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Alteration of components of chlorophyll-protein complexes and distribution of excitation energy between the two photosystems in two new rice chlorophyll *b*-less mutants

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Abstract

Two new yellow rice chlorophyll (Chl) *b*-less (lack) mutants VG28-1 and VG30-5 differ from the other known Chl *b*-less mutants with larger amounts of soluble protein and ribulose-1,5-bisphosphate carboxylase/oxygenase small sub-unit and smaller amounts of Chl *a*. We investigated the altered features of Chl-protein complexes and excitation energy distribution in these two mutants, as compared with wild type (WT) rice cv. Zhonghua 11 by using native mild green gel electrophoresis and SDS-PAGE, and 77 K Chl fluorescence in the presence of Mg^{2+}. WT rice revealed five pigment-protein bands and fourteen polypeptides in thylakoid membranes. Two Chl *b*-less mutants showed only CPI and CPa pigment bands, and contained no 25 and 26 kDa polypeptides, reduced amounts of the 21 kDa polypeptide, but increased quantities of 32, 33, 56, 66, and 19 kDa polypeptides. The enhanced absorption of CPI and CPa and the higher Chl fluorescence emission ratio of F685/F720 were also observed in these mutants. This suggested that the reduction or loss of the antenna LHC1 and LHC2 was compensated by an increment in core component and the capacity to harvest photon energy of photosystem (PS) 1 and PS2, as well as in the fraction of excitation energy distributed to PS2 in the two mutants. 77 K Chl fluorescence spectra of thylakoid membranes showed that the PS1 fluorescence emission was shifted from 730 nm in WT rice to 720 nm in the mutants. The regulation of Mg^{2+} to excitation energy distribution between the two photosystems was complicated. 10 mM Mg^{2+} did not affect noticeably the F685/F730 emission ratio of WT thylakoid membranes, but increased the ratio of F685/F720 in the two mutants due to a reduced emission at 685 nm as compared to that at 720 nm.

Additional key words: chlorophyll fluorescence; chlorophyll-protein complexes; Mg^{2+}; photosystems 1 and 2; *Oryza*.

Introduction

Chlorophyll (Chl) *b*-deficient mutants have been widely used as the important material for understanding the genetic and biochemical control of Chl *b* biosynthesis and the function of Chl *b* in the assembly of light-harvesting Chl *a/b* protein complexes (Anderson *et al.* 1978, Eggink *et al.* 2001). Various Chl *b*-deficient mutants were found in several species of higher plants (Falbel *et al*. 1996). In rice, two groups of Chl *b*-deficient mutant were distinguished based on their different Chl *a/b* ratios. They were named Chl *b*-less mutant (Chl *a/b* ratio ≥ 10-15 or even no Chl *b*, yellow leaves) and Chl *b*-deficient mutant (yellowish green appearance containing normal Chl *a* amount) (Terao *et al.* 1985a,b). All the Chl mutants are multiple allelic nuclear gene mutants, but the lesion of Chl *b*-deficient mutant is able to genetically complement and is suggested to be in a different gene of the Chl *b*-less mutant (Markwell *et al.* 1986, Chunaev *et al.* 1991). Therefore, the distinct features among the several Chl *b*-deficient mutants depending on mutation site, mutation way, and environmental factors are important for advanced studies.

Recently, two new Chl *b*-less rice mutants were selected during the tissue culture of transformed rice by inserting a maize Ds transposon element (Wang *et al.* 2000), and were named VG28-1 and VG30-5. Our primary study on their photosynthetic features showed

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that they differed somewhat from the other Chl *b*-less mutants, they particularly contained larger amounts of soluble proteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) protein, and less Chl *a* than the wild type (WT) rice (Lin *et al.* 2003). Thus, they provide an interesting and useful experimental material for studying the regulation between the synthesis of Chls and proteins, and to illuminate the presence of multiple characters within the Chl *b*-less mutants. In the present study we investigated the components of Chl-protein complexes in thylakoid membranes, and the effect of Mg^{2+} on the excitation energy distribution between PS2 and PS1 in WT rice and the two new Chl *b*-less mutants.

