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Protease-stable integration of Lhcb1 into thylakoid membranes is dependent on chlorophyll *b* in allelic *chlorina-f2* mutants of barley (*Hordeum vulgare* L.)

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Abstract. Allelic *chlorina-f2* mutants of barley (*Hordeum vulgare* L.) growing under different light and temperature conditions demonstrated that the chlorophyll *b*-free *chlorina-f2*^{f2} and *chlorina-f2*^{101} express a stable phenotype. Only 3 out of 10 light-harvesting chlorophyll *a*/*b*-binding proteins, Lhca4 (photosystem I), and Lhcb1 and Lhcb6 (photosystem II), required chlorophyll *b* for accumulation. The other light-harvesting proteins were found in all *chlorina-f2* alleles, indicating that the integration pathway of these proteins into mutant thylakoid membranes was not affected. *Chlorina-f2* alleles with a thylakoid membrane capable of fullfilling photosynthesis and transport demands, but with various amounts of chlorophyll *b*: *chlorina-f2*^{101} (chlorophyll *b*-free), *chlorina-f2*^{123} (27% of chlorophyll *b* compared with the wild type) and *chlorina-f2*^{122} (70% chlorophyll *b*), were chosen to investigate whether chlorophyll *b* is necessary for the protease-stable insertion of Lhcb 1 into mutant thylakoid membranes. The Lhcb1 was affected in almost all alleles and was most sensitive to chlorophyll *b* deficiency. The Lhcb1 antibody confirmed the heterogeneity of the polypeptides of the light-harvesting chlorophyll *a*/*b*-binding protein II (LHCII) and detected in wild-type membranes, two protease-resistant, mature forms of Lhcb1 with apparent molecular masses of 28 and 29 kDa. Only one band reacting with the Lhcb1 antibody could be detected in chlorophyll *b*-free *chlorina-f2*^{f2}. It co-migrated with the 29-kDa band, but was completely digested after treatment of the isolated mutant membranes with exogenous protease. This showed that in *chlorina-f2*^{f2} the Lhcb1 precursor was processed at one cleavage site only. The resulting 29-kDa Lhcb1 was not provided with chlorophyll *b*, and, consequently, not properly folded and inserted into the membrane. It remained susceptible to protease and was inconvertable to a 28-kDa form.

Key words: *Chlorina-f2* – Chlorophyll *b* deficiency – *Hordeum* – Light-harvesting protein – Mutant (barley) – Thylakoid (integration of Lhcb1)

Introduction

Chlorophyll-deficient mutants of barley (*Hordeum vulgare* L.) have been known since the first decades of this century (Nilsson-Ehle 1922; Robertson 1937; Robertson et al. 1941). The term "*chlorina*" is used to characterize pale-green to yellow-green viable seedlings capable of growing to maturity. In 1950, Highkin described a barley *chlorina* stock # 2 which completely lacked chlorophyll *b* (Highkin 1950). Two decades later, the lack of chlorophyll *b* in *chlorina-f2*^{f2} was linked to the absence of a pigment-protein complex (Thornber and Highkin 1974). One out of two separated pigment-protein complexes, originally named photosystem II chlorophyll-protein, was missing in *chlorina-f2*^{f2} compared with barley wild type (WT) after electrophoresis of dodecyl-sulfate-treated thylakoid membranes. This missing protein was not essential for photosynthesis, and was renamed light-harvesting chlorophyll *a*/*b*-binding protein.

At least 10 light-harvesting chlorophyll *a*/*b*-binding (LHC) proteins (LHCPs) have since been identified in thylakoid membranes of plants: Lhcal–4 are associated with PSI, Lhcb3–6 are associated with PSII, and light-harvesting chlorophyll *a*/*b*-binding protein II (LHCII) trimers consisting of Lhcb1 and -2 are attached to both photosystems (Jansson et al. 1997). Consequently, this prompted the question of whether the lack of chlorophyll *b* affects all antenna proteins in *chlorina-f2*^{f2} in the same way as the major light-harvesting chlorophyll

Abbreviations: LHC = light-harvesting chlorophyll *a*/*b*-binding; LHCP = light-harvesting chlorophyll *a*/*b*-binding protein; LHCII = light-harvesting chlorophyll *a*/*b*-binding protein II; Lhca = light-harvesting chlorophyll *a*/*b*-binding protein of photosystem I; Lhcb = light-harvesting chlorophyll *a*/*b*-binding protein of photosystem II; PVDF = polyvinylidenedifluoride; WT = wild type

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a/*b*-binding protein. Recently, almost another 25 years after the first report that *chlorina* $f2^{f2}$ lacks not only chlorophyll *b* but also chlorophyll *a*/*b*-binding protein, Bossmann et al. (1997) used monospecific antibodies against all plant light-harvesting proteins to identify the individual proteins affected by chlorophyll *b* deficiency. The investigation showed that only three out of ten antenna proteins fail to accumulate in the absence of chlorophyll *b* in *chlorina-*$f2^{f2}$: one of each of the chlorophyll *a*/*b*-binding proteins of PSI and PSII, Lhca4 and Lhcb6, respectively, and Lhcb1, one of the trimer-constituting LHCII proteins. The proteins Lhcb2–4 were reduced compared with the WT, but Lhca1–3, Lhcb5 and PsbS (subunit of PSII) were not affected.

