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Photosystem 2 photochemistry and pigment composition of a yellow mutant of rice (*Oryza sativa* L.) under different irradiances

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Abstract

A yellow leaf colouration mutant (named *ycm*) generated from rice T-DNA insertion lines was identified with less grana lamellae and low thylakoid membrane protein contents. At weak irradiance [50 μmol(photon) m^{-2} s^{-1}], chlorophyll (Chl) contents of *ycm* were ≈20 % of those of WT and Chl *a/b* ratios were 3-fold that of wild type (WT). The leaf of *ycm* showed lower values in the actual photosystem 2 (PS2) efficiency (Φ_{PS2}), photochemical quenching (q_P), and the efficiency of excitation capture by open PS2 centres 1 (F_v'/F_m') than those of WT, except no difference in the maximal efficiency of PS2 photochemistry (F_v/F_m). With progress in irradiance [100 and 200 μmol(photon) m^{-2} s^{-1}], there was a change in the photosynthetic pigment stoichiometry. In *ycm*, the increase of total Chl contents and the decrease in Chl *a/b* ratio were observed. Φ_{PS2}, q_P, and F_v'/F_m' of *ycm* increased gradually along with the increase of irradiance but still much less than in WT. The increase of xanthophyll ratio [(Z+A)/(V+A+Z)] associated with non-photochemical quenching (q_N) was found in *ycm* which suggested that *ycm* dissipated excess energy through the turnover of xanthophylls. No significant differences in pigment composition were observed in WT under various irradiances, except Chl *a/b* ratio that gradually decreased. Hence the *ycm* mutant developed much more tardily than WT, which was caused by low photon energy utilization independent of irradiance.

Additional key words: β-carotene; chlorophyll; photochemical and non-photochemical quenching; xanthophyll cycle.

Introduction

Leaf colouration mutants are usually caused by the breakdown of chlorophyll (Chl) synthetic pathway such as protochlorophyllide oxidoreductase (Masuda *et al.* 2003), Mg-chelatase (Zhang *et al.* 2006), and Chl *a* oxygenase (Lee *et al.* 2005). In rice, Chl mutants are usually classified as *albino*, *chlorina* (Zhang *et al.* 2006), *stripe*, *virescent* (Sugimoto *et al.* 2004), *yellow-green*, and *zebra* (Kusumi *et al.* 2000). Although several genes were identified from these mutants, the genetic mechanisms are largely unknown. T-DNA insertion mutagenesis has been successfully used to isolate a number of genes from *Arabidopsis*. Recently, an *OsCHLH* gene encoding the largest subunit of the rice Mg-chelatase (Jung *et al.* 2003) was identified using T-DNA gene-trap system. However, the complexities of T-DNA insertion mutants hinder the potential application of this technique to explore desired genes at large scale.

We recently established a large pool of T-DNA insertion lines in rice (*Oryza sativa* ssp. *japonica* cv. Nipponbare). Among them, more than 20 leaf colouration mutants were found. A yellow leaf colouration mutant (*ycm*) was found shorter than wild-type (WT) in plant height. The Chl contents (especially Chl *b*) of this mutant were less than those of WT. Because Chl *b* is indispensable for the stability of thylakoid membrane, it is still unclear whether this mutant could suffer from high irradiance. Irradiation is important for the formation of Chl and chloroplast, and excess photons cause oxidation

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Abbreviations: A – antheraxanthin; Chl − chlorophyll; F_0 − minimal fluorescence in dark-adapted leaves; F_m − maximal fluorescence in dark-adapted leaves; F_v − maximum variable fluorescence in dark-adapted leaves; F_0' − minimal fluorescence in light-adapted leaves; F_m' − maximal fluorescence in light-adapted leaves; F_v' − maximum variable fluorescence in light-adapted leaves; F_v/F_m − maximal efficiency of PS2 photochemistry; F_v'/F_m' − efficiency of excitation energy capture by open PS2 reaction centres; PS – photosystem; q_N − non-photochemical quenching; q_P − photochemical quenching; V – violaxanthin; Z – zeaxanthin; Φ_{PS2} − actual PS2 efficiency.

