Exchange of Pigment-Binding Amino Acids in Light-Harvesting Chlorophyll *a*/*b* Protein†

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Received March 30, 1999; Revised Manuscript Received September 8, 1999

ABSTRACT: Four amino acids in the major light-harvesting chlorophyll (Chl) *a*/*b* complex (LHCII) that are thought to coordinate Chl molecules have been exchanged with amino acids that presumably cannot bind Chl. Amino acids H68, Q131, Q197, and H212 are positioned in helixes B, C, A, and D, respectively, and, according to the LHCII crystal structure [Kühlbrandt, W., et al. (1994) *Nature 367*, 614−621], coordinate the Chl molecules named a_5, b_6, a_3, and b_3. Moreover, a double mutant was analyzed carrying exchanges at positions E65 and H68, presumably affecting Chls a_4 and a_5. All mutant proteins could be reconstituted in vitro with pigments, although the thermal stability of the resulting mutant versions of recombinant LHCII varied significantly. All complexes reconstituted with the mutant proteins contained fewer chlorophyll molecules per two lutein molecules than complexes reconstituted with the wild-type protein. However, the chlorophyll-binding amino acids could not be unambiguously assigned to binding either chlorophyll *a* or *b*, as in most cases more than one chlorophyll molecule was lost due to the mutation. The changes in Chl stoichiometries suggest that in LHCII some chlorophyll positions can be filled with either Chl *a* or *b*. Only some of the point mutations in LHCII affected the ability of the apoprotein to assemble into trimeric LHCII upon insertion into isolated thylakoid membranes. Among these were exchanges of H68 with either F or L, suggesting that the stability of the LHCII trimer significantly depends on this amino acid or the Chl molecule named a_5 that is attached to it and is located close to the center of the trimeric complex. The ion pair bridge between E65 and R185 in LHCII does not appear to be essential for the proper folding of the protein.

Light-harvesting chlorophyll (Chl)[1] *a*/*b* complexes enable the photosynthetic apparatus of higher plants to utilize solar light energy efficiently by capturing light quanta and conducting the energy to the photosynthetic reaction centers. The most abundant component of this light-harvesting system is the major light-harvesting complex of photosystem II (LHCII). It contains apoproteins Lhcb1 and Lhcb2 (27 and 25 kDa, respectively) and, noncovalently associated with these proteins, 7−8 Chl *a* and 5−6 Chl *b* molecules and 2−3 xanthophylls. Twelve of the chlorophyll molecules and 2 of the carotenoids are seen in a structural model at 3.4 Å resolution that is based on the electron crystallographic analysis of two-dimensional crystals of LHCII isolated from pea (*1*). The 2 xanthophylls closely interact with a super helix formed by the first and third transmembrane α-helices of the apoprotein. These 2 xanthophylls are generally thought to be luteins since 2 luteins have been reported to be present in LHCII complexes isolated from a large number of plant species and also in other Chl *a*/*b* complexes related to LHCII. Most of the chlorophylls in LHCII interact, through their central Mg ion, with nucleophilic side chains in the protein, such as histidine, glutamine, and asparagine. Chl *a* and Chl *b* cannot be distinguished yet in the structural model presented here. However, the Chl molecules at a more central position, in close contact with the 2 xanthophylls, have been assigned as Chl *a* molecules, as Chl *a* needs xanthophylls as scavengers for its potentially harmful triplet excited state, more so than Chl *b* that is less likely to adopt a triplet excited state.

A proper positioning of the light-harvesting pigments in LHCII is essential for the functions that the complex fulfills in photosynthesis. The chlorophylls must be positioned closely enough and oriented such that they can be involved in rapid pigment−pigment energy transfer. On the other hand, they must be prevented from forming, in an uncontrolled fashion, aggregates that would dissipate light energy into heat and, thus, cause loss of some of the captured energy. Chlorophylls and xanthophylls must be brought into contact such that singlet excitation energy can migrate from xanthophylls to chlorophylls and that triplet excitation energy can migrate vice versa, from chlorophylls to xanthophylls. And finally, xanthophylls and chlorophylls must interact such that the complex can switch to an increased level of heat dissipation of captured energy under excess light conditions that would otherwise harm the photosynthetic apparatus.

The structural information presently available about LHCII is not detailed enough to describe all the molecular interactions that hold the light-harvesting pigments in the right

† This work has been funded by the Deutsche Forschungsgemeinschaft (Pa 324/5-1), Stiftung Rheinland-Pfalz für Innovation, and Fonds der Chemischen Industrie.

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[1] Abbreviations: Chl, chlorophyll; LHCII, major light-harvesting Chl *a*/*b*-binding complex of photosystem II; Lhcb, light-harvesting Chl *a*/*b*-binding protein of LHCII; LDS, lithium dodecyl sulfate; DTE, dithioerythritol; LM, dodecyl maltoside; OG, octyl glucoside; DP and DP*, protease digestion products of Lhcb1; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

10.1021/bi990738x CCC: $18.00
Published on Web 11/12/1999

place. Even when a higher-resolution structural model will be available, this will not tell us which of the molecular interactions, which may then be visible, are the most important ones responsible for stabilizing the functional structure. This question will have to be approached experimentally by changing some of the structural features and testing whether these changes affect functions of the light-harvesting complex.

Structural alterations have been introduced to Lhcb1,2 by expressing point-specifically mutated genes; the resulting peptides have then been tested for their assembly into LHCII in vivo or upon import into isolated plastids (*2*, *3*) or insertion into isolated thylakoids (*4*−*6*) in vitro. A particularly useful assay specifically directed at the pigment-binding properties of Lhcb1,2 is its reconstitution with pigments in vitro (*7*). When bacterially expressed protein is used in this assay, structural alterations are easily introduced into the protein by site-directed mutagenesis (*8*−*10*). Progressive deletions from the N- or C-terminus have been introduced into recombinant Lhcb1 protein so protein domains involved in pigment binding and/or stabilization of the pigment−protein complex could be identified (*8*, *9*). Typically, these deletions had all-or-nothing effects on pigment binding; either virtually all pigments were still bound to the shortened apoprotein, or the mutant protein was no longer able to form any stable complexes at all, indicating a highly cooperative binding of pigments.