The differences in LHCP composition compared with those published earlier could be due to different growth conditions (Bossmann et al. 1997). Sensitivity to light and temperature was shown for a number of *chlorina* mutants (Simpson et al. 1985; Knoetzel and Simpson 1991). Król et al. (1995) showed that *chlorina-*$f2^{f2}$ lacks all LHCPs under intermittent light conditions. Thus, we have grown *chlorina-*$f2^{f2}$ and *chlorina-*$f2^{101}$ mutant seedlings under different light and temperature conditions to test whether these changes have an influence on the LHCP composition.

Chlorina-$f2^{f2}$ is defective in chlorophyll *b* biosynthesis, probably in the oxygenation of chlorophyll *a* by an oxygenase (von Wettstein et al. 1995). Support for this assumption comes from the observation that 10 chlorophyll *b*-deficient mutants of barley belong to a single complementation group (Simpson et al. 1985), as is the case for 54 chlorophyll *b*-less *Chlamydomonas* mutants (Chunaev et al. 1991). *Chlorina-*$f2^{f2}$ capable of transcription and translation of LHC genes (Preiss and Thornber 1995). In-vitro translation of *chlorina-*$f2^{f2}$ mRNA indicates normal concentrations of the messages for the missing LHCPs (Bellemare et al. 1982). The translation products of these proteins are imported into mutant chloroplasts, where the transit peptides are removed and the LHCPs are integrated into the thylakoids (Bellemare et al. 1982). Chitnis et al. (1988) reported that radioactively labelled precursor of the major light-harvesting chlorophyll *a*/*b*-protein (pLhcb1 or pCab-2) was imported into *chlorina-f2* chloroplasts and concluded that the protein was as efficiently integrated into the thylakoid membrane of the mutant as into that of the WT. Taken together, these results led to the widely accepted conclusions that (i) the mutant is able to perform the complete sequence of events from biosynthesis to assembly of Lhcb1, and that (ii) Lhcb1 fails to accumulate in thylakoid membranes, because chlorophyll *b* is needed to stabilize the protein and to prevent its degradation after insertion into the membrane.

In vivo and in vitro studies on the binding of chlorophylls and carotenoids to LHCII apoproteins were performed to identify the stage at which they bind to the protein and to find out whether they are needed for the insertion of the protein into the membrane and/or its stabilization following insertion. In the absence of carotenoids, the LHCPs of both photosystems failed to accumulate in mutants of *Chlamydomonas reinhardtii* (Herrin et al. 1992). Absence of chlorophyll had the same effect, and these results were taken as a demonstration of a direct role for the pigments in the stabilization of the LHC apoproteins (Herrin et al. 1992). A carotenoid-free mutant of *Scenedesmus obliquus* was shown to lack the major LHCII proteins, which led to the conclusion that lutein is necessary for their assembly (Heinze et al. 1997). Other studies reveal that LHCP was able to insert into the membranes without attached pigments. The precursor of LHCP preferentially inserted into stroma lamellae from which it migrated to grana regions (Yalovsky et al. 1990, 1992). This was taken as support for the idea that LHCP inserts as a pigment-free apoprotein into stroma membranes. When LHCP was fused to a bacterial signal peptide it could be inserted into *Escherichia coli* inner bacterial membranes (Kohorn and Auchincloss 1991). These studies were used to support the view that binding of pigments to LHCP occurs in a second step after lateral migration to the grana regions. After in vitro reconstitution experiments, Paulsen et al. (1993) and Kuttkat et al. (1997) suggested that pigments protect *E. coli*-overexpressed Lhcb1 against protease attack, that pigments induce folding of SDS-denatured Lhcb1 apoprotein, and that chlorophyll is needed for protease-resistant insertion of Lhcb1 into etioplast membranes.

To test whether chlorophyll *b* is required for protease-stable insertion of Lhcb1 into thylakoid membranes, we used three *chlorina-f2* mutants with different chlorophyll *b* concentrations: *chlorina-*$f2^{101}$ (chlorophyll b-free), *chlorina-*$f2^{123}$ (27% chlorophyll *b* compared with the WT), and *chlorina-*$f2^{122}$ (70% chlorophyll *b*). Thermolysin treatment of thylakoids was used to test for stable integration of Lhcb1 into the membrane. An antibody to Lhcb1 confirmed the heterogeneity of LHCII polypeptides and detected in WT membranes two protease-resistant mature forms of Lhcb1 with apparent molecular masses of 28 and 29 kDa. Only one band reacting with the Lhcb1 antibody could be detected in chlorophyll *b*-free *chlorina-*$f2^{f2}$. This protein band co-migrated with the 29-kDa form and was highly sensitive to protease treatment of the membranes. This might mean that in *chlorina-*$f2^{f2}$ the Lhcb1 precursor is only processed at one cleavage site, resulting in a 29-kDa polypeptide. The failure to provide this protein with chlorophyll *b* leads to its improper insertion in the membrane, its susceptibility to protease and its inconvertibility to the 28-kDa form.