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and photoinhibition. Usually, xanthophyll cycle pigments undergo a de-epoxidation process to quench excess photon energy in senescence (Lu *et al.* 2001), under heat (Lu and Zhang 2000a,b) and photoinhibition (Demmig-Adams and Adams 1992) which lead to the decomposition of chloroplast. The purpose of this study was to find the influence of irradiance on this mutant.

Materials and methods

Growth: WT and *ycm* were grown on moist vermiculite for 16 d under irradiation of 160 μmol(photon) m^{-2} s^{-1}. To achieve this, natural irradiation in a greenhouse was supplemented by metal halide and sodium vapour lamps.

Chl and xanthophyll contents: Chl was extracted with 80 % acetone from 0.1 g samples of fresh leaves. The extract was measured spectrophotometrically at 475, 645, and 663 nm. Specific Chl contents were determined according to Lichtenthaler (1987) with a *DU800* spectrophotometer (*Beckman*, Coulter, USA). Xanthophyll cycle pigments were separated and quantified by HPLC essentially as described by Thayer and Björkman (1990). The samples were extracted in ice-cold 100 % acetone and the pigment extracts were filtered through a 0.45 μm membrane filter before assay with *LC-MS2010* (*Shimadzu*, Tokyo, Japan).

Thylakoid membranes were prepared according to Peng *et al.* (2006) with little modification. Thylakoids were prepared from rice leaves homogenized in 0.4 M sorbitol, 100 mM Tricine-KOH (pH 7.5), 10 mM NaCl, and 5 mM $MgCl_2$. After the sample was filtered through 500, 195, and 20 μm nylon mesh and centrifuged for 5 min at 4 000×*g*, the thylakoid pellet was lysed by re-suspending in 5 mM Hepes-KOH, pH 7.5, 10 mM NaCl, and 5 mM $MgCl_2$. The thylakoids were pelleted by centrifugation (5 min, 4 000×*g*). Finally, thylakoids were washed in 5 mM Hepes-KOH, pH 7.5, 10 mM EDTA, centrifuged, and re-suspended in the same buffer with 10 % glycerol added. Samples were stored at –80 °C in small aliquots.

SDS-PAGE electrophoresis: Denaturing gels were composed of 15 % acrylamide/0.4 % bis-acrylamide with Tris-glycine (pH 8.3) as a buffer based on the system described by Laemmli (1970). Gels were surrounded by electrophoresis buffer and typically run without cooling at a constant current between 20 and 40 mA per gel. The concentrations of Chl-protein complex were adjusted to 1 kg m^{-3} Chl and 50 mm^3 were added to each gel. SDS-PAGE was performed using a Mini-protein II Electrophoresis Cell (*Bio-Rad*, USA). Polypeptides were stained with Coomassie Brilliant Blue R-250.

Electron microscopy was done according to Wang *et al.* (2006). The intact leaves were thoroughly rinsed with tap water and distilled water, and then dried with filter paper. The samples (1 mm^3) were fixed in 3 % (m/v) glutaraldehyde and 0.1 M phosphate buffer (pH 7.2) for 4 h, and washed with the phosphate buffer for 1 h. Then the samples were fixed with 1 % OsO_4 (pH 7.2) for 1 h, and rinsed with a buffer for three 10-min periods. After washing, the samples were dehydrated in a graded acetone and ethanol in series, and then embedded in Spurr's resin for 3 d. Dry sections (1–2 μm) were cut with a diamond knife using an ultra-microtome, and mounted on copper grids. Electron microscope observation was made at 100 kV with a *JEM1230* tranmission electron microscope (*JEOL*, Tokyo, Japan).

Modulated Chl fluorescence was measured in attached leaves with a *PAM-2000* portable fluorometer (*Walz*, Effeltrich, Germany) connected to a notebook computer with data acquisition software (*DA-2000*). The experimental protocol of Demmig-Adams *et al.* (1996) was essentially followed.

Irradiance of 50, 100, or 200 μmol(photon) m^{-2} s^{-1} was provided by a 1 000 W tungsten bulb. A water tank with recycled water was used between radiation source and samples to absorb heat. Data are means ± SE of 3–5 independent measurements.