These experiments are extended in this paper to mutation of single amino acids that, in the structural model of LHCII, are seen to interact with Chl molecules. The exchange of Chl-binding amino acids has been performed with another member of the Chl *a*/*b* protein family, Lhcb4 (*11*). All 8 supposed Chl-binding amino acids in Lhcb4 have been exchanged, and most of the resulting mutant protein versions have been reported to bind 1 less Chl than the wild-type protein (*11*). We show here that in the case of Lhcb1, too, the exchange of Chl-complexing amino acids affects complex stability to various degrees, and that the pigment−protein complexes formed with these mutant proteins lack one or several Chl molecules compared to wild-type Lhcb1. In the accompanying paper (*12*), consistent results are obtained with a set of Lhcb1 mutants that have been reconstituted to form trimeric LHCII.

MATERIALS AND METHODS

Mutagenesis. The mutagenesis of Lhcb1 started from an expression plasmid of the Lhcb1*2 gene (AB80) from pea (*13*) expressing the mature protein except for 2 amino acids near the amino terminus (*14*). Site-directed mutagenesis of Lhcb1 was carried out according to the PCR method described by Chen and Przybyla (*15*). The mutagenesis primers designed for the mutations were as follows: H212L, 5′-ctg atc ttc ttg cag acc c-3′; H212F, 5′-gct gat ttt ctt gca gac cca g-3′; Q197E, 5′-cgt tga agc tat tgt aac tgg-3′; Q197S, 5′-cgt ttc agc tat tgt aac tgg-3′; Q131E, 5′-gcc act gag gtt atc ttg atg-3′; Q131S, 5′-gcc act tcg gtt atc ttg atg-3′; H68L, 5′-cca tct gga gag gat gac ttc gag-3′; and H68F, 5′-cca tct gga gaa gat gac ttc gag-3′. All PCRs were carried out with Pfu DNA polymerase (Stratagene, Heidelberg, Germany). In the mutant expression clone, the entire section that had been amplified by PCR during the mutagenesis procedure was verified by DNA sequencing.

Reconstitution with Pigments and Isolation of Recombinant Complexes. Wild-type and mutated Lhcb1 were overexpressed in *Escherichia coli* as described previously (*10*). Reconstitution of proteins with pigments was as described by Paulsen et al. (*16*) with the following modifications. Reconstitution mixtures contained 1 μg/μL protein, 2.5 μg/μL Chl (Chl *a*:Chl *b* ratio of 1 if not mentioned otherwise), and 0.4 μg/μL xanthophyll, and the reconstitution buffer was 2% (w/v) LDS, 100 mM Tris-HCl (pH 9), 12.5% (w/v) sucrose, and 10 mM DTE; the latter was added only after the heat denaturation step. The sucrose was omitted if the complexes were to be isolated by sucrose density centrifugation. Complexes were isolated on a partially denaturing polyacrylamide gel (*10*) or by centrifugation through a sucrose density gradient. Sucrose gradients were generated in 4 mL of a solution containing 0.6 M sucrose, 0.1% (w/v) LM, and 5 mM tricine (pH 7.8) in a 11 mm × 60 mm centrifuge tube (Beckman, Palo Alto, CA) by three subsequent freeze−thaw cycles (−20 and 4 °C, respectively). Fifty microliters of the reconstitution mix was loaded on the gradient which was then centrifuged at 45000*g* for 16 h at 4 °C. The second band from the bottom contained the reconstituted complexes, and it was removed from the gradient by using an injection needle attached to a peristaltic pump (Abimed, Langenfeld, Germany).

Stability of Reconstituted Complexes. The thermal stability of reconstituted LHCII was measured by observing the decrease in the extent of energy transfer between complex-bound Chl *b* and Chl *a* upon gradual dissociation of the complexes at 37 °C. The assay was conducted by diluting the reconstitution solution 100-fold with buffer [0.1 M Tris (pH 7), 1% (w/v) OG, and 10% glycerol] and measuring the decay kinetics of Chl *b*-stimulated Chl *a* fluorescence (excitation and emission wavelengths of 460 and 680 nm with slit widths of 1 and 5 nm, respectively) in a fluorimeter (Fluoromax 2, Spex/ISA, Grasbrunn, Germany) thermostated to 37 °C. Measurements were taken for 6 min at 1 s intervals. The rate constant of the fluorescence decay was calculated by fitting to first-order kinetics (Table Curve 2D, SPSS Inc., Chicago, IL), and used to calculate the lifetimes of the complex at the elevated temperature (37 °C).

Pigment Analysis in Reconstituted LHCII. Pigment compositions were analyzed in reconstituted complexes by extraction into 2-butanol (*17*). When complexes were isolated by gel electrophoresis, the gel was run just long enough that the band of reconstituted complexes was completely separated from the band of unbound pigments. The former band was excised from the gel and extracted. Sucrose gradient fractions were directly used for extraction. Extracted pigment solutions in 2-butanol were applied to an RP-C8 HPLC column (MZ Analysentechnik, Mainz, Germany) and separated in a gradient from 70 to 100% acetone (20 min, at a rate of 1 mL/min; Gynkotec, Germering, Germany). Pigments were quantified by comparing integrated peak areas to calibrated ones of known pigment amounts; calibration was performed on the basis of Chl and xanthophyll absorption (*10*).

Preparation of Chloroplasts, Lysates, Thylakoids, Stroma, and Radioactively Labeled Protein. Chloroplasts were isolated from 8-day-old pea seedlings (*Pisum sativum* var. Golf) grown on vermiculite with 16 h:8 h light:dark periods as described by Kuttkat et al. (*5*). The stromal extract was

