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Letters

AtCYP20-2 is an auxiliary protein of the chloroplast NAD(P)H dehydrogenase complex

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ABSTRACT

AtCYP20-2 is one of 16 immunophilins in thylakoid lumen. The presence of the isomerase domain in AtCYP20-2, an enrichment of AtCYP20-2 in the stroma membranes and it's co-migration with NAD(P)H dehydrogenase (NDH) in native gels provide evidence that AtCYP20-2 is an auxiliary protein of NDH. When different NDH mutants were studied, AtCYP20-2 was found to be strongly reduced especially in mutants deficient in the membrane domain of NDH, thus suggesting a role in the assembly of NDH hydrophobic domain. Lack of AtCYP20-2, however, did not lead to severe malfunction of NDH, indicating redundancy in the function of lumenal immunophilins.

1. Introduction

Photosystem (PS)I and PSII, cytochrome (cyt) b_6f and ATP synthase, all embedded in the thylakoid membrane, form the basis for light harvesting and solar energy conversion into chemical form. While PSI and the ATP synthase are located in unstacked thylakoid membranes, the stroma membranes, PSII is enriched in the stacked grana membanes and cyt b_6f is evenly distributed in both locations. The stroma thylakoid membrane also contains a low abundance protein complex, the NAD(P)H dehydrogenase (NDH) complex, which functions in PSI cyclic electron flow and chlororespiration [1]. Blue native gel (BN-PAGE) separation of the thylakoid membrane protein complexes has recently revealed that NDH interacts with PSI and thus forms a supercomplex [2,3]. Several auxiliary proteins regulate the biogenesis and stability of NDH both at the transcriptional and post-transcritional level [1].

During the past decade, significant progress has been made towards understanding the complicated processes of the assembly, maintenance and turnover of the photosynthetic protein complexes. High quality proteomics studies have, however, revealed that the thylakoid membrane network of plant chloroplasts contains a number of functionally unknown proteins. Up to 200 proteins are predicted to be present in the inner space of the thylakoid membrane, the lumen, and proteomic studies have identified over 80 different proteins in this compartment [4]. Interestingly, 16 proteins located in the lumenal compartment comprise a peptidyl prolyl isomerase (PPIase) domain [5]. Members of the PPIase family are believed to function as auxiliary proteins facilitating the rate limiting *cis–trans* isomerisation of the peptidyl prolyl bond during protein folding. Cyclophilins and FK506 binding proteins (FKBP) belong to PPIases and are collectively referred to as immunophilins. Recently, we characterized the thylakoid lumen cyclophilin AtCYP38 and showed that AtCYP38 guides folding of the D1 protein into PSII, thereby enabling the correct assembly of the water-splitting Mn_4–Ca cluster [6]. Here we focused on another thylakoid lumen cyclophilin, AtCYP20-2, which likewise was previously assigned to function in PSII assembly [7]. Nevertheless, we show that AtCYP20-2 is associated with another thylakoid protein complex, the NDH complex.

2. Materials and methods

2.1. Plant material and growth conditions

Three-weeks old full-size *Arabidopsis thaliana* (*Arabidopsis*), ecotype Columbia, plants were used in all of experiments. *cyp20-2* mutant lines [8], SALK_009552 and SALK_024971, containing T-DNA insert within the *AT5G13120* gene, were screened by immunoblotting. *ndho* (SALK_068922), *crr2-2*, *ndh45* (SALK_ 111363), *psad* (*psad1-1*) and *fnr1* (SALK_085403) lines were used in specific

experiments [3,9–13]. Plants were grown under a photon flux density of 150 μmol photons m^{-2} s^{-1} in 16 h light regime at 23 °C. For specific experiments, intact plants were illuminated for 2 h at 1000 μmol photons m^{-2} s^{-1}.

2.2. Isolation and fractionation of the thylakoid membrane

Thylakoid membranes were isolated [14] and chl was determinated according to [15]. Chl per leaf area was calculated as described in [16]. Digitonin solubilization combined with differential centrifugation steps was used to subfractionate the thylakoid membrane into grana stacks and stroma-exposed membranes as in [17].

2.3. Separation and detection of thylakoid proteins and protein complexes

The polypeptides were separated with SDS-PAGE (15% polyacrylamide, 6 M urea) [18] and after electrophoresis the proteins were electroblotted to a PVDF membrane (Millipore, Watford, Herts, UK). Gels were loaded on chl basis. BN-PAGE was performed essentially as described in [19]. Immunoblotting using chemiluminescence detection was performed according to standard procedures. AtCYP20-2 antibody was produced by immunizing rabbits with synthetic peptides GDFEKGNGTGGKSVYC and CEEQETDRGDRPRKKV (Eurogentec, Seraing, Belgium).

