Mutation mechanism of chlorophyll-less barley mutant *NYB*

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Abstract

NYB is chlorophyll-less barley mutant, which is controlled by a recessive nuclear gene. The mutation mechanism is revealed. The activities of enzymes transforming 5-aminolevulinic acid into protochlorophyllide were the same in both *NYB* and the wild type (WT), but the activity of the protochlorophyllide oxidoreductase (POR) in WT was much higher than that of *NYB*. Most of the photosystem 2 apoproteins were present in both WT and *NYB*, suggesting that the capability of protein synthesis was probably fully preserved in the mutant. Thus chlorophyll (Chl) biosynthesis in *NYB* was hampered at conversion form protochlorophyllide (Pchlide) into chlorophyllide. The open reading frame of *porB* gene in *NYB* was inserted with a 95 bp fragment, which included a stop codon. The *NYB* mutant is a very useful material for studies of Chl biosynthesis, chloroplast signalling, and structure of light-harvesting POR-Pchlide complex (LHPP).

Additional key words: chlorophyll; fluorescence emission spectra; light-harvesting complex 2; Nanchong yellow barley (*NYB*); nucleotide sequences; photosystem 2; proteins; protochlorophyllide oxidoreductase (POR).

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).

NYB is a new Chl-deficient mutant obtained by ^{60}Co-γ ray treatment (Tang *et al*. 1994). Its phenotype is stable and not affected by irradiation or temperature. The chloroplast of *NYB* contains fewer thylakoids and grana than the wild type (WT), with a lower total Chl content and a higher Chl *a*/*b* ratio in old leaves (Lin *et al*. 1999). When *NYB* was hybridized with WT, the ratio of character segregation was 3 : 1, and the ratio of testcross was 1 : 1 (Cheng *et al*. 2001). Therefore, the yellowish colour of *NYB* leaves is most likely controlled by a recessive nuclear gene.

We have researched on the mutant for many years. A study by Tan *et al*. (1997) demonstrated that the *NYB* has a higher photosystem (PS) 2 photochemical efficiency (F_v/F_m), which may be due to the lesser excitation energy transfer from PS2 to PS1. The first identification of qualitative and quantitative differences in the harvesting complexes of PS2 (LHC2) between *NYB* and its WT was done by Lin *et al*. (1998, 1999). According to Yuan *et at.* (2007), this mutant is insensitive to osmotic stress. However, the mechanism of mutation in *NYB* remains unclear so far.

In the present study, the biosynthetic system to perform the transformations from 5-aminolevulinic acid (ALA) to protochlorophyllide (Pchlide), and Pchlide to chlorophyllide (Chlide) was analyzed. The apoprotein accumulation in the early period of greening of etiolated leaves was also investigated. The method we used is suitable for other Chl-less mutants as well. In addition, the prospective studies of the light-harvesting POR-Pchlide complex (LHPP) and chloroplast signalling with this mutant were analysed.

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Abbreviations: ALA – 5-aminolevulinic acid; Chl – chlorophyll; Chlide – chlorophyllide; Coprogen III – coproporphyrinogen III; LHC2 – light-harvesting complex of photosystem 2; LHPP – light-harvesting POR-Pchlide complex; Mg-proto – Mg-protoporphyrin IX; *NYB* – Nanchong Yellow Barley; ORF – open reading frame; PBG – porphobilinogen; Pchlide – protochlorophyllide; POR – protochlorophyllide oxidoreductase; Proto IX – protoporphyrin IX; PS – photosystem; Urogen III – uroporphyrinogen; WT – wild type.
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Materials and methods

Plants: The experiments were performed with the Chl *b*-less barley (*Hordeum vulgare* L.) mutant *NYB* and its WT. Seeds were soaked overnight, and seedlings were grown in vermiculite in a greenhouse at 25 ºC for 7 d under complete darkness and, if necessary, were subsequently continuously irradiated for times specified.

Chl contents were estimated as described by Lichtenchaler and Wellburn (1983). 7-d-old etiolated seedlings were continuously irradiated for 1 h. Chls were extracted with 80 % acetone and spectrophotometrically evaluated.

Contents of Chl synthesis precursors: Leaves selected for analysis were weighed. 5-aminolevulinic acid (ALA) was extracted as described by Dei (1985). Isolations of porphobilinogen (PBG) and of uroporphyrinogen (Urogen III) were performed using the method of Bogorad (1962); coproporphyrinogen III (Coprogen III), protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-proto), and protochlorophyllide (Pchlide) were determined according to the methods of Rebeiz *et al*. (1975).

Introduction of exogenous ALA: According to Rudoi and Shcherbakov (1998), etiolated leaves were cut off under water and placed in darkness with the cut side immersed in an ALA solution in phosphate buffer (0.067 M, pH 7.7). After fixed time intervals the samples were taken for determination of pigment contents.

Efficiency of conversion of Pchlide to Chlide was determined as described by Lay *et al*. (2000) with some modifications. The non-irradiated leaves were ground on ice in 80 % acetone saturated with $NaHCO_3$. The suspension was passed through a filter, and then one-half volume of hexane was added to extract the acetone phase. After vigorous shaking, Chlide concentration in the acetone phase was measured by the fluorescence intensity excited at 400 nm and scanned at 600–720 nm. The seedlings were transferred to incandescent light for 60 s [100 μmol(photon) m^{-2} s^{-1}]. The treated leaves were extracted immediately and then scanned according to the above method. Subsequently, the treated seedlings were darkened for 90 min, and then extracted and scanned again. All the procedures were done under darkness.

Isolation of thylakoid membranes, fully-denaturing SDS-PAGE, and Western blot analysis: Thylakoid membranes were isolated as described by Lin *et al*. (2003). Polypeptide composition of isolated thylakoids was fractionated using the gel system of Laemmli (1970). For Western blotting, electrophoresed proteins without staining were immediately electro-transferred onto nitrocellulose films according to Mohanty *et al*. (2006). Membranes were blocked with non-fat milk for 1 h at room temperature before applying antisera to D1/D2 (gifts from Dr. Eva-Mari Aro), CP43/CP47 (gift from Dr. Hong-Guo Liang), and LHC2s (*AgriSera Comp*., Sweden). Then signals were revealed by using alkaline phosphatase-conjugated goat-anti-rabbit secondary antibodies.

Reverse transcription and PCR: Preparation of total RNA from barley seedlings was carried out by the method of Zhang *et al.* (2004). PolyA-containing RNA was transcribed into single-stranded DNA (Krawetz *et al*. 1989), which was then amplified by PCR using the primers 1 (5'-AATTCCGCAGCACAGTGCAC-3' and 5'-ACGACAAGTTGAGCATCGGC-3') and 2 (5'-GCG CTATCTGATACGCTCAC