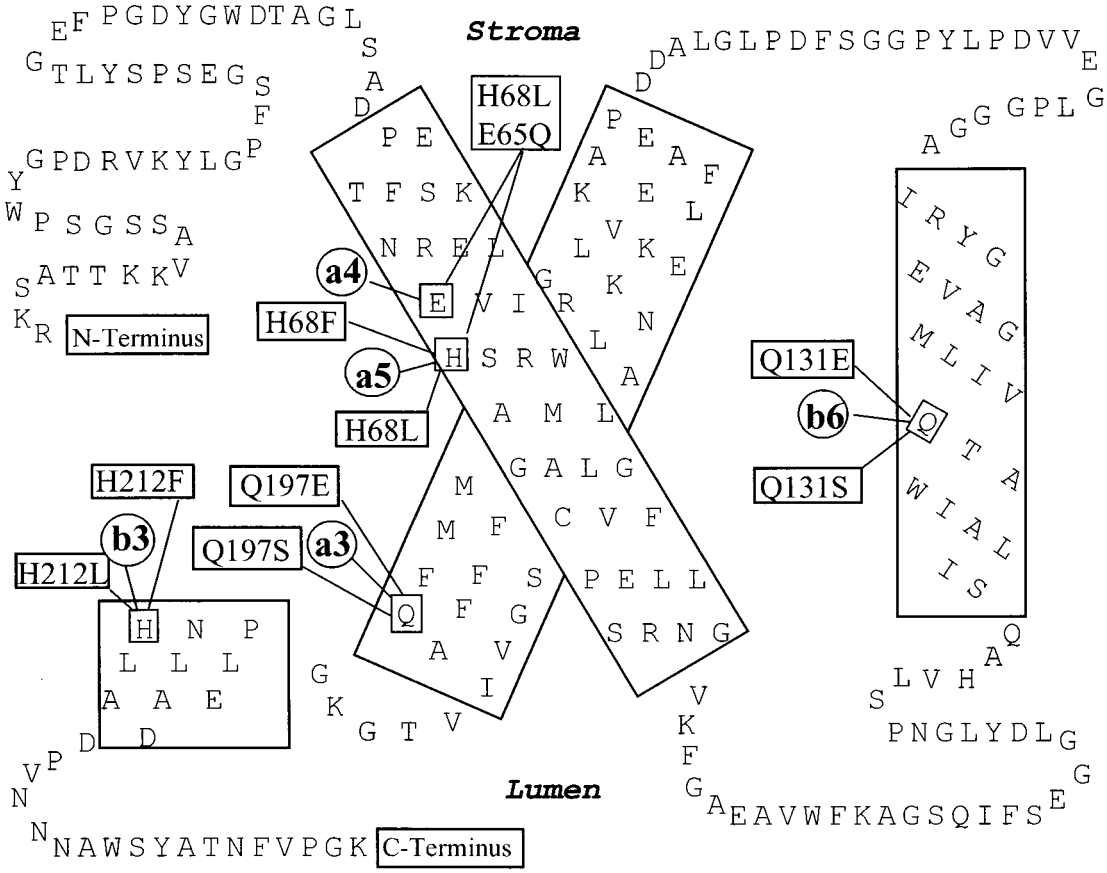

FIGURE 1: Localization of amino acid exchanges and chlorophyll binding sites affected in the Lhcb1 amino acid sequence, according to the LHCII crystal structure (*1*).

prepared by resuspending chloroplast pellets to a Chl concentration of 3 mg/mL in 10 mM Hepes/KOH (pH 8). After centrifugation of the lysate for 10 min at 4 °C and 8000*g*, the stroma fraction in the supernatant was removed. The thylakoid pellet was resuspended to a Chl concentration of 2 mg/mL in 0.33 M sorbitol and 50 mM Hepes/KOH (pH 8). Proteins were labeled in vivo with ^{35}S as described in ref *5*.

Thylakoid Protein Insertion Assay. Insertion of Lhcb1 into isolated thylakoids and subsequent treatment of the thylakoids with thermolysin were carried out following the procedure of Kuttkat et al. (*5*), with the modification that the Mg^{2+} concentration was reduced to 15 mM. Proteins of thermolysin-treated membranes were separated by polyacrylamide gel electrophoresis either on partially denaturing gels upon solubilization of the thylakoids to a final Chl concentration of 0.45 mg/mL in a slightly modified buffer (*18*) containing 0.45% (w/v) decyl maltoside, 0.45% (w/v) OG, 0.1% (w/v) LDS, 10% (v/v) glycerol, 2.5 mM EDTA, and 5 mM Hepes/KOH (pH 8) or on fully denaturing gels after precipitation and solubilization of the proteins in 1.3% SDS. Gels were analyzed by fluorography on a phosphoimaging analyzer (Fujix BAS 1500, Raytest, Straubenhardt, Germany).

RESULTS

Exchange of Chlorophyll-Associating Amino Acids Differently Affects LHCII Stability. Four amino acid positions in Lhcb1 were mutated in this study (Figure 1). These positions are located in all four α-helical domains, and are thought to represent binding sites of both Chl *a* (H68 and Q197) and Chl *b* (Q131 and H212). Each of these amino acids was exchanged with 2 amino acids that are not thought to be able to form a complex with chlorophyll: one of these having some structural resemblance with the amino acid exchanged (E and F replacing Q and H, respectively) and the other one being smaller (S and L replacing Q and H, respectively). Care was taken that neither of the newly introduced amino acids was likely to break the local α-helix (*19*). In addition to the 8 single-amino acid exchanges, a double mutant (H68L/E65Q) was obtained by chance in one of the mutagenesis reactions, carrying exchanges in 2 supposed Chl *a* binding sites.

All mutant versions of Lhcb1 were found to be able to bind pigments in an in vitro reconstitution with pigments so that Lhcb1−pigment complexes could be isolated by partially denaturing polyacrylamide gel electrophoresis (Figure 2). However, the intensity of the green bands representing reconstituted pigment−protein complexes differed greatly between various mutants, although the amount of protein used in the reconstitution and then applied to the gel was the same in each experiment, as checked by subsequent protein staining with Coomassie Blue (not shown). The mutant Q131S does not exhibit a band of reconstituted complex (M) in Figure 2; however, this band was visible after shorter electrophoresis times, when the complex was just separated from the free pigment band (FP), and then disintegrated during further electrophoresis (not shown). This difference in band intensities suggests that the various recombinant complexes exhibit different stabilities. The less

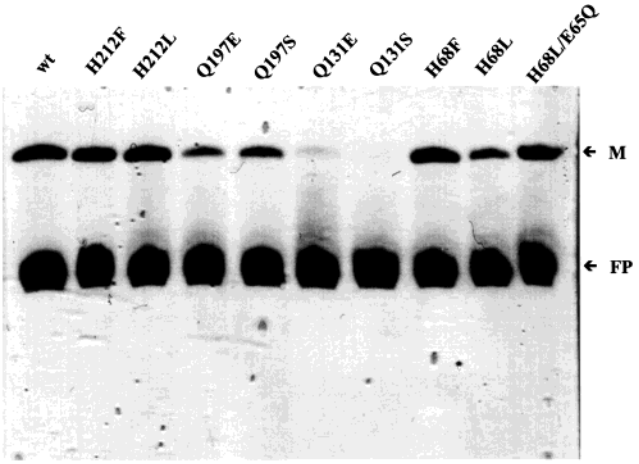

FIGURE 2: Gel electrophoretic analysis of wild-type (wt) and mutant Lhcb1 reconstituted with pigments. Proteins were reconstituted and then applied to a partially denaturing polyacrylamide gel (Materials and Methods). M, monomeric LHCII. FP, unbound pigments.