Materials and methods

Plant material. Ten chlorophyll-deficient *chlorina-f2* mutants (*chlorina-*$f2^{f2}$,-$f2^{101}$, $f2^{102}$, -$f2^{105}$, -$f2^{107}$, -$f2^{108}$, -$f2^{109}$, $f2^{122}$, $f2^{123}$ and -$f2^{133}$) from the Carlsberg mutant collection (Department of Physiology, Carlsberg Laboratory, Copenhagen, Denmark) were grown at 22 °C and a photon flux density (PFD) of 75 μmol m^{-2} s^{-1} in a 14 h light/10 h dark cycle for 7–8 d (normal growth conditions). To test the composition of LHCP, *chlorina-*$f2^{f2}$ was grown under these normal growth conditions but at different

Table 1. Influence of light and growth temperature on the antenna protein composition of *chlorina-f2*^{f2} (clo-*f2*^{f2}) and *chlorina-f2*^{101} (clo-*f2*^{101}). *Chlorina-f2*^{f2} was grown at different PFDs from 30 to 225 μmol m^{-2} s^{-1}. *Chlorina-f2*^{101}was grown at 15 °C or 25 °C and 75 μmol m^{-2} s^{-1}. Immunoblot analyses were done with antibodies against all antenna proteins of PSI (Lhca l–4) and PSII (Lhcb l–6). (xxx, WT level; xx, reduced amounts; x, trace amounts; 0, not detectable)

	Lhca1	Lhca2	Lhca3	Lhca4	Lhcb1	Lhcb2	Lhcb3	Lhcb4	Lhcb5	Lhcb6
clo-*f2*^{f2} (30 μmol)	xxx	xxx	xxx	0	0	xx	xx	xx	xxx	0
clo-*f2*^{f2} (75 μmol)	xxx	xxx	xxx	0	0	xx	xx	xx	xxx	0
clo-*f2*^{f2} (125 μmol)	xxx	xxx	xxx	0	0	xx	xx	xx	xxx	0
clo-*f2*^{f2} (225 μmol)	xxx	xxx	xxx	0	0	xx	xx	xx	xxx	0
clo-*f2*^{101} (15 °C)	xxx	xxx	xxx	0	0	xxx	xxx	xxx	xxx	0
clo-*f2*^{101}(25 °C)	xxx	xxx	xxx	0	0	xxx	xxx	xxx	xxx	0

PFDs of 30, 75, 125 and 225 μmol m^{-2} s^{-1}. In addition, the chlorophyll *b*-free *chlorina-f2*^{101} was cultivated under normal conditions but at different temperatures of 15 °C and 25 °C.

Isolation of thylakoid membranes and immunoblot assays. Intact chloroplasts of *chlorina-f2* mutants were isolated according to Nielsen et al. (1994), subsequently broken and the thylakoid membrane proteins subjected to denaturing SDS-PAGE on 16–21% gradient gels (Fling and Gregerson 1986). To prepare samples from the apical and basal leaf areas the distal 2 cm and the proximal 2 cm of WT leaves, respectively, were harvested and thylakoid membranes isolated according to Nielsen et al. (1994). Protein was transferred to a polyvinylidenedifluoride (PVDF) membrane (Immobilon; Millipore, Bedford, Mass. USA) with a Mini-Trans-Blot-Transfer Cell (Biorad, Munich, Germany) at 20 V for 12 h.

Polyclonal monospecific antibodies against the proteins Lhca1, -3, -4 and Lhcb6 (Kròl et al. 1995), Lhcb1–3 (Sigrist and Staehelin 1992, 1994), Lhcb5 (Falbel and Staehelin 1992) and a monoclonal antibody detecting Lhca2 and Lhcb4 (Høyer-Hansen et al. 1988) were used. The Lhcb1 antibody was raised against the amino acid sequence KAKPVSSGSPW following the N-terminus MRKTVT of mature petunia Lhcb1*8 (*cab*13) of Dunsmuir (1985).

Pigment analysis. Reversed-phase high-performance liquid chromatography of pigment extracts from the thylakoid membranes was carried out as described by Knoetzel et al. (1988).