Materials and methods

Plants: Seeds of wild type rice (*Oryza sativa* L. cv. Zhonghua No. 11) and of the mutants VG28-1 and VG30-5 at third generation were grown in an incubator at 28 °C and a photosynthetic photon flux density (PPFD) of 30 μmol m^{-2} s^{-1}. Seedlings with two leaves and one expanding leaf were transplanted in pot soil and grown outdoors. The young mature leaves were sampled for analysis at anthesis stage.

Preparation of thylakoid membranes: Rice leaves were homogenised with a blender in 0.4 M sucrose, 50 mM Tricine, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.6, and then filtered through four layers of gauze. The homogenate was centrifuged at 500×*g* for 2 min to remove the cell debris; chloroplast pellets were collected by centrifugation of the supernatant at 4 000×*g* for 10 min, and resuspended twice in a Tricine buffer (50 mM, pH 7.6) containing 10 mM NaCl and 5 mM $MgCl_2$. The thylakoid membranes were obtained after 10-min centrifugation at 10 000×*g* and suspended in 10 % glycerol with the above medium. The isolated samples were stored at liquid nitrogen before use.

Analysis of protein complexes: The procedure of native green gel electrophoresis at low ionic strength for determining Chl-protein complexes of thylakoid membranes was conducted by using the method of Anderson *et al.* (1978) and Wang *et al.* (1989). Electrophoretogram was evaluated *in situ* by absorbance densitometry at 675 nm. Absorption spectra of CP1 and CPa were carried out with the excised gel. SDS-PAGE of thylakoid membrane proteins was performed following the method of Laemmli (1970). The stacking gel and the separating gel contained 4 and 12 % acrylamide, respectively. Polypeptides were stained with Coomassie Brilliant Blue R-250 and scanned at 590 nm.

Chl fluorescence emission spectra at 77 K: Low temperature Chl fluorescence emission spectra of chloroplasts and the bands of CPI and CPa excised from green gel electrophoretogram were recorded with a *Hitachi F-4500* spectrofluorometer at 77 K. The excitation wavelength was 436 or 480 nm. The fluorescence emission was recorded in the range of 650–770 nm. The slit width of emission was 5 nm, Chl concentration in each sample was 10 μg per cm^3. The effect of Mg^{2+} on the excitation energy distribution between PS2 and PS1 was estimated from the 77 K Chl fluorescence spectra of thylakoid membrane suspensions after incubation at 25 °C for 2 min in the absence or presence of 5-10 mM $MgCl_2$.

Photosynthetic pigments: Chls and carotenoids were extracted with 90 % acetone and analysed by HPLC (*Waters*, USA) according to Gilmore and Yamamoto (1991).

Results

Composition of Chl-protein complexes: HPLC analysis of photosynthetic pigments showed that the two new rice mutants VG28-1 and VG30-5 had no Chl *b* (Fig. 1), and the Chl *a* content was just at 36–48 % of the corresponding content of the WT rice. This severe Chl *b*-deficiency was identified as group I (Chl *b*-less) to be distinguished from the group II (with different extents of Chl *b*-deficiency) according to Terao *et al.* (1985a).

Comparison of the pigment-protein complexes in thylakoid membranes between WT and Chl *b*-less mutants by green gel electrophoresis (Fig. 2) showed that WT rice contained five pigment-proteins, which are the PS1 complexes CPI, LHCP1, LHCP2, CPa, LHCP3, and some free pigment. In the mutants, only two pigment bands, CPI and CPa were resolved. The absorption peaks of CPI at 672 and 435 nm (Fig. 3*B*) and CPa at 670 and 433 nm (Fig. 3*A*) were similar to those separated from wheat chloroplast membranes (Hao *et al.* 1981). The absorption level of CP1 and CPa in the mutants was somewhat higher than that in WT. Moreover, two shoulders at 471 and 650 nm exhibited in CPa of WT were not present in the mutants. Comparison of the 77 K Chl fluorescence excitation spectra of CPa and CP1 in the mutants with these of WT showed that the excitation peak of Chl *b* did not occur in CPa and CP1 of the two mutants (Fig. 3*C,D*). The 77 K Chl fluorescence emission spectra excited at 436 nm showed that CPa had an emission peak at 682 nm, while that of CPI exhibited two emission peaks at 722 and 676 nm (Fig. 3*E,F*). The Chl fluorescence yields of CPI and CPa in WT were higher than in

Chl *b*-less mutants. Harrison *et al.* (1993) pointed out that barley mutant *chlorina* f_2 with no Chl *b* failed to accumulate great amounts of Chl *a/b* binding LHCs. Terao *et al.* (1985b, 1989) reported that rice Chl *b*-less mutant *chlorina* 2-10 lost LHC2 and LHC1. Our experiment on two new Chl *b*-less rice mutants confirmed their conclusion.