Results and discussion

Phenotype of *ycm* and WT: The *ycm* mutant showed yellow colour phenotype at all developmental stages, its plant height was about half of that of WT at the same stage (Fig. 1). The chloroplast of mesophyll cells from WT had normal thylakoid membrane with well arranged abundant grana lamella (Fig. 2*E*). In contrast, cells at early developmental stages were found in *ycm* mesophyll leaf (Fig. 2*A*). In fully emerged leaves of *ycm* (Fig. 2*B*,*C*,*D*), there were much less thylakoid membranes than in WT, especially in the grana lamellae.

SDS-PAGE also showed in PS2 reaction centre of *ycm* less 43 and 47 kDa proteins than in WT. The contents of LHC2 light-harvesting pigment-protein complex were also decreased. These results were consistent with ultrastructure observations, which indicated a great loss of functional pigment-protein complexes in *ycm*.

The total Chl content of *ycm* was 82.4 mg kg^{-1}(f.m.), which was much lower than in WT at an irradiance of 50 μmol(photon) m^{-2} s^{-1}. The significant increase of

Fig. 1. Phenotypes of *ycm* mutant and wild type.

Chl *a/b* ratio showed that this is a Chl *b* deficient mutant. Similar phenomenon was found in WT, but at a less degree. At the same time, a great reduction of β-carotene content was found both in WT and mutant leaves (Table 1). The formation of Chl *b* helped in the formation of LHC2, which would in turn increase the photon absorption of mutant. Chl *b*-less or -deficient mutants are common in barley, *Arabidopsis*, and rice (Terao *et al.* 1985, Hsu and Lee 1995). Because Chl *b* is found in the light-harvesting pigment-protein complex, limiting the supply of Chl *b* affects antenna complexes that both Chl *a* and Chl *b* need for stability. Our observations showed not only a loss of PS2 reaction centre polypeptides, but also a great loss of LHC2 proteins which were Chl *b*-containing antenna complexes.

Absorption spectra of *ycm* **and WT**: Absorption peaks of *ycm* and WT were both at 432 nm of the Soret band and 663 nm of the Q band. However, the absorbance per pigment unit was higher in *ycm* than in WT. Along with the increase in irradiance, the absorbance of mutant Chl was decreased. At 200 μmol(photon) m^{-2} s^{-1}, it was nearly identical with that of WT (Fig. 3).

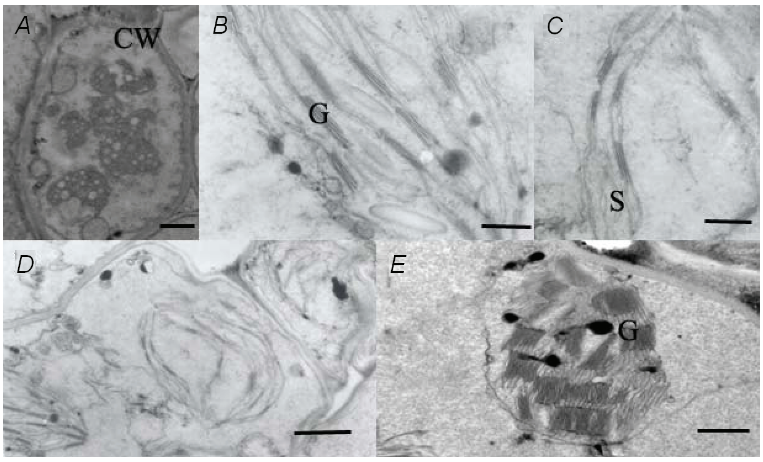

Fig. 2. Ultrastructure of chloroplasts in *ycm* and wild type (WT). *A*: Mutant cell at 2 d. *B, C, D*: Mutant chloroplasts at full growth. *E*: WT chloroplast. G – granum, S – stroma, CW – cell wall. *Bars* = 10 μm (*A*), 1 μm (*E*), 0.5 μm **(***B, C, D*).