Protease treatment of thylakoid membranes. A 100-μg aliquot of total thylakoid membrane protein was centrifuged for 1 min at 15 000 rpm in a Biofuge 15 (Heraeus, Hanau, Germany). The pellet was resuspended in 500 μl of 10 mM HEPES-KOH, 0.5 mM $MgCl_2$ (pH 8.0), mixed with thermolysin (Protease X, P-1512; Sigma) as indicated in the figure legends, and stored for 30 min at 0 °C. After incubation, membranes were pelleted and resuspended in 50 μl sample buffer, according to Fling and Gregerson (1986), for electrophoresis.

Results

Growth conditions have no influence on LHCP composition in chlorina-f2^{f2}. *Chlorina-f2*^{f2} was cultivated under a normal growth temperature of 22 °C and a 14-h photoperiod, but at different PFDs of 30, 75, 125 and 225 μmol m^{-2} s^{-1}. The results of the immunoblot assays of the thylakoid membrane proteins against the LHC antibodies are shown in Table 1. The mutant expressed the same antenna phenotype at all light levels. No differences could be seen with respect to our earlier results, in which mutant seedlings were grown at our standard PFD of 75 μmol m^{-2} s^{-1} (Bossmann et al. 1997). To test whether different growth temperatures have an influence on LHCP composition, the chlorophyll *b*-free *chlorina-f2*^{101} was cultivated at 15 °C and 25 °C (Table 1). Again, no changes in the LHC pattern could be observed. In summary, the results in Table 1 show that the antenna patterns in *chlorina-f2*^{f2} and *chlorina-f2*^{101} were not influenced by the light levels or temperature changes applied.

Thylakoid membranes of chlorina-f2 alleles have unaltered protein integration and translocation capacity. The GTP- and 54CP (54CP being the chloroplast homologue to the 54-kDa subunit of the signal-recognition particle) protein-dependent integration pathway of LHCPs functions in *chlorina* mutants, as was demonstrated from the presence of almost all Lhca and Lhcb proteins in the alleles (Bossmann et al. 1997). Immunoblots, in which *chlorina-f2* alleles were assayed with antibodies against PsaD, -E, -F, -H, -L and -N show that each of the alleles

Table 2. Pigment composition (mol/100 mol chlorophyll *a*) and chlorophyll *a*/*b* ratios (mol/mol) of thylakoid membrane preparations from the WT and ten *chlorina-f2* mutants of barley. Seedlings were grown at 22 °C and a PFD of 75 μmol m^{-2} s^{-1}. Data from two experiments. Chl, chlorophyll; clo, *chlorina*

	Neoxanthin	Violaxanthin	Lutein	Chlorophyll *b*	*β*-Carotene	Chl *a*/*b* ratio
WT	3.8	3.2	17.8	41.9	9.3	2.4
clo-*f2*^{f2}	0.6	3.1	7.5	0.3	10.3	344.8
clo-*f2*^{102}	1.1	4.3	10.8	0.3	9.4	303.1
clo-*f2*^{105}	0.6	3.3	10.9	0.4	12.7	270.3
clo-*f2*^{108}	0.6	3.6	9.3	0.4	9.4	227.3
clo-*f2*^{101}	0.3	2.1	8.5	0.8	12.1	119.1
clo-*f2*^{123}	1.5	2.8	8.7	11.5	8.1	8.7
clo-*f2*^{109}	1.7	2.8	10.1	14.9	8.1	6.7
clo-*f2*^{133}	2.7	4.1	12.1	17.1	10.2	5.9
clo-*f2*^{107}	2.7	3.6	12.7	21.1	10.8	4.8
clo-*f2*^{122}	3.9	5.7	13.6	29.1	11.4	3.4

contained WT levels of all these PSI subunits (data not shown).

Chlorophyll b*-dependent insertion of Lhcb1 into mutant thylakoid membranes*. In order to gain more insight into the role of chlorophyll *b* in the integration of Lhcb1 into thylakoid membranes, we chose to analyze allelic *chlorina-f2* mutants with different chlorophyll *b* amounts. Table 2 gives the pigment compositions of the ten allelic *chlorina-f2* mutants in comparison to the pigment pattern of WT barley. The chlorophyll *b* content ranged from 42 mol chlorophyll *b*/100 mol chlorophyll *a* in the WT to 0.3 mol chlorophyll *b*/100 mol chlorophyll *a* in *chlorina* $f2^{f2}$. *Chlorina-*$f2^{101}$,-$f2^{108}$, -$f2^{102}$, and -$f2^{105}$ were almost chlorophyll *b*-free. Increasing chlorophyll *b* amounts from 11 to 29 mol chlorophyll *b*/100 mol chlorophyll *a* were measured for the leaky alleles *chlorina-*$f2^{123}$, -$f2^{109}$, -$f2^{133}$, -$f2^{107}$, and -$f2^{122}$.