Fig. 4 shows the composition pattern of thylakoid

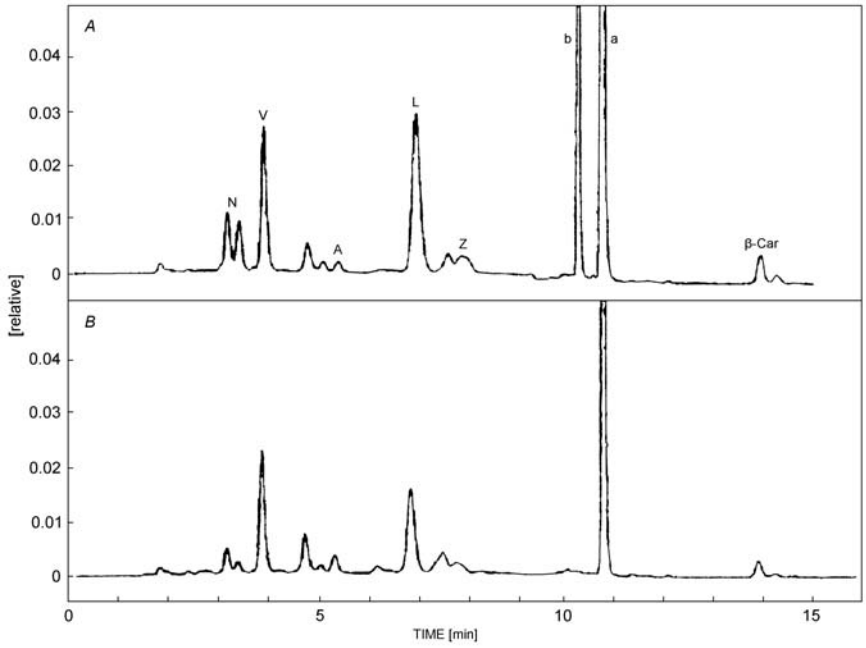

Fig. 1. Composition of photosynthetic pigments analysed by HPLC in wild type (WT) rice (*A*) and mutant VG30-5 (*B*). a, Chl *a*; b, Chl *b*; A, antheraxanthin; L, lutein; N, neoxanthin; Z, zeaxanthin; β-Car, β-carotene.

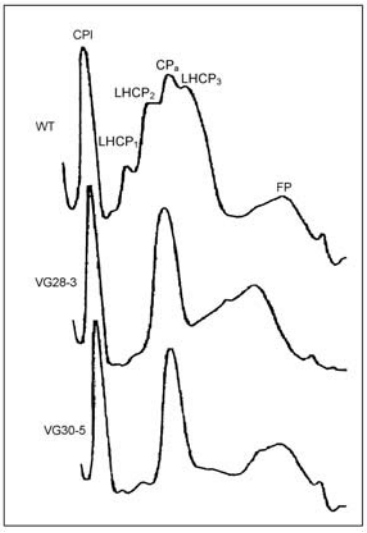

Fig. 2. Densitometry absorbance of a native green gel electrophoretogram of chlorophyll-protein complexes of thylakoids of wild type (WT) and mutants of rice.

membrane proteins by SDS-PAGE, which revealed 29 d after transplantation fourteen polypeptides in WT rice. In the two new mutants, two polypeptides of 25 and 26 kDa were missing, amount of 21 kDa polypeptide was reduced, but 66, 56, 33, 32, and 19 kDa proteins were significantly amplified. This indicates that the mutation resulted in the loss of the minor peripheral antenna proteins of PS2 (25-26 kDa) and the reduction of PS1 antenna (21 kDa). Contents of both D_1 and D_2 proteins (32-33 kDa) in the PS2 reaction centre increased to a much higher level than in the WT. The higher amount of the 66 kDa polypeptide suggested that the PS1 reaction centre (CPI) in the two mutants was stable.