2.4. Measurements of photosynthetic parameters

The maximal photochemical efficiency of PSII was determined as F_V/F_M using Hansatech Plant Efficiency Analyzer (King's Lynn, UK) after 30-min dark incubation. Post-illumination increase in chl fluorescence, the "F_0-rise", was monitored as described in [20].

2.5. Statistical analyses

The numerical data were subjected to statistical analysis by Student's t-test with statistical significance at the P values <0.001.

3. Results

3.1. AtCYP20-2 is associated with the NDH complex

The exact location of the lumenal AtCYP20-2 protein was addressed by fractionating the thylakoid membrane into grana and stroma thylakoids, followed by immunoblot analysis. The D1 protein enriched in grana thylakoids and Atpβ located in stroma thylakoids were used as control proteins to indicate the success of fractionation. AtCYP20-2 protein was found to be strongly enriched in stroma thylakoids and it was shown to migrate as two forms with distinct molecular mass indicating a post-translational modification (Fig. 1A).

To clarify, whether AtCYP20-2 is associated with any of the thylakoid membrane protein complexes, both the intact thylakoid membrenes and the stroma membrane fraction were subjected to BN-PAGE enabling the separation of the protein complexes according to their size. Immunoblot analysis of the BN-PAGE gel using antibodies raised against the NDHH, D1 and AtCYP20-2 proteins revealed the association of AtCYP20-2 with the PSI/NDH supercomplex (Fig. 1B). No free form of AtCYP20-2 was observed in gels after separation of thylakoid protein complexes by BN-PAGE (data not shown). Since AtCYP20-2 is predicted to be a hydrophilic protein, it seems to interact tightly with some thylakoid membrane protein, most likely a subunit of the NDH complex.
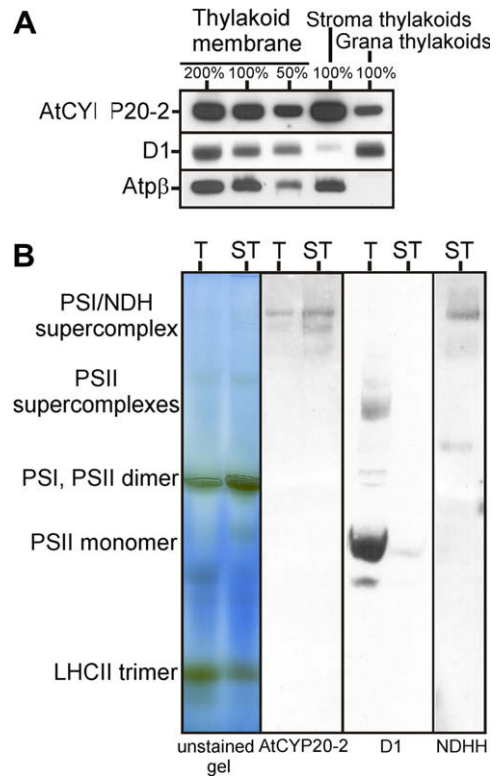

Fig. 1. Localization of AtCYP20-2. (A) Immunoblot from thylakoids fractionated into stroma and grana membranes by digitonin and subsequently subjected to denaturing SDS–PAGE. (B) Non-denaturing BN-PAGE of thylakoids (T) and stroma thylakoids (ST). Representative blots are shown.

In order to verify the association of the AtCYP20-2 protein specifically with the NDH complex, the accumulation of AtCYP20-2 was studied in WT and different mutant lines. The mutants included *ndho* lacking the hydrophilic NDHO subunit, plants defective in nucleus-encoded factor, CRR2, essential for proper expression of the hydrophobic NDHB subunit, as well as *ndh45* lacking the NDH45 protein embedded into the hydrophobic domain of the NDH complex. In addition to the NDH mutants, *psad* and *fnr1* lines were used as controls. As shown in Fig. 2, AtCYP20-2 was present in strongly reduced amounts in thylakoids isolated from NDH mutant plants with defective hydrophobic domain (*crr2-2* and *ndh45*), while the amount of AtCYP20-2 was only slightly reduced in the *ndho* line with defective hydrophilic domain. On the contrary, AtCYP20-2 was present in similar amounts in *psad* and *fnr1* thylakoids as in the WT thylakoids.