None of the mutants although severely chlorophyll *b*-depleted, seemed to be affected in carotenoid accumulation. Reductions in the levels of neoxanthin and lutein mainly reflected deficiencies in Lhcb1 in the respective *f2* alleles (Table 2). This is consistent with the assumption that only chlorophyll *b* biosynthesis is affected at the oxygenase step (von Wettstein et al. 1995). Unaltered carotenoid availability is a prerequisite for Lhcb1 accumulation in thylakoid membranes (Herrin et al 1992). Reconstitution of Lhcb1 from heat-denatured or acetone-extracted thylakoid membranes or from *E. coli*-overexpressed Lhcb1 apoprotein has shown that stable association of chlorophyll with apoproteins requires xanthophylls (Plumley and Schmidt 1987; Paulsen et al. 1990). The alleles are able to code for carotenoids, but can only code for limited amounts of chlorophyll *b* and, thus, represent in-vivo systems in which to follow Lhcb1 integration into thylakoid membranes where chlorophyll *b* is the only limiting factor.

Thylakoid membranes from (i) *chlorina-*$f2^{122}$ with a chlorophyll *a*/*b* ratio of 3.4, (ii) *chlorina-*$f2^{123}$ with a ratio of 8.7, and (iii) the almost chlorophyll *b*-free mutant *chlorina-*$f2^{101}$, corresponding to chlorophyll *b* concentrations of 69%, 27% and 2%, respectively, relative to the WT (chlorophyll *a*/*b* ratio = 2.4), were isolated and treated with the protease thermolysin. After treatment, membrane proteins were subjected to denaturing SDS-PAGE, after which proteins were transferred onto PVDF membranes, that were incubated with antibodies against Lhcb1.

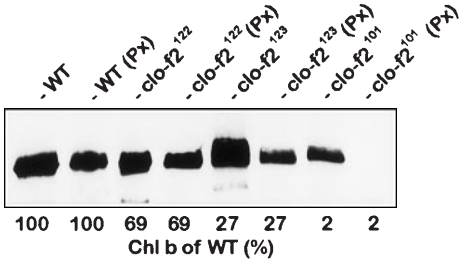

Fig. 1. Immunoblot analysis. Thylakoids membranes were isolated from barley wild type (*WT*) and the chlorophyll-*b*-deficient *chlorina* mutants clo-$f2^{101}$, clo-$f2^{122}$ and clo-$f2^{123}$ grown at 22 °C and a PFD of 75 μmol m^{-2} s^{-1}. In each lane, 100 μg total thylakoid membrane protein was treated with 10 μg of thermolysin (*Px*). Different amounts of protein (WT: 1 μg, clo-$f2^{122}$: 1 μg, clo-$f2^{123}$: 6 μg, and clo-$f2^{101}$: 9 μg) were loaded onto each track, separated by 16% SDS-PAGE and blotted onto PVDF membrane. Proteins were detected with the antibody against Lhcb1. Chl *b*, Chlorophyll *b*

Figure 1 presents the results of the protease treatments of thylakoid membranes from barley WT compared with those from *chlorina-*$f2^{101}$, -$f2^{123}$, and -$f2^{122}$, followed by an immunoblot assay for the detection of proteins reacting with the Lhcb1 antibody. The lanes show the immunoreaction of the non-treated thylakoids and of membranes treated with 10 μg thermolysin per 100 μg thylakoid membrane protein (Fig. 1, lanes Px). In the WT, Lhcb1 should be stably integrated into the thylakoid membranes. The immunoreaction of protease-treated membranes, however, was reduced, indicating that part of the N-terminal stroma-exposed Lhcb1 region recognized by the antibody is accessible to protease under our experimental conditions. In *chlorina-*$f2^{122}$ which contains about 70% of chlorophyll *b* compared with the WT and in *chlorina-*$f2^{123}$ (27% chlorophyll *b* of WT), the antibody-reacting protein band was more affected. Untreated thylakoid membranes of the nearly chlorophyll *b*-free mutant *chlorina-*$f2^{101}$ showed an Lhcb1 protein. This residual Lhcb1 in *chlorina-*$f2^{101}$ occurred at a level equal to about 10% of that in the WT. Thus, a 9-fold amount of mutant thylakoid membrane protein had to be loaded onto the gel and blotted onto PVDF membrane to give equivalent Lhcb1 immunoreactions in the WT and in *chlorina-*$f2^{101}$. This protein band disappeared after protease treatment (10 μg thermolysin per 100 μg thylakoid membrane protein), indicating that the protein was completely degraded (Fig. 1).