The 77 K Chl fluorescence emission spectrum of WT thylakoid membranes exhibited two peaks, at 685 and 730 nm (Fig. 5), which has been attributed to the antenna pigment-protein complexes of PS1 and PS2 (Krause and Weis 1991). The Chl fluorescence spectrum was characterised by a prominent emission at 685 nm and a lower emission at 730 nm in both WT and mutants. It differed in the prominent emission at 740 nm of normal WT rice cv. Norin No. 8 reported by Terao *et al.* (1985a), but was consistent with emission of normal wheat thylakoids in the presence of electron transport acceptor $K_3Fe(CN)_6$, and with wheat CD3 Chl deficient mutant (Duysen *et al.* 1984). For the two new rice mutants, the fluorescence emission at 685 nm was strongly reduced as compared with WT, whereas the long wavelength emission band F730 was blue-shifted to shorter wavelength of F720 nm (F720) and its fluorescence intensity decreased. CP1 represents the PS1 reaction centre and exhibits a low-temperature Chl fluorescence emission at 722 nm, while

CP1a (the oligomer of CP1 containing CP1 and LHC1) shows a F730 maximum emission at 77 K (Falbel *et al.* 1994). Hence, the blue shift of F730 to F720 in the two rice mutants might demonstrate the loss of most of the PS1 antenna during mutation, and F720 was ascribed to the emission by PS1 core. This is in agreement with the changes in pigment and polypeptide components as mentioned above.

Effect of Mg^{2+} on excitation energy distribution between PS1 and PS2: The 77 K Chl fluorescence emission feature of WT thylakoid membranes excited by 436 or 480 nm was similar (Fig. 6*A,B*). However, in the Chl *b*-less mutants the emission spectra excited at 480 nm differed significantly from those excited at 436 nm. They showed very little Chl fluorescence emission and almost equals amounts of F720 and F685 (data not shown). This together with the low emission yield at F685 and F720 in the mutants than in WT again proved the absence of Chl *b* in the two new rice mutants.

Addition of 5-10 mM Mg^{2+} to the Mg^{2+} depleted thylakoid membrane suspension resulted in different

