 showed that PGR5 was enriched in the stroma lamellae fraction in both F. pringlei and F. bidentis (Fig. 3B). Identical results were obtained with tobacco (data not shown). From these results, we concluded that PGR5 is localized in stroma lamellae.

Localization of PGR5 in stroma lamellae indicates that PGR5-related cyclic electron transport takes place in non-appressed thylakoid membranes. It also explains why NADP-ME-type C₄ species developed agranal chloroplasts in bundle sheath cells to promote PSI cyclic electron transport. Interestingly, while expression of the PSI subunit was slightly higher in C₄ species than in C₃ species, expression of PGR5 was four times higher in C₄ species than in C₃ species (Fig. 1). We propose that the increased expression of PGR5 and the NDH complex directly promotes cyclic electron transport activity to drive ATP production for C₄ photosynthesis in C₄ Flaveria species.

Materials and Methods

Plants of F. trinervia, F. bidentis, F. anomala and F. pringlei were grown in pots on soil for 6–8 weeks in a growth chamber (200–300 μmol photons m⁻² s⁻¹, 16 h light/8 h dark, 25–28°C). A cDNA library was prepared from leaves of F. bidentis. Total RNA was extracted from leaves using the RNeasy Maxi Kit (Qiagen, Courteboeuf, France) and poly(A) mRNA was isolated with the PolyATtract® mRNA Isolation System IV (Promega, Madison, WI, USA). cDNA synthesis and rapid amplification of cDNA ends (RACE) (3′ and 5′) PCR was performed using the Marathon™ cDNA Amplification Kit (Clontec, Ozyme, France) according to the manufacturer’s instructions. Primers for RACE PCR are listed in Supplementary Table S1.

Leaf samples were blended in liquid N₂ and the powder was suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 50 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. The membrane fraction was sedimented by centrifugation at 27,000 × g for 15 min and the pellet was resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 1% SDS.

Stromal and granal thylakoid membrane separation was performed essentially as described by Rumeau et al. (2005). Leaves were homogenized at high speed using a Polytron homogenizer to grind mesophyll and bundle sheath cells. Isolated thylakoids were resuspended in 100 mM Tricine/NaOH buffer (pH 7.8) containing 10 mM NaCl and 10 mM MgCl₂ to a chlorophyll concentration of 1.5 mg ml⁻¹ and incubated for 3 min with digitonin (Sigma-Aldrich) added to give a final concentration of 8.25 mg ml⁻¹. The samples were centrifuged at 10,000 × g for 30 min to separate grana membranes. After centrifugation at 40,000 × g for 30 min to remove contaminating grana vesicles, the supernatant (stroma lamellae) was sedimented at 150,000 × g for 1 h.

Denaturing SDS–PAGE was performed using 15% (w/v) polyacrylamide gels. Proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and probed with the following antibodies. The anti-NDH-H antibody raised from recombinant proteins and the anti-PGR5 antibody raised from a synthetic peptide antigen are
described in Horvath et al. (2000) and Munekage et al. (2002), respectively. The anti-PsaC antibody and anti-Lhcb1 antibody were purchased from Agrisera (Vannas, Sweden). Antibodies against Rieske were raised from recombinant proteins (Sanda et al. unpublished data). Immunocomplexes were detected using the ECL Plus Western Blot Detection Reagent (GE Healthcare).

Small leaf tissue fragments were first fixed in 3.7% formaldehyde, 5% acetic acid and 50% ethanol, dehydrated with ethanol, impregnated with xylene and then embedded in paraffin. Embedded tissue was sliced into 8 μm sections and mounted onto glass slides, deparaffinized and stained with toluidine blue for anatomical observation. For immunolabeling, deparaffinized sections were incubated in blocking and mounting solution with Tween (TBST) with 3% BSA. Emission was observed with a 510–560 nm filter. Emission was observed with a 510–560 nm filter.

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