Figure 2A shows the results of treating WT and *chlorina-*$f2^{101}$ thylakoids with 10, 25 and 50 μg thermolysin per 100 μg total thylakoid membrane protein (lanes WT and lanes 101, 10–50 μg Px). In the WT, a continuous reduction in the levels of two Lhcb1 forms with apparent masses of 29 kDa and 28 kDa was apparent, indicating that degradation of Lhcb1 by protease is dependent on the protease/Lhcb1 ratio. The immunoreaction of untreated chlorophyll *b*-free *chlorina-f*2^{101} thylakoid membranes as shown in Fig. 2A (lane 101). To apply equivalent amounts of Lhcb1 onto the gels, a 9-fold greater amount of total protein compared with the WT was loaded and blotted onto the PVDF membrane. Only one form of Lhcb1 was recognizable, co-migrating with the 29-kDa form of the WT. Protease digestion led to the virtual disappearance of the 29-kDa form, even at the lowest thylakoid membrane protein/protease ratio of 10:1 (Fig. 2A, lane 101, 10 μg Px).

The experiment shown in Fig. 2B was performed in order to use comparable protease/Lhcb1 ratios in the WT and *chlorina-*$f2^{101}$. A range of protease concentrations was applied from 1, 2.5, to 5 μg thermolysin per 100 μg mutant thylakoid membrane protein. This

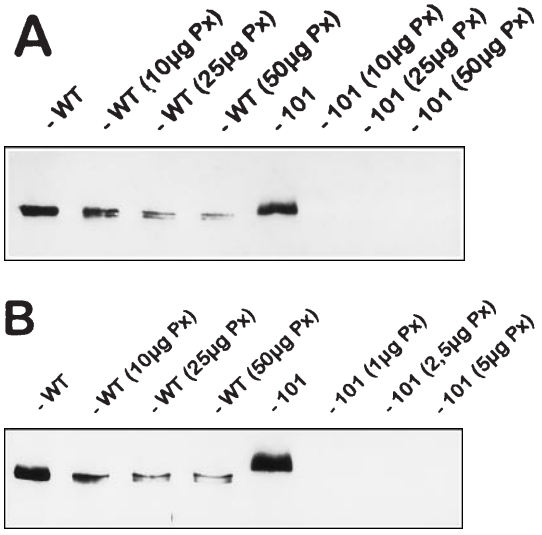

Fig. 2. Immunoblot analysis. Thylakoid membranes were isolated from the barley wild type (*WT*) and the chlorophyll *b*-lacking *chlorina-f2*^{101} (*101*) grown at a PFD of 75 µmol m^{-2} s^{-1} and 22 °C. **A** Samples (100 µg each) of total thylakoid membrane protein were treated with 0, 10, 25 or 50 µg of thermolysin (*Px*). Different amounts of protein (WT: 1 µg and *chlorina-f2*^{101}: 9 µg) were loaded onto each track, separated by 16% SDS-PAGE and blotted onto PVDF membrane. Proteins were detected with the antibody against Lhcb1. The antibody recognized a 28-kDa and a 29-kDa form of mature Lhcb1. **B** Samples (100 µg each) of total thylakoid membrane protein were treated with 0, 10, 25 or 50 µg (*WT*) or with 0, 1, 2.5 or 5 µg (*chlorina f2*^{101}; 101) of thermolysin (*Px*) to achieve equivalent Px/Lhcb1 ratios. Different amounts of protein (WT: 1 µg and *chlorina-f2*^{101}: 9 µg) were loaded onto each track, separated by 16% SDS-PAGE and blotted onto PVDF membrane. Proteins were detected with the antibody against Lhcb1. The antibody recognized a 28-kDa and a 29-kDa form of mature Lhcb1

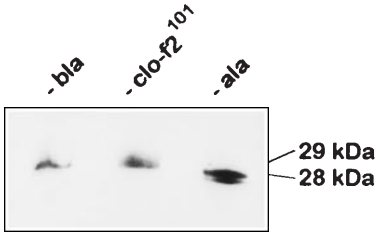

Fig. 3. Immunodetection of the LHCII protein Lhcb1 in thylakoid preparations from basal leaf areas (*bla*) and apical leaf areas (*ala*) of WT barley and in thylakoid preparations from *chlorina-f2*^{101} (clo-*f2*^{101}) after Western blotting. For ala preparations the top 2 cm of the WT leaves were used; bla preparations were done with the lowest pale green 2 cm of the leaves. *Chlorina-f2*^{101} thylakoids were isolated from whole-leaf material. Wild type and *chlorina-f2*^{101} plants were grown at a PFD of 75 µmol m^{-2} s^{-1} and 22 °C. Different amounts of protein (ala: 1 µg, bla: 5 µg and *chlorina-f2*^{101}: 5 µg) were loaded onto each track, separated by 16% SDS-PAGE and blotted onto PVDF membrane. Membranes were incubated with the antibody against Lhcb1 recognizing a 28-kDa and a 29-kDa form of mature Lhcb1

experiment gave the same result as the protease treatment at equivalent protease/thylakoid membrane ratios shown in Fig. 2A. In summary, we have shown that chlorophyll *b* has a substantial influence on the resistance of mature Lhcb1 to exogenous protease treatment in *chlorina-f2* thylakoid membranes.