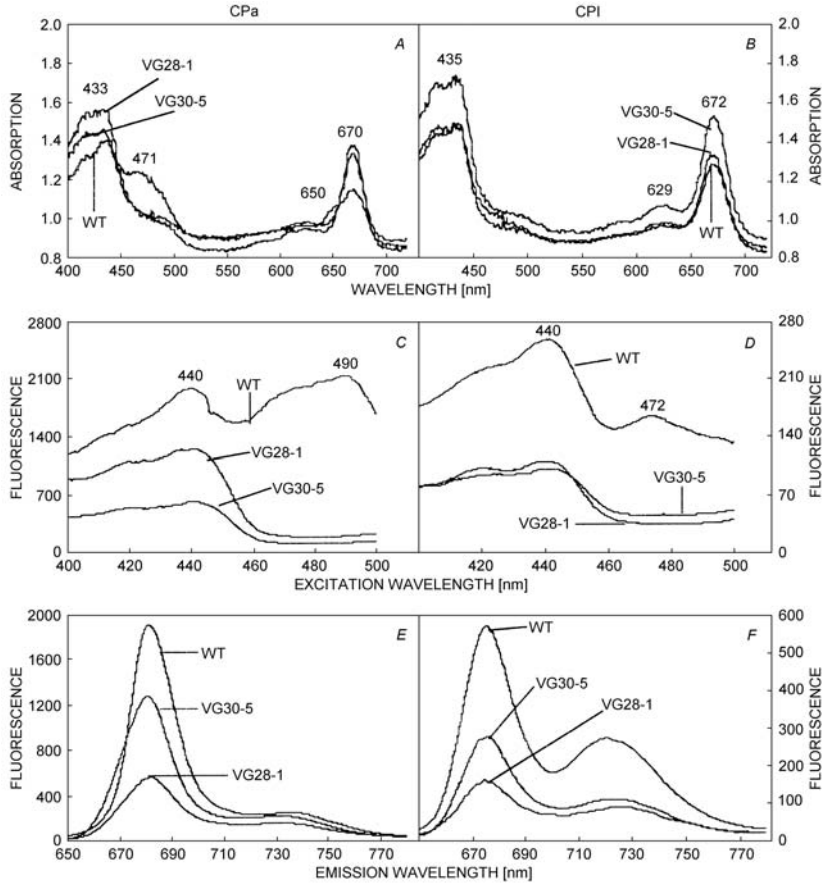

Fig. 3. Absorption spectra (*A, B*), 77 K chlorophyll fluorescence excitation spectra (*C, D*), and Chl fluorescence emission spectra (*E, F*) of CPa (*left panels*) and CPI (*right panels*) in wild type (WT) and the two new mutants. The detection wavelengths for excitation spectra of CPa and CP1 were 682 and 728 nm, respectively.

changing trends of the 77 K Chl fluorescence emission between WT and two new mutants (Fig. 6). Upon adding 5 mM Mg^{2+}, the F685 and F730 excited by 436 nm in thylakoid membranes of WT decreased by 46 and 43 %, respectively. However, with increase in Mg^{2+} concentration to 10 mM, both F685 and F730 were restored to 84 and 91 % of the initial level (Fig. 6*A*). In the mutant VG28-1, F685 and F720 declined with increase in the Mg^{2+} concentration, and the decrease in F720 was greater than in F685 (Fig. 6*C*). The response of the mutant VG 30-5 to Mg^{2+} showed certain different Chl fluorescence emission features (Fig. 6*D*): both its F685 and F720 increased after 5 mM Mg^{2+} addition, but when the Mg^{2+} concentration rose to 10 mM, the intensity of F685 remained but the F720 band was reduced to 82 % of the control value.

The fluorescence emission ratio of F735/F685 is a relative indicator of the excitation energy distribution between the two photosystems (Duysen *et al.* 1984). Table 1 describes the effect of Mg^{2+} on the ratios of

PS2 (F685)/PS1 (F730 or F720). The PS2/PS1 emission ratio of WT rice was lower than that of the two mutants in the thylakoid membranes suspended with or without Mg^{2+}, when excited at 436 nm, but was higher by exciting at 480 nm. This implied more excitation energy distributed to PS2 in the mutants by the Chl *a* excitation wavelength of 436 nm. This was in contrast to that of the 480 nm excitation due to the lack of Chl *b*. In the presence of Mg^{2+} the PS2/PS1 ratio of WT thylakoid membranes slightly decreased by 9 %, while in the two mutants it increased by 6–21 % under the 436 nm excitation. The effect of Mg^{2+} on the F685/F720 ratio of Chl *b*-less mutants was pronounced in 10 mM Mg^{2+}. It seemed that Mg^{2+} enhanced the fraction of excitation energy at PS2 and reduced the fraction of excitation energy of PS1 in the two mutants. This is consistent with the result for thylakoids isolated from normal wheat reported by Wen *et al.* (1999). The small but negative effect of Mg^{2+} on the excitation energy distribution in WT rice was out of expectation, it might reflect the insensitive response of WT rice thylakoids to Mg^{2+}. Moreover, the decreases in both F685 and F720 in thylakoid membranes of the new mutants (except the VG30-5 in 5 mM Mg^{2+}) with Mg^{2+} addition was contrary to the view that Mg^{2+} could significantly enhance the PS2 emission at 77 K (Wen *et al.* 1999).

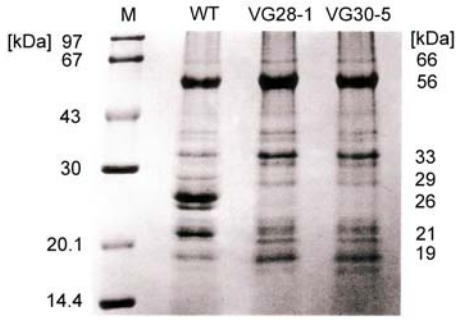

Fig. 4. SDS-PAGE of polypeptide profile of thylakoid membranes from wild type (WT) and the two new Chl *b*-less rice mutants.