3.2. Characterization of cyp20-2 mutant plants

Both *cyp20-2* lines, SALK_009552 and SALK_024971, totally lacked AtCYP20-2 (Fig. 3). The *cyp20-2* plants did not show a
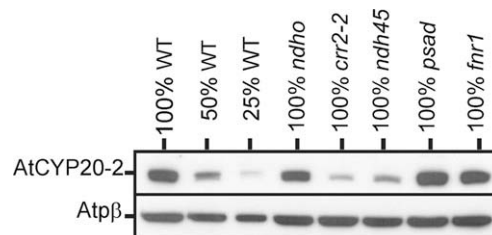

Fig. 2. Accumulation of AtCYP20-2 in *Arabidopsis* mutants. Atpβ is used as a reference protein. Representative blots are shown.
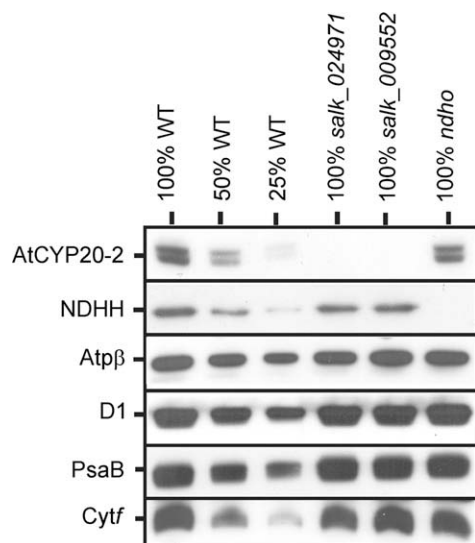
Fig. 3. Immunoblot demonstrating the amounts of representative subunits of the major thylakoid membrane protein complexes in *cyp20-2* (*salk_024971*; *salk_009552*) plants. WT and *ndho* are used as control.

phenotype when growing under standard growth conditions (data not shown). Neither there were any statistically significant differences in the chl measurements. The total amount of chl/leaf area (μg chl/cm^2) of *salk_024971*, *salk_009552* and WT plants were 20.1 ± 0.8, 18.1 ± 0.6 and 19.0 ± 0.7, while the average chl a/b ratios were 3.7 ± 0.0, 3.7 ± 0.0 and 3.6 ± 0.1, respectively.

The amounts of different thylakoid membrane protein complexes were analyzed from the *cyp20-2* thylakoid membranes by immunoblot analysis of a representative protein in each complex using WT and *ndho* thylakoids as controls. No distinct differences could be observed in the amounts of the PSI, PSII and cyt b_6f or in the ATP synthase, represented by PsaB, D1, Cytf and Atpβ, respectively (Fig. 3). Only the amount of NDHH (representing NDH) was slightly ($\sim$20%) reduced in *cyp20-2* compared to WT (Fig. 3).

Finally, the functional characteristics of the thylakoid membrane protein complexes were studied from the WT and *cyp20-2* plants. The photochemical efficiency of PSII (F_V/F_M) was slightly increased in the *cyp20-2* mutant plants both under constant growth light conditions and after short-term high light treatment (Fig. 4). The post-illumination rise in chl fluorescence (F_0-rise) [21], which is dependent on NDH-mediated reduction of the plastoquinone pool in darkness [22], was reduced only moderately in the *cyp20-2* plants compared to WT (Fig. 5).

4. Discussion

AtCYP20 protein is the only lumenal cyclophilin in *Arabidopsis* shown to possess PPIase activity in vitro [23,24]. However, the exact role of the AtCYP20-2 protein has remained poorly characterized. It was earlier reported that AtCYP20-2 is associated with a supercomplex composed of PSII core dimer and its light harvesting complex (LHCII) and functions as an auxiliary protein assisting the insertion of LHCII [7]. Nevertheless, we found AtCYP20-2 strongly enriched in stroma membranes (Fig. 1A), which is in sharp contrast with earlier results, since the PSII/LHCII supercomplexes are present only in grana thylakoids [25]. To solve this discrepancy, we analyzed the co-migration profile of AtCYP20-2 with thylakoid membrane protein complexes, using both intact thylakoid membranes and stroma thylakoid membranes. In these experiments AtCYP20-2 was found to co-migrate with the PSI/NDH supercomplex, instead of the PSII/LCHII supercomplex (Fig. 1B). In accordance
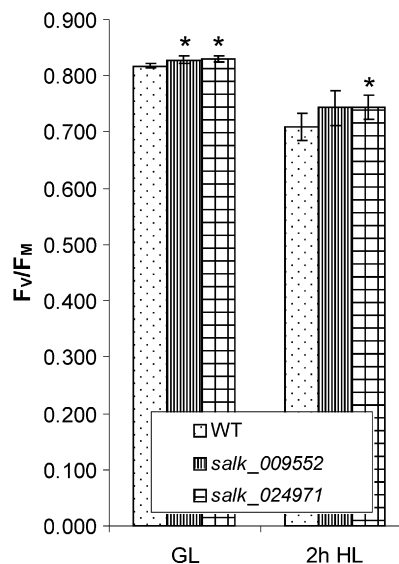