Processing of Lhcb1 depends on correct insertion into the thylakoid membrane. In WT barley, the antibody against Lhcb1 detected two forms of the mature chlorophyll *a*/*b*-binding protein in thylakoid membranes, with apparent masses of 29 kDa and 28 kDa (Fig. 2A, B, lanes WT). The 29-kDa Lhcb1 in *chlorina-f2*^{101} was highly unstable in the absence of chlorophyll *b* and in the presence of protease (Fig. 2A, B, lanes 101).

Figure 3 shows the immune reaction of the Lhcb1 antibody against thylakoid membrane proteins from apical and basal leaf tissue of WT barley and from leaves of *chlorina-f2*^{101}. The apical preparation showed the presence of two forms of Lhcb1 with apparent molecular masses of 29 kDa and 28 kDa. Only the 29-kDa form was detected in the basal part of the leaves, and in *chlorina-f2*^{101} thylakoid membranes.

Discussion

Chlorina-f2^{f2} is the most well-characterized chlorophyll *b*-free mutant, having nearly normal amounts of chlorophyll *a*. Chlorophyll *b* and the few missing light-harvesting chlorophyll *a*/*b*-binding proteins are not essential for the photosynthetic performance of the mutant (Boardman and Highkin 1966). Deficiency in LHCII, however, leads to an overexcitation of PSI relative to PSII in *chlorina-f2* mutants, indicating the role of LHCII in balancing excitation energy (Andrews et al. 1995). Recent investigations on the thermo- and photostability of PSII in *chlorina-f2*^{f2} showed that the LHCII deficiency destabilizes the oxidizing side, indicating that light-harvesting proteins support the active conformation of the water oxidation complex (Havaux and Tardy 1997). Parameters such as temperature and light often play an essential role in the expression of organismal phenotypes. The mutants respond especially sensitively to changing parameters. Thus, we analyzed the LHCP composition of the chlorophylly *b*-free alleles *chlorina-f2*^{f2} and *chlorina-f2*^{101} grown under different irradiance and temperature conditions. There were no differences in the pattern of the chlorophyll *a*/*b*-binding proteins in *chlorina-f2*^{f2} and *-f2*^{101} under these conditions. The *chlorina-f2* mutants express a reasonably stable phenotype. Thus, chlorophyll *b* is required for accumulation of Lhcb1, Lhca4 and Lhcb6 only (Bossmann et al. 1997). In vitro reconstitutions by Schmid et al. (1997) have led to an explanation of why Lhca4 could be lacking in the chlorophyll *b*-free *chlorina-f2*^{f2}. The experiments indicate that chlorophyll *b* is needed for heterodimerization of Lhca1 and Lhca4 to generate the subcomplex LHCI-730.

The Lhcb1 protein is affected in almost all alleles and is one of the plant antenna proteins most sensitive to chlorophyll *b* deficiency (Bossmann et al. 1997). Thus, it seemed the prime candidate to use to focus on the question of whether the integration of a light-harvesting protein in thylakoid membranes is already chlorophyll *b* dependent. We used thermolysin treatment of

membranes isolated from barley WT and *chlorina-f2* mutants followed by detection of proteins reacting with an antibody against an N-terminal amino-acid sequence of Lhcb1 (Sigrist and Staehelin 1994). The use of proteases such as thermolysin or trypsin is still a standard assay for the control of integration in vivo and in vitro, although it was shown recently that trypsin resistance indicated pigment binding rather than insertion in the case of LHCP reconstituted in vitro (Paulsen et al. 1993; Kuttkat et al. 1997).

The *chlorina-f2* mutants offer interesting model plants in which the mutation leads to a lack of chlorophyll *b* or to different degrees of chlorophyll *b* deficiency, enabling them to be used to study the role of chlorophyll *b* in the assembly of light-harvesting proteins. Non-integrated Lhcb1, or Lhcb1 that is only associated with the membrane should be totally degraded by exogenous protease treatment (Hoober et al. 1990, 1994).