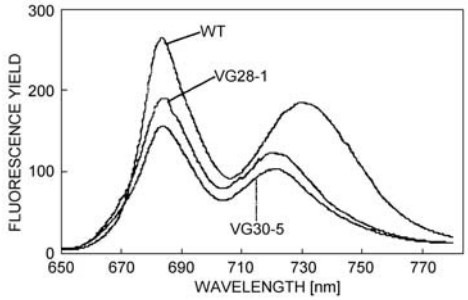

Fig. 5. Chlorophyll fluorescence emission spectra at 77 K of chloroplasts of wild type (WT) and the two Chl *b*-less mutants of rice excited at 436 nm.

Discussion

Light harvesting complexes of PS2 are accounted for half the Chl and a third of the protein in the Chl-protein complexes of the thylakoid membranes (Kühlbrandt and Wang 1991). LHCPs participate in thylakoid stacking; the limited supply of Chl *b* affects the stability of Chl *b*-binding protein of antenna complexes (Bellemare *et al.* 1982, Falbel *et al.* 1996, Hoober and Eggink 1999). PS2 has at least four different Chl *b*-containing antenna

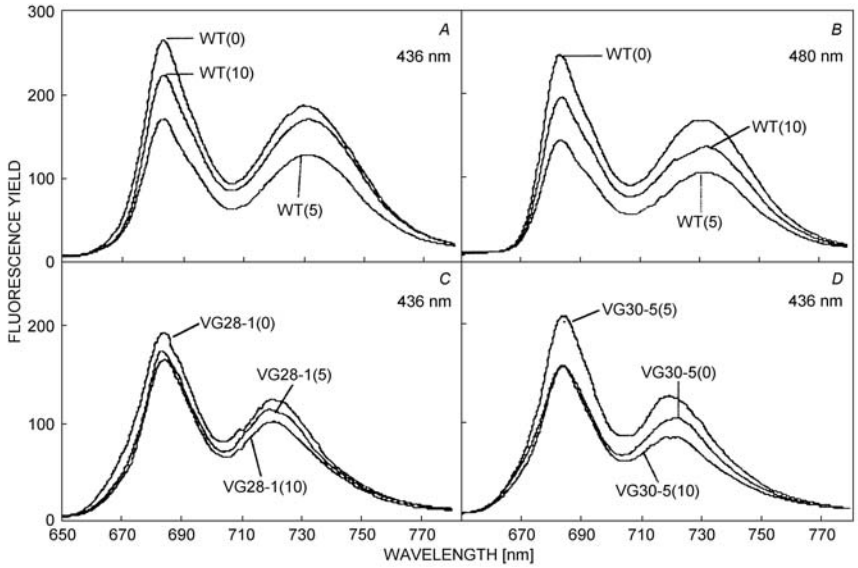

Fig. 6. Effect of Mg^{2+} on the 77 K Chl fluorescence emission spectra of thylakoid membranes of wild type (WT) and the two Chl *b*-less rice mutants excited with 436 nm. The numbers in parenthesis represent the mM concentration of Mg^{2+}.

complexes, *i.e.* LHC2, CP29, CP26, and CP24. The Chl *a/b* antenna proteins differ in their affinity for Chl *b*, so the stability and accumulation of Chl *a/b* antenna proteins are varied in the absence of Chl *b* (Knoetzel and Simpson 1991). Two rice Chl *b*-less mutants tested in the present paper lost polypeptides of 25 and 26 kDa, reduced the amount of 21 kDa polypeptide (Figs. 2 and 4), increased the amounts of 66 and 31-32 kDa polypeptides and the absorption of CPa and CP1, and revealed a 720 nm Chl fluorescence emission (Fig. 5) of their thylakoid membranes. These alterations were due to a change in organisation and stabilisation of Chl-protein complexes and implied that the mutants had a truncated light-harvesting antenna of PS2 and PS1. This is consistent with the deficiency in the peripheral antenna complex of PS1 and PS2 in barley Chl *b*-less mutant (Burke *et al.* 1979, Mullet *et al.* 1980). It implied that Chl *a* in the two new rice Chl *b*-less mutants bears a key action for photon capture and energy transformation. Nevertheless, increase in the amounts of 32-33 kDa polypeptides and photon absorption of CPI and CPa (Fig. 4) as well as the PS2/PS1 ratio (Table 1) evidenced the enhancement of size and function of PS2 and PS1 core components in the Chl *b*-less rice mutants. And these effects are inferred as compensation in the centres of PS2 and PS1 for the reduction of antenna and relative apoprotein when Chl *b* was lacking.