Table 1. Chlorophyll (Chl) and β-carotene (β-car) contents in *ycm* mutant and wild type (WT) [mg kg^{-1}(f.m.)] at 50, 100, and 200 μmol(photon) m^{-2} s^{-1}.

Type and irradiance	Chl *a*	Chl *b*	β-car	Chl (*a*+*b*)	Chl *a/b*
ycm-50	81.4±0.5	6.4±0.0	27.5±1.3	82.4±0.9	12.70
ycm-100	77.4±0.6	11.8±0.1	22.3±0.9	89.2±2.6	6.57
ycm-200	106.1±0.9	54.2±0.4	18.2±0.3	160.3±3.9	1.96
WT-50	425.7±10.3	114.2±5.6	78.8±2.4	539.9±26.7	3.70
WT-100	440.4±15.6	120.4±9.7	74.3±3.4	560.7±22.8	3.48
WT-200	405.3±12.7	126.4±4.3	70.0±2.9	531.7±19.7	3.21

Chl contents and Chl *a/b* ratio of *ycm* mutant fluctuated with irradiance. Table 2 shows the light-induced Chl *a* fluorescence kinetic parameters (Genty *et al.* 1989) of the leaves from mutant and WT. The maximal efficiency of PS2 photochemistry (F_v/F_m) of *ycm* slightly decreased with irradiance increase. The actual photochemical efficiency of PS2 (Φ_{PS2}), the efficiency of excitation energy trapped by open PS2 reaction centre in the light-adapted state (F_v'/F_m'), electron transport rate (ETR), and photochemical quenching (q_P) showed

Table 2. Photosystem 2 photochemical parameters of *ycm* mutant and wild type (WT).

Type and irradiance	F_v/F_m	ETR	q_P	q_N	F_v'/F_m'	Φ_{PS2}
ycm-50	0.83±0.01	15.00±1.30	0.26±0.02	0.37±0.02	0.62±0.01	0.16±0.01
ycm-100	0.79±0.01	17.00±0.80	0.33±0.01	0.39±0.03	0.57±0.03	0.19±0.01
ycm-200	0.74±0.01	18.00±0.70	0.31±0.02	0.84±0.02	0.64±0.04	0.20±0.02
WT-50	0.80±0.01	17.00±0.70	0.26±0.01	0.31±0.04	0.72±0.02	0.19±0.04
WT-100	0.80±0.01	28.00±0.90	0.43±0.03	0.34±0.01	0.71±0.02	0.31±0.03
WT-200	0.81±0.02	24.50±1.20	0.38±0.02	0.45±0.05	0.70±0.01	0.27±0.02

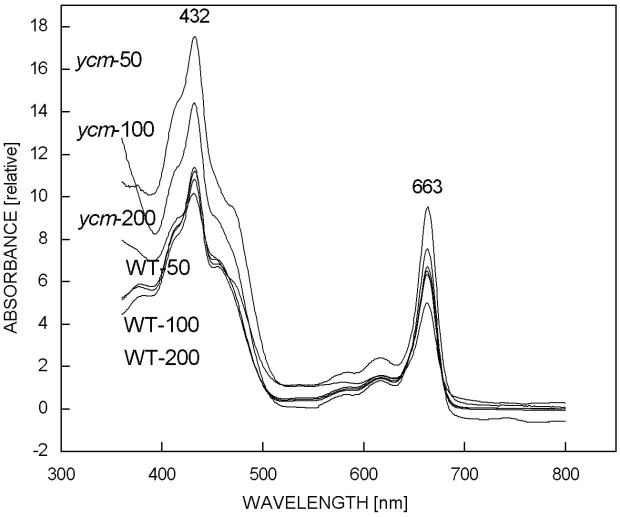

Fig. 3. Chlorophyll absorption spectra in *ycm* mutant and wild type (WT) at 50, 100, and 200 μmol(photon) m^{-2} s^{-1}.

increasing trends. The WT showed no difference in F_v/F_m and other parameters showed increasing trends. Meanwhile, the non-photochemical quenching (q_N) which reflects the process competing with PS2 photochemistry for absorbed excitation energy (Campbell *et al.* 1998) increased in the leaves of both *ycm* and WT.