Table 1: Stability of Reconstituted Complexes toward Thermal Denaturation[a]

Lhcb1	τ (s)	Lhcb1	τ (s)
wild-type	70.3 ± 8.6	Q131E	24.9 ± 2.2
H212L	54.1 ± 6.3	Q131S	20.0 ± 1.8
H212F	67.4 ± 9.1	H68F	27.4 ± 2.4
Q197E	33.0 ± 4.5	H68L	18.4 ± 2.3
Q197S	34.9 ± 3.6	H68L/E65Q	16.8 ± 2.2

[a] The dissociation kinetics of reconstituted Lhcb1−pigment complexes of the wild-type (wt) and mutant proteins at 37 °C was followed by measuring the decrease in Chl *b*-stimulated Chl *a* fluorescence emission. The data represent the lifetimes (τ) of pigment−protein complexes ± standard deviations of three to five measurements. Lifetimes were calculated from first-order rate constants (see Materials and Methods).

stable versions either reconstitute at a lower yield or dissociate to a greater extent during the isolation procedure.

To test this notion, we assayed the stability of the recombinant complexes by measuring their dissociation kinetics at an elevated temperature. Only within the reconstituted complexes did energy transfer from Chl *b* to Chl *a* take place. Therefore, the decay of Chl *b*-sensitized Chl *a* fluorescence upon raising the temperature reflects heat-induced dissociation of the complexes. The lifetime of the complex in the dissociation procedure is taken as a relative measure of complex stability. Table 1 shows that the complexes reconstituted from mutant proteins exhibit a less stability than complexes with the wild-type protein. The smallest loss in complex stability is seen when H212 is exchanged with either F or L. The most pronounced destabilization is caused by exchanging Q131 with E or S, by replacing H68 with L, and by a double exchange at positions 65 and 68. The replacement of Q197 with E or S and of H68 with F gives rise to intermediate effects. Interestingly, the exchange of H with F in both positions appears to be less of a disturbance for complex stability than with L. In position 131, the replacement of Q with either E or S is equally detrimental, whereas in the case of Q197, the exchange with S has less of an effect than that with E.

Exchange of Chlorophyll-Associating Amino Acids Causes the Loss of 1 or 2 Chlorophylls. To test whether Chl *a* or Chl *b* can be assigned to the Chl binding amino acids that are exchanged, we compared the Chl stoichiometries in the recombinant complexes with mutated protein with those in recombinant complexes with the unaltered protein. We have been unable to measure the protein amounts in detergent-dissolved recombinant LHCII with sufficient reproducibility to deduce reliable Chl:protein ratios. Therefore, we used Chl:(2 lutein) ratios as an approximation for Chl:protein ratios, assuming that the recombinant complexes invariably contain 2 lutein molecules. This appears to be justified for the following reasons. (a) Native LHCII has repeatedly been reported to contain 2 lutein molecules per apoprotein (see, for example, ref *20*). (b) A loss of a lutein molecule from one of the mutated recombinant LHCII would be expected to cause a larger change in the Chl:(2 luteins) ratio than those that are observed. (c) Lutein possibly adopts a stoichiometry of slightly more or less than 2 in recombinant LHCII. However, since we are using the stoichiometry of Chl:(2 luteins) only to compare Chl stoichiometries in recombinant complexes with and without mutations, this would not affect our evaluations as long as the lutein:LHCII ratios stay constant. The neoxanthin:lutein ratio was found to stay constant around 0.5 in all reconstitution products (not shown), indicating that the mutations do not affect the carotenoid composition of the recombinant complexes. From the Chl *a*:Chl *b* ratios and the Chl:(2 lutein) ratios (Table 2), we calculated the number of Chl *a* and Chl *b* molecules lost due to amino acid exchanges in the apoprotein (Table 3).

As explained in more detail in the Discussion, it is difficult to decide on the basis of our present knowledge which reconstitution procedure yields the most authentic recombinant monomeric LHCII. If reconstitution and isolation are performed at high stringency, only those complexes are selected that are about as stable as wild-type monomeric LHCII but tend to have a Chl *a*:Chl *b* ratio lower than the one found in native LHCII isolated under mild conditions. Recombinant monomeric LHCII can be reconstituted with a Chl *a*:Chl *b* ratio closer to the native one by using milder reconstitution and isolation procedures; however, these may contain less stable complexes in which some less specific Chl binding sites are occupied with the "wrong" Chl. Therefore, we chose to employ four diffent procedures for the reconstitution and isolation of all mutant proteins and the wild-type Lhcb1. The reconstitutions were performed using a Chl *a*:Chl *b* ratio of 1 or 2, and the resulting complexes were isolated either on a partially denaturing polyacrylamide gel or by ultracentrifugation on a sucrose density gradient. The nonmutated protein reconstituted with equal concentrations of Chl *a* and *b* and isolated by partial denaturing gel electrophoresis yielded complexes with a Chl *a*:Chl *b* ratio of 0.8 and a Chl:(2 lutein) ratio of 13, suggesting that the reconstituted complexes contain 6 Chl *a* and 7 Chl *b* molecules. After isolation on the sucrose density gradient, both the Chl *a*:Chl *b* ratio (1.0) and the Chl:(2 lutein) ratio (15) were higher, suggesting 7−8 Chl *a* and 7−8 Chl *b* molecules per complex. Reconstitution with an elevated Chl *a*:Chl *b* ratio also increased the Chl *a*:Chl *b* ratios in the recombinant complex. The Chl *a*:Chl *b* ratio is 1.1 upon gel electrophoresis and 1.3 upon sucrose gradient centrifugation. In this case, the Chl:(2 lutein) ratios suggest about 8 Chl *a* and 7 Chl *b* molecules per complex upon gel isolation and 8 Chl *a* and 6−7 Chl *b* molecules after isolation on a sucrose density gradient.