Table 1. PS2/PS1 ratio of chlorophyll fluorescence intensity at 77 K.

Excitation wavelength [nm]	Mg^{2+}concentration [mmol mol^{-1}]	WT (Zonghua 11)	VG28-1	VG30-5
436	0	1.43	1.54	1.51
	5	1.34	1.50	1.65
	10	1.32	1.63	1.83
480	0	1.47	0.89	0.86
	5	1.36	0.94	1.01
	10	1.43	0.87	1.06

Chl *b* is derived from Chl *a* during the biosynthesis of Chl (Tanaka *et al.* 1998). On the other hand, Ohtsuka *et al.* (1997) reported that also Chl *b* can be released from LHC2 apoprotein and the free Chl *b* converts rapidly to Chl *a* in thylakoid membranes *via* 7-hydroxymethyl Chl. Then the converted Chl *a* incorporates into CPI and participates in the formation of CPa during the re-organisation of the photosynthetic apparatus. Therefore, the compensation changes in amounts and components of Chl-protein complexes of the two new rice mutants, together with their levels of F_v/F_m and apparent quantum yield comparable with WT rice (Lin *et al.* 2003), are considered to be a modulated re-assembly for alleviating the depression of photon energy utilisation and plant survival in the absence of Chl *b*.

The action of Mg^{2+} on thylakoid membrane is through multiple ways and LHC2 plays an important role on the induced changes of PS2 fluorescence (Lin *et al.* 1980). LHC2 requires cations to induce thylakoid stacking. Mg^{2+} could induce the LHC2 trimer stacking to form oligomers, and results in more excitation energy distribution to PS2 in wheat thylakoids (Wen *et al.* 1999). Vani *et al.* (2001) showed that addition of 5 mM $MgCl_2$ to the cation depleted thylakoids at 25 or 40 °C significantly increased the F685/F735 ratio. However, a rice mutant *chlorina*-7 with no Chl *b* or LHC2 showed no response to Mg^{2+} depletion (Hsu and Lee 1995). Burke *et al.* (1979) found that the Mg^{2+} dependent regulation of excitation energy distribution in chloroplasts of Chl *b*-less barley mutant required 3-fold higher concentration of Mg^{2+} than in WT barley. In our experiments, the enhanced effect of PS2/PS1 fluorescence emission ratio was notable at 10 mM Mg^{2+} in the two rice mutants lacking LHC2. This indicates an altered redistribution of excitation energy tending towards the PS2 in mutants by the action of Mg^{2+}. The increment of F685/F720 in the two rice mutants in the presence of Mg^{2+} was due to a lower decrease in F685 and a large decrease in F720. It did not result from the increase in F685 content as the normal wheat thylakoids (Wen *et al.* 1999) and barley Chl *b*-less mutant (Burke *et al.* 1979) did. The induction of increase in Chl fluorescence yield was only found in the mutant VG30-5 thylakoids when adding 5 mM Mg^{2+}, indicating some difference between mutants VG30-5 and VG28-1 in response to Mg^{2+}. The 23 and 25 kDa polypeptides in thylakoid membranes were thought to be the specific acting sites responding to Mg^{2+}, and the sensitivity of chloroplasts to Mg^{2+} might be affected by Chl *a/b* (Li *et al.* 1992). Therefore, the altered patterns of excitation energy distribution between the two photosystems in two rice mutants were probably referred to the loss of Chl *b* and 25 kDa polypeptide of thylakoid membranes. The details of the effect of Mg^{2+} on the regulation of excited energy distribution in WT rice and the two new mutants and the reason of their different response sensitivities remain to be solved by a future study.

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