In the present report, we have shown that protease added to thylakoid membranes isolated from *chlorina-f2* mutants leads to an increasing instability of Lhcb1 concomitant with the decreased amount of chlorophyll *b* in the *chlorina-f2* seedlings (Fig. 1). *Chlorina-f2*^{122}, with a chlorophyll *a*/*b* ratio similar to that of the WT and an unaffected antenna protein pattern, obviously contains enough chlorophyll *b* to integrate Lhcb1 to WT level. *Chlorina-f2*^{123}, with a reduced level of Lhcb1 and a chlorophyll *a*/*b* ratio of 8.7, is more affected by protease treatment than the WT or *chlorina-f2*^{122}. The chlorophyll *b* content of this mutant seems high enough to supply the two photosystems with a complete antenna system at the WT level, but initial deficiencies appear in the most sensitive antenna protein Lhcb1. In *chlorina-f2*^{101}, essentially devoid of chlorophyll *b*, Lhcb1 is detectable with our antibody, but extensively digested when thermolysin is added (Figs. 1, 2). Apparently, digestion occurs because the protein is only associated with the membrane surface, or is not properly integrated into the membrane. This is in agreement with the findings of Hoober et al. (1990, 1994) in the chlorophyll *b*-free *Chlamydomonas cbn*1–113. Due to the lack of a membrane-bound protease, the *Chlamydomonas* mutant, in contrast to barley *chlorina-f2*^{f2}, is capable of accumulating nearly normal levels of a full complement of LHCPs. The LHCPs in thylakoid membranes isolated from *Chlamydomonas cbn*1–113 were degraded by exogenous proteases, which was taken as evidence that the proteins are not correctly inserted into the membrane. Etioplasts are capable of importing in vitro-synthesized Lhcb1, but not of inserting proteins into membranes (Chitnis et al. 1987). Consistent with our conclusion, it was recently shown that chlorophyll is the only factor needed for stable integration of Lhcb1 into the membrane (Kuttkat et al. 1997). Taken together, this supports speculations that folding of Lhcb1, membrane insertion, and the binding of chlorophyll *a*, chlorophyll *b* and xanthophylls have to be coupled to allow the development of the native, protease-resistant, membrane-embedded structure of Lhcb1 (Paulsen 1995).

In WT barley, the Lhcb1 antibody cross-links with mature 29-kDa and 28-kDa forms of Lhcb1 which are both protease-stably integrated into the thylakoid membrane. In *chlorina-f2*^{101}, only one Lhcb1, co-migrating with the 29-kDa form, can be detected; this Lhcb1 is unstable in the presence of thermolysin (Fig. 2A, B). Multiple forms of LHCPs have been found in vivo in the thylakoids and the problem of their origin has not been entirely resolved. Upon in vitro import into chloroplasts, precursor Lhcb1(pLhcb1) was shown to be cleaved at two sites, termed the primary and secondary sites into thermolysin-resistant proteins of 26 and 25 kDa, respectively (Lamppa and Abad 1987; Oblong and Lamppa 1992). In an organelle-free assay, the chloroplast-processing enzyme, with characteristics of the stromal-processing peptidase, cleaves pLhcb1 to one mature 25-kDa form, with no evidence that the 26-kDa form might be a transient intermediate (Clark et al. 1989; Vander Vere et al. 1995). The two forms seem to arise from two independent processing events, which remove the transit peptides at two sites approximately 1 kDa apart. Interestingly, after the import of pLhcb1 into isolated pea chloroplasts, the mature form of Lhcb1 in stroma lamellae was shown to have a slightly greater apparent molecular mass than the mature protein located in the grana membranes (Yalovsky et al. 1992). These two forms resemble the 26-kDa and 25-kDa Lhcb1 forms and our 29-kDa and 28-kDa Lhcb1. Yalovsky and co-workers (1992) speculated that the two mature forms in the two membrane fractions could reflect processing at two sites or a covalent modification of the Lhcb1 protein. Binding of phosphatidylglycerol is probably involved in trimerization of Lhcb1/2 (Garnier et al. 1990; Hobe et al. 1994). However, the differential distribution between grana and stroma suggests a functional aspect. Consistently, the experiment illustrated in Fig. 3 shows that the 29-kDa Lhcb1 is found in an early stage of leaf development in WT barley whereas in older apical leaf tissues, both Lhcb1 forms are found. This, together with our finding that in *chlorina-f2*^{101} only the Lhcb1 with the greater apparent molecular mass is found, could mean that a stepwise processing or modification occurs in the course of Lhcb1 integration into the thylakoid membrane and membrane biogenesis.

We presume that in WT chloroplasts the 29-kDa protein is cleaved, mainly integrated into stroma lamellae (Yalovsky et al. 1990,1992) and, subsequently, can be digested or covalently modified to 28-kDa Lhcb1. The 29-kDa Lhcb1 is not only a transient intermediate form, but is also found in the WT in amounts similar to the 28-kDa Lhcb1. In *chlorina-f2*^{101}, Lhcb1 is not converted to the 28-kDa Lhcb1, probably because the lack of chlorophyll *b* inhibits its complete integration into the thylakoid membrane. The 29-kDa Lhcb1 is unable to stably integrate into the thylakoid membrane. is rapidly degraded, and processing or modification to the 28-kDa form does not take place.

The function of the two different forms of Lhcb1 is not apparent and needs further investigation. Two subsequent cleavage events would mean that processing at the secondary site creates an Lhcb1 protein with lower molecular mass, with the phosphorylation site missing.

Attachment of phosphatidylglycerol to Lhcb1 after insertion would support heterogeneity in terms of the abundance of trimeric complexes. In any case, the product of modification would be important in the assembly of multiprotein complexes and/or in balancing the distribution of excitation energy between the two photosystems in separate membrane regions.

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