Table 2: Pigment Stoichiometries of the Wild-Type (wt) and Mutant Lhcb1−Pigment Complexes[a]

	Chl *a*:Chl *b*				Chl(*a*+*b*):(2 lutein)			
	gel		UC		gel		UC	
Lhcb1	*a*:*b* of 1:1	*a*:*b* of 2:1	*a*:*b* of 1:1	*a*:*b* of 2:1	*a*:*b* of 1:1	*a*:*b* of 2:1	*a*:*b* of 1:1	*a*:*b* of 2:1
wild-type	0.82 ± 0.04	1.10 ± 0.09	1.02 ± 0.1	1.34 ± 0.19	13.3 ± 1.2	15.3 ± 1.8	14.8 ± 1.5	14.6 ± 3.4
H212L	0.76 ± 0.03	0.94 ± 0.13	1.01 ± 0.16	1.26 ± 0.20	11.4 ± 0.2	13.2 ± 2.0	13.0 ± 1.4	13.2 ± 2.6
H212F	0.77 ± 0.05	0.90 ± 0.09	0.93 ± 0.06	1.29 ± 0.17	11.5 ± 0.2	13.0 ± 0.8	13.3 ± 0.9	11.9 ± 0.7
Q197E	0.83 ± 0.09	1.02 ± 0.13	1.07 ± 0.19	1.26 ± 0.33	12.5 ± 0.3	13.9 ± 1.6	14.1 ± 1.6	13.1 ± 3.7
Q197S	0.84 ± 0.03	0.88 ± 0.13	0.99 ± 0.06	1.22 ± 0.24	12.5 ± 0.3	14.1 ± 2.0	13.9 ± 1.0	12.5 ± 1.5
Q131E	1.00 ± 0.06	1.33 ± 0.11	1.02 ± 0.03	1.43 ± 0.23	11.2 ± 0.4	12.7 ± 0.6	13.5 ± 2.7	13.0 ± 3.8
Q131S	1.01 ± 0.04	1.46 ± 0.00	1.10 ± 0.02	1.60 ± 0.46	11.5 ± 0.6	12.4 ± 1.3	13.6 ± 1.7	12.1 ± 2.9
H68L	0.71 ± 0.05	0.79 ± 0.02	0.91 ± 0.05	1.23 ± 0.18	10.7 ± 0.4	11.5 ± 1.5	12.1 ± 0.1	13.3 ± 3.0
H68F	0.73 ± 0.03	0.94 ± 0.02	0.81 ± 0.03	1.08 ± 0.12	11.4 ± 0.3	13.9 ± 1.1	12.9 ± 1.2	13.1 ± 0.7
H68L/E65Q	0.75 ± 0.03	0.89 ± 0.05	0.77 ± 0.06	1.21 ± 0.21	11.2 ± 0.4	12.5 ± 0.7	12.4 ± 2.1	14.2 ± 2.5

[a] The protein−pigment complexes were reconstituted as described in Materials and Methods, at Chl *a*:Chl *b* ratios of 1:1 or 2:1, and isolated either by partially denaturing gel electrophoresis (gel) or on sucrose density gradients (UC). Given are averages and standard deviations, obtained from four (gel) or six to ten experiments (UC), of Chl *a*:Chl *b* ratios and Chl(*a*+*b*):(2 lutein) ratios.

Table 3: Number of Chl *a* Molecules/Chl *b* Molecules Missing in Mutated Recombinant LHCII as Compared to Wild-Type Recombinant LHCII[a]

	Chl *a*:Chl *b* ratio of 1		Chl *a*:Chl *b* ratio of 2	
mutant	gel	UC	gel	UC
H212L	1.1/0.8	1.0/0.9	1.6/0.5	1.0/0.4
H212F	1.0/0.9	1.1/0.4	1.8/0.4	1.7/1.1
Q197E	0.3/0.5	0.2/0.5	1.0/0.4	1.1/0.5
Q197S	0.3/0.5	0.5/0.3	1.4/−0.2	1.5/0.6
Q131E	0.4/1.7	0.7/0.7	0.8/1.8	0.7/0.9
Q131S	0.2/1.6	0.3/0.8	0.6/2.2	0.9/1.6
H68L	1.6/1.1	1.7/1.0	2.9/0.8	1.0/0.3
H68F	1.2/0.7	1.7/0.2	2.1/0.7	1.6/0.0
H68L/E65Q	1.2/0.9	2.1/0.3	1.2/0.1	0.6/−0.2

[a] The calculation was based on the data shown in Table 2.

All mutant recombinant complexes contain fewer Chl per 2 luteins than the wild-type ones, regardless of the Chl *a*:Chl *b* ratio in the reconstitution mixture and the complex isolation procedure chosen. The calculated number of molecules lost ranges between 1 and 2 in most cases. Several mutant proteins lose about equal amounts of Chl *a* and Chl *b* when compared to the wild-type protein; both H212 mutants, when reconstituted at a Chl *a*:Chl *b* ratio of 1, lose about 1 Chl *a* and 1 Chl *b*, and both Q197 mutants lose about 0.5 Chl *a* and 0.5 Chl *b*. In the H68 mutants, the amount of Chl *a* missing is higher than that of Chl *b* in all instances. Only the 2 Q131 mutants lose more Chl *b* than Chl *a*. This preferential loss of Chl *b* in Q131 mutants is detected even upon reconstitution at a Chl *a*:Chl *b* ratio of 2, although the mutant complexes reconstituted with a higher Chl *a*:Chl *b* ratio tend to lose more Chl *a* than those reconstituted with equal amounts of Chl *a* and Chl *b*.

The results in Tables 2 and 3 are very similar for parallel mutants that have different amino acid exchanges in the same position. Moreover, the mutant H68L/E65Q, having E65 exchanged with Q in addition to the H68L exchange, does not seem to suffer further Chl losses compared to the mutants carrying only the H68L or H68F mutation. Upon reconstitution at a Chl *a*:Chl *b* ratio of 2, Chl losses in the double mutant H68L/E65Q seem even smaller than those in the single mutant H68L.

Insertion of Mutant Lhcb1 in Isolated Thylakoids and Assembly into Trimeric Complexes. To test whether the mutant versions of Lhcb1 studied here are able to bind

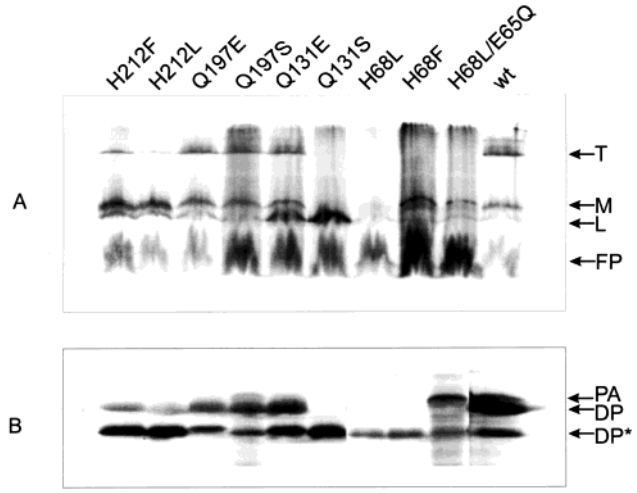

FIGURE 3: Insertion of mutated and wild-type Lhcb1 into isolated pea thylakoids. [^{35}S]Methionine-labeled proteins were inserted into isolated thylakoids, subsequently treated with thermolysin, solubilized, and analyzed by (A) partially denaturing gel electrophoresis or (B) fully denaturing SDS gel electrophoresis, as described in Materials and Methods. Exposure times were 5 days (A and B, lanes 1−6 and 10) and 4 days (B, lanes 7−9). T, trimeric LHCII; M, monomeric LHCII; L, nonpigmented Lhcb1; FP, unbound pigments; PA, nondigested Lhcb1 due to the formation of protein aggregates; DP, digestion product of trimeric LHCII; DP*, digestion product of monomeric LHCII.

pigments not only in detergent solution but also in the thylakoid membrane and whether they are even able to assemble into trimers, we labeled the proteins with ^{35}S in vivo, inserted them into isolated pea thylakoids, and identified their assembly status by assaying the protease sensitivity of the proteins. After solubilization of the thylakoids, either the proteins and pigment−protein complexes were separated on a partially denaturing gel (Figure 3A) or the fully denatured proteins were separated on an SDS gel (Figure 3B).

The partially denaturing gel (Figure 3A) separates monomeric LHCII (M) from nonpigmented Lhcb1 (L). All mutant versions of Lhcb1 show up in the monomeric LHCII band, suggesting that the proteins are assembled into LHCII in thylakoids. The nonpigmented protein band presumably is due to dissociation of part of the LHCII during the isolation procedure. Consistently, the band labeled M is rather prominent for proteins forming stable complexes such as the

wild-type protein and the H212 mutants, whereas it is weak compared to the L band for mutant proteins whose pigment complexes dissociated more rapidly (Table 1), such as the Q131 mutants.

Only some of the thylakoid-inserted Lhcb1 mutants comigrate with trimeric LHCII on the partially denaturing gel, marked as T in Figure 3A. The mutant Q131S and all mutants with H68 being exchanged do not, suggesting that these point mutations abolish the ability of the protein to assemble into trimeric LHCII. This notion is confirmed by the analysis of the fully denatured proteins in the same protease-treated thylakoids (Figure 3B). Typically, upon treatment of thylakoids with thermolysin, Lhcb1 forms two digestion products: a longer one, termed DP, representing Lhcb1 assembled into trimeric LHCII in which all of the protein except 1 kDa at the N-terminus is protected against the protease, and a shorter one, called DP*, representing monomeric LHCII in which an N-terminal fragment of about 4 kDa is protease-sensitive (*5*). All mutants comigrating with LHCII trimers in the partially denaturing gels form the higher-molecular mass digestion product DP, supporting the notion that part of the inserted protein is assembled into trimeric LHCII. The mutants that do not comigrate with trimeric LHCII form only the shorter digestion product DP* and, thus, are organized into monomeric LHCII exclusively. An additional band (PA in Figure 3B) shows up in several lanes. As judged from its electrophoretic mobility, it represents the full-length Lhcb1. It is most likely due to protein that aggregates during the insertion reaction and, thus, becomes inaccessible to protease. We often see it in experiments with proteins that do not efficiently insert into isolated thylakoids (not shown).

The LHCII trimer bands of the H212 mutants in Figure 3A are rather weak compared to the LHCII monomer bands, whereas the trimer signals for the wild-type protein, the Q197 mutants, and Q131E are much more prominent. This, again, is consistent with the analysis of the protein lengths after protease treatment; the DP band in the H212 mutants is weaker compared to the DP* band than those for the other proteins that form DP bands at all. Taken together, this strongly suggests that the amino acid exchanges at position 197 and the Q131E mutation have little or no effect on the assembly of these proteins into LHCII trimers, whereas the amino acid exchanges at position 212 partially impair trimerization.

DISCUSSION

Stability of Recombinant LHCII Carrying Point Mutations. Four amino acids in LHCII that are supposedly binding Chl molecules have been exchanged with amino acids that are thought to be unable to coordinate chlorophylls. All mutants can still be reconstituted with pigments in vitro to form pigment−protein complexes stable enough to be isolated. Moreover, all mutant proteins insert into isolated thylakoid membranes and become assembled with pigments, as assayed by their protection against protease (*5*). However, the mutant complexes exhibit significant differences in their stability as exemplified by the yield of green complexes obtained upon isolation on a partially denaturing polyacrylamide gel and by the dissociation kinetics of the reconstituted complexes at an elevated temperature. Interestingly, the amino acid replacing the exchanged one has only little (positions 68, 131, and 212) or no (position 197) influence on complex stability. If the amino acid has an effect, then the mutant complexes in which the new and the replaced amino acids share some structural similarity (F and H, and E and Q) are slightly more stable than when the difference between the two amino acids is greater (L and H, and S and Q). We conclude that the amino acid side chains in these positions contribute less to complex stability than the Chl molecule that is or is not bound to these amino acids.

Both assays of recombinant LHCII stability, thermal dissociation kinetics as well as the comparison of complex yields in partially denaturing gel electrophoresis, show that the exchange of amino acids H212, Q197, and Q131 cause minor, intermediate, and strong decreases, respectively, in complex stability. The results from the two assays are not as congruent with regard to the exchange mutants in position H68; according to thermal dissociation kinetics, both H68F and H68L are rather unstable, whereas the complex yield in partially denaturing gel electrophoresis is comparable, at least in the case of H68F, to those of the rather stable mutant complexes H212F and H212L. One possible explanation is that the relative stability of at least some of the mutant complexes may be differently dependent on the different conditions in the two assays, for instance, the presence or absence of dodecyl sulfate in the reaction solution. Kohorn (*2*) exchanged histidines in Lhcb2 equivalent to H68 and H212 with A or R and, upon insertion into isolated thylakoids, found that the proteins assembled into LHCII with lowered efficiency; this may also be due to a decreased stability of these complexes.