The (Z+A)/(Z+A+V) ratio increased in the leaves of *ycm* and WT plants (Fig. 4). This ratio in *ycm* increased much more sharply from 0.33 to 0.58, whilst that of WT only from 0.41 to 0.48.

In the present study, we characterized PS2 photochemistry of mutant and WT at different irradiances and showed that changes in F_v/F_m in the dark-adapted state were of opposite trend in *ycm* and WT. A straight-down of F_v/F_m in *ycm* suggested a potential damage effect of photon energy on PS2. We also showed that an increase in q_N was accompanied by an increase in (Z+A)/(Z+A+V) during the increment of irradiance. Exposure of plants to fluctuations in irradiance in excess of that required for photosynthesis generally induces xanthophyll-cycle activity characterized by the reversible, light-dependent de-epoxidation of V to A and Z. A strong correlation has been established between the non-photochemical dissipation of excess photon energy and Z content, which protects PS2 reaction centres from over-excitation (Demmig-Adams and Adams 1992). No difference was found in the values of F_v'/F_m' both in *ycm* and WT. Slight increases of ETR, q_P, and Φ_{PS2} were found in *ycm*, which suggests that the significant increase of Chl contents did not directly contribute to the photon energy utilization in *ycm*. However, the increases of ETR, q_P, and Φ_{PS2} found in WT indicated that Chl of WT chloroplast can use photon energy at high efficiency. Φ_{PS2} is probably the product of q_P and F_v'/F_m' and the differences in Φ_{PS2} among *ycm* and WT are the result of the differences in q_P only (Table 2). The q_P indicates the oxidation-reduction state of the primary acceptor for PS2, which is determined by the rate of photoreduction of the acceptor by PS2 and by its rate of re-oxidation linked to CO_2 reduction *via* PS1 (Krause and Cornic 1987). The q_P values also can be used to estimate the fraction of the reduction state of Q_A, which reflects the excitation pressure on PS2 (Öquist and Huner 1993). An increase of q_P in WT suggested a decrease in the fraction of the reduction state of Q_A of PS2, which means there was a decrease in susceptibility to photoinhibition in WT. In contrast, q_P values remained stable in *ycm* which indicated a tendency to photoinhibition. The increase in q_N in *ycm* suggested that the lower Φ_{PS2} values in *ycm* were due to the decreased rate of utilization of electrons generated from water splitting and an increased capacity to divert photon energy to non-photochemical quenching pathways.

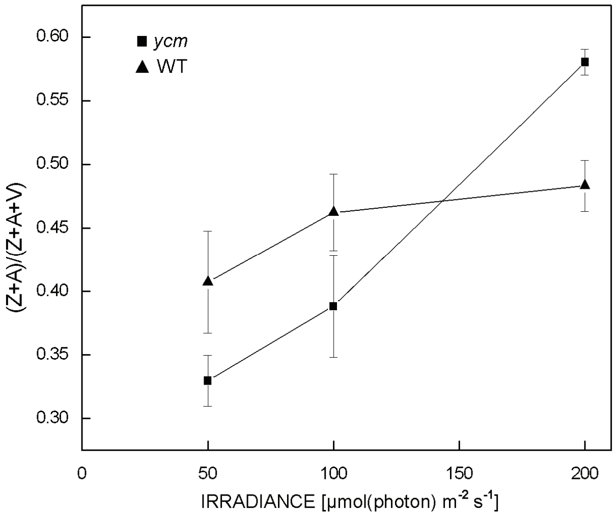

Fig. 4. (Z+A)/(Z+A+V) of *ycm* mutant and wild type (WT) under 50, 100, and 200 μmol(photon) m^{-2} s^{-1}.

In conclusion, *ycm* mutant did not show any green leaf colour under different irradiances. Thus it could not use photon energy efficiently and independent of irradiance. The stable q_P and Φ_{PS2} values and great increase in q_N values suggested that most photon energy was quenched in xanthophyll cycle in this mutant to avoid photoinhibition.

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