Fig. 4. Photochemical efficiency of PSII (F_V/F_M) in WT and *cyp20-2* (*salk_024971*; *salk_009552*) plants. Measurements were carried out using plants from GL (150 μmol photons m^{-2} s^{-1}) 2 h after the lights were turned on. Plants were then transferred to HL (1000 μmol photons m^{-2} s^{-1}) and samples were taken after 2 h illumination. The values are the means $\pm$ S.D. of $n = 20$. Statistically significant differences are marked with asterisk (*).
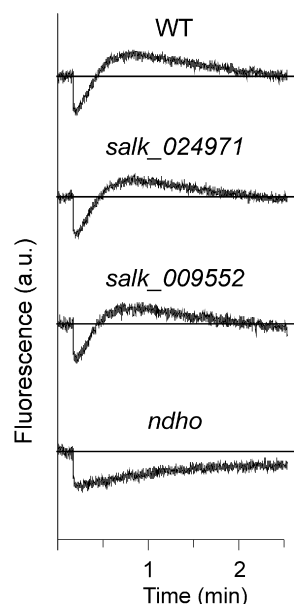

Fig. 5. Functional status of the NDH complex in WT, *ndho* and *cyp20-2* plants (*salk_024971*; *salk_009552*). "F_0-rise" in darkness after termination of actinic light (100 μmol photons m^{-2} s^{-1}). An average curve of five measurements is shown.

with our results, the AtCYP20-2 homolog in maize bundle sheath chloroplasts has been found to co-migrate with NDH [26]. Indeed, the presence of supercomplexes of over 1000 kDa other than PSII/LHCII in the thylakoid membrane has been unknown until recently [2,3], thus explaining why it was previously [7] taken for granted that any protein associated with a high molecular mass complex in the thylakoid membrane interacts with the PSII/LHCII supercomplex.

Specific association of AtCYP20-2 with the NDH complex instead of PSII/LHCII is further supported by analysis of mutants with reduced amounts of the NDH complex. Indeed, when NDH was severely reduced in the thylakoid membrane, particularly in the

absence of the thylakoid membrane embedded domain (e.g. in *crr2-2*), also AtCYP20-2 become dispensable and largely disappeared (Fig. 2). On the contrary, functional analysis of PSII in *cyp20-2* plants did not reveal any malfunction of PSII when measured from plants incubated either at growth light conditions or from plants exposed to short high light treatment (Fig. 4), which renders the PSII complexes susceptible to photoinhibition [27]. Based on results above and on the presence of the PPIase domain in AtCYP20-2, it is likely that AtCYP20-2 is an auxiliary protein of NDH, which is important especially for the accumulation of the hydrophobic domain of the NDH complex.

Disruption of the *AtCYP20-2* gene did not result in any visual phenotype, which could be expected since none of the NDH mutants has so far been reported to show any specific phenotype. Neither was there any difference in the accumulation of the PSI, PSII, cyt b_6f or ATPase in the *cyp20-2* plants compared to WT (Fig. 3), which is also a typical feature of NDH mutants. On the contrary, both the accumulation of NDHH and the F_0-rise, generated by NDH-mediated reduction of the plastoquinone pool in darkness, were slightly decreased in the *cyp20-2* plants compared to WT (Fig. 5) However, only minor decrease in these components suggests a redundancy in the function of AtCYP20-2. Out of 18 immunophilins located in the chloroplast, the thylakoid lumen comprises 11 FKBP proteins and five cyclophilins possibly resulting in a such redundancy of the PPIase properties [5]. Indeed, the PPIase activity of another lumenal immunophilin, AtFKBP13, has been shown to accumulate in *Arabidopsis cyp20-2* mutant [28]. The real degree of redundancy within the lumenal immunophilins remains to be studied – it is likely that also other lumenal immunophilins than AtCYP20-2 are redundant. Thus, the thylakoid lumen seems to contain both highly specific, indispensable immunophilins like AtCYP38 specific for the PSII complex [6] and redundant immunophilins like AtCYP20-2 specific for the NDH complex.

Acknowledgments

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