A surprising observation is that the double mutant H68L/E65Q forms pigment−protein complexes at all, although these are the least stable ones in the thermal dissociation kinetics. In the LHCII crystal structure, E65 is seen to form a transhelix linkage with R185 in the parallel transmembrane helix. This ionic bond, together with the similar bond between R70 and E180, is thought to cross-link the two helices and, thus, to be a major element in the stabilization of the protein structure in LHCII. It has been shown that the exchange of the corresponding E in an Lhcb protein of *Lemna gibba* leads to less efficient membrane integration of this protein upon importing the precursor form into isolated *Lemna* chloroplasts (*21*). Our data show that, after the H68 mutation, the additional E65 exchange does not lead to further destabilization of the complex. We conclude that the salt bridge between E65 and R185 is not an absolutely essential element for proper folding of the protein.

Pigment Stoichiometries. Reconstitution of recombinant LHCII and its isolation at high stringency yields complexes containing Chl *a* and Chl *b* at a ratio of 1.1 ± 0.2 (*8*, *10*). This Chl *a*:Chl *b* ratio is relatively stable even when the Chl *a*:Chl *b* stoichiometry in the reconstitution mix is varied over a wider range. The resulting pigment−protein complexes are about as stable as native monomeric LHCII; however, a Chl *a*:Chl *b* ratio of 1.1 clearly is below the one reported for native LHCII, 1.4 (*22*). This is most likely due to the relatively harsh conditions employed during complex isolation by partially denaturing gel electrophoresis, causing dissociation of some Chl molecules, predominantly Chl *a*, from the complex. Preferential loss of Chl *a* during isolation has been observed with native LHCII (*23*).

Recombinant LHCII complexes containing a more native-like Chl *a*:Chl *b* stoichiometry of around 1.4 can be produced by reconstituting and isolating the complexes under milder conditions. Under these conditions, the Chl *a*:Chl *b* stoichiometry in the recombinant complexes can be varied over a very wide range (*24*), depending on the Chl *a*:Chl *b* composition of the reconstitution mixture, up to complexes that contain only Chl *b* but no Chl *a*. Thus, complexes isolated under these less stringent conditions are not selected for wild-type-like stability, so one cannot be sure that Chl molecules are in fact bound to their cognate binding sites. Therefore, an average Chl *a*:Chl *b* ratio of these less stable reconstitution products close to the native value of 1.4 may or may not be meaningful. In fact, it has been suggested that some Chl binding sites in Lhcb6 (CP24) (*25*) and Lhcb4 (CP29) (*26*) can accommodate either Chl *a* or Chl *b*. Pigment titrations performed in our laboratory with recombinant Lhcb1 in vitro suggest that LHCII, too, contains some binding sites that make virtually no distinction between Chl *a* and Chl *b*; however, several binding sites exclusively accept either Chl *a* or Chl *b*, and some exhibit partial specificity for one pigment or the other (S. Hobe and H. Paulsen, unpublished observations).

On the basis of these considerations, we decided to isolate mutant Lhcb1 by using two different procedures. One of these, partially denaturing gel electrophoresis, is very stringent, whereas the other one, sedimentation by ultracentrifugation in detergent-containing sucrose gradients, is a very mild procedure. The latter procedure is, however, still able to distinguish protein-bound Chl from Chl molecules nonspecifically attached to the complex, as indicated by Chl stoichiometries per complex that are close to the stoichiometry found in native LHCII. Moreover, we used two different Chl *a*:Chl *b* ratios in each reconstitution experiment to test whether the individual binding sites that were analyzed are specific for either Chl.

As expected, the reconstitutions with wild-type Lhcb1 under the various conditions yielded different Chl stoichiometries (Table 2). Therefore, the pigment stoichiometries of recombinant LHCII with single Chl-associating amino acids mutated can be compared only with that of the reconstitution product of the wild-type protein obtained under the same conditions (single columns in Table 2). However, these comparisons do not allow unambiguous assignment of Chl *a* or Chl *b* to the mutated binding sites, as in most cases both Chl *a* and Chl *b* seem to disappear, and in many mutants more than one Chl molecule is missing compared to recombinant LHCII reconstituted with the wild-type protein (Table 3). On the other hand, the stoichiometries obtained for parallel mutations, i.e., two different amino acid exchanges in the same position, are strikingly similar throughout. Therefore, we believe that the differences in pigment stoichiometries between the wild type and the mutant recombinant complexes in fact reflect the loss of a mixture of Chl *a* and Chl *b* and/or the loss of more than 1 Chl molecule, and are not due to imprecision or scattering of the measurements. Possible explanations are that some of the Chl binding sites that were analyzed can accommodate either Chl *a* or Chl *b*, as has been indicated by other measurements (see above), and that at least some Chl molecules are bound cooperatively, so that the loss of one Chl molecule destabilizes one or more other ones.

Point mutations of Chl-binding amino acids in the recombinant CP29 complex have been reported to lead to the loss of exactly one chlorophyll each per apoprotein (*11*). This is quite different from what we have observed with point-mutated LHCII. Possibly, CP29 differs from LHCII in that there is less pigment−pigment interaction involved in complex stabilization, so that pigments are bound (and lost) more independently of each other.

Although, for the reasons discussed above, Chl *a* and Chl *b* cannot be unambiguously assigned to the mutated Chl binding sites, the following conclusions can be drawn from our data.

Position 212. Upon reconstitution at a Chl *a*:Chl *b* ratio of 1, the mutant complexes lack about 1 Chl *a* and 1 Chl *b* (except when the H212F complex is isolated by ultracentrifugation when less than 1 Chl *b* is missing). When the Chl *a*:Chl *b* ratio in the reconstitution mixture is 2, the loss of Chl *a* generally increases to values between 1 and 2 molecules and the loss of Chl *b* decreases to values between 0.4 and 1. One possible explanation is that 2 Chl binding sites are affected by the mutation, one associating with Chl *a* and one binding preferentially Chl *b* when the protein is reconstituted with equal amounts of Chl *a* and *b* but is occupied in part with Chl *a* and in part with Chl *b* when the Chl *a*:Chl *b* ratio in the reconstitution is 2. According to the Chl assignment on the basis of the electron crystallographic analysis, H212 associates with Chl b_3 which in turn interacts with Chl a_3. Our results are consistent with the notion that H212 is essential for stabilizing both of these Chls, and that one of them is a Chl *a* and the other one can be either Chl *a* or Chl *b*.

Position 197. The Chl assigned to Q197 is Chl a_3 which has been discussed above as possibly being also affected by mutations at position 212. However, the exchange of Q197 changes pigment stoichiometries in a different way than the exchange of H212. Clearly, the number of Chl molecules lost, due to Q197 exchanges, is lower, at least when the reconstitution is performed with equimolar amounts of Chl *a* and Chl *b*. In these reconstitutions, the mutations at position 197 cause the loss of between 0.2 and 0.5 molecule each of Chl *a* and Chl *b*. Possible explanations are (a) that Q197 can bind either Chl *a* or Chl *b* and (b) that the mutation at position 197 causes the loss of both Chl molecules assigned to Q197 and H212 but only in part of the reconstituted complexes. When this protein is reconstituted with an excess amount of Chl *a*, its pigment complement differs from that of the wild-type protein by more than 1 Chl *a* and between 0 and 0.6 Chl *b* molecule, suggesting either that the Chl binding site assigned to position 197 binds more Chl *a* than Chl *b* at higher Chl *a*:Chl *b* ratios in the reconstitution or that the same is true for position H212 if these two Chls in fact stabilize one another. Of course, it is also possible that the mutation labilizes some other Chl *a* that is bound to the complex only when the reconstitution mixture contains an excess amount of Chl *a*, and that may not be visible in the electron crystallographic structure of LHCII.

Position 131. These are the only mutants whose pigment complexes lack more Chl *b* than Chl *a* compared to the complexes with the wild-type protein, suggesting but certainly not proving that Q131 preferably binds Chl *b*. This is consistent with the assignment in the LHCII structure of Chl

b_6 to this amino acid. Under all experimental conditions, the difference between the mutant and wild-type versions of the recombinant complex amounts to more than 1 Chl molecule, suggesting that a mutation at position 131 destabilizes more than one Chl binding site. Candidates, according to the LHCII structure, are the Chl molecules termed Chl a_6 and Chl a_7 that do not seem to be associated with an amino acid side chain and that may rely, in part, on Chl b_6 for stabilization.

In Lhcb4 and Lhcb5, the amino acids corresponding to Q131 in Lhcb1 are glutamates. It has been suggested that such glutamates are the points where the proton gradient over the thylakoid induces energy quenching in the light-harvesting complexes; protonation of Chl-binding E residues is proposed to modulate the interaction between E and the Chl such that energy-trapping Chl dimers are formed (*27*, *28*). We do not know whether Q131 is protonated under reconstitution conditions, but our pigment stoichiometries give no indication that E in position 131 is able to contribute to Chl binding. Approximately the same number of Chl molecules is lost from the complex when Q131 is replaced with either E or S, the latter being very likely unable to coordinate a Chl molecule.

Position 68. The exchange of H68 causes the loss of between 1.5 and more than 3 Chl molecules under the various experimental conditions, with the amount of Chl *a* always exceeding that of Chl *b*. According to the molecular structure of LHCII, H68 associates with Chl a_5 which in turn is neighboring Chl a_4 (bound by E65) and Chl b_5 (bound by E139). The loss of two or three of these Chl molecules due to the exchange of H68 would be consistent with the pigment stoichiometries measured in reconstituted complexes with the mutant protein. A loss of several Chl molecules may also explain the low thermal stability of the mutant complexes. Of course, it is also possible that the exchange of H68 causes a strong decrease in the overall stability of the pigment protein complexes, triggering the loss of Chl molecules in more distant binding sites. However, the fact that the exchange of E65 in addition to H68 does not appear to cause additional loss of pigment may be explained by assuming that mutations at position 68 destabilize not only Chl a_5 but also Chl a_4 and Chl b_5. Therefore, the behavior of the double mutant favors the interpretation that the H68 exchange affects the binding of a cluster of Chl molecules that stabilize each other in the complex.

LHCII Trimer Formation. There are clear differences between the point mutations in recombinant LHCII with regard to their impact on LHCII trimer formation. Trimerization is completely abolished when H68 is exchanged with L or F and Q131 with S, whereas the Q197 exchanges show little or no influence. The other mutations partially impair trimer formation. The strong effect of mutations at position 68 may be explained by the fact that H68 and Chl a_5 attached to it are thought to be located in the very center of the trimer at the interface to the other 2 subunits (*29*). Q197 and H212 are located further toward the periphery of the trimer. Although these amino acids with their attached pigments Chl a_3 and Chl b_3, respectively, are not too far from the interface with a neighboring subunit, they do not seem to contribute to trimer stability as much as H68 and/or its attached Chl molecule do. The accompanying paper (*12*) shows that the mutant H68A also fails to form trimers upon refolding on a solid surface.

Amino acid Q131 and the Chl molecule attached to it (b_6) are located on the surface of helix C that is not adjacent to the neighboring subunit in the trimer and, therefore, are unlikely to be directly involved in trimer-stabilizing intersubunit interactions. In position 131, trimer formation is abolished only when Q is replaced with S but not with the structurally more closely related amino acid E. The pigment stoichiometries in the mutant monomeric complexes do not suggest that the two different replacements show any difference in Chl coordination. Therefore, the effect of the Q131S exchange on trimer formation is most likely due to a structural distortion of the protein which is expected to be more pronounced in this than in the Q131E mutant.

Amino acids H68 and Q131 add to previously known amino acids in LHCII whose exchange can abolish trimer formation, namely, those in the trimerization motif, W16, Y17, and R21 (*30*) as well as W222 near the C-terminus (*6*). We cannot determine whether exchange of H68 and Q131 directly alters a site of interaction between subunits in the trimer or whether it induces long-range structural changes in the monomeric complex that then affect oligomerization. However, the sensitivity of LHCII trimerization to point mutations in different domains of the apoprotein supports the view that multiple contacts between monomeric subunits cooperatively contribute to the stability of the LHCII trimer.

ACKNOWLEDGMENT

We thank Stephan Hobe and Volkmar Schmid for critically reading the manuscript.

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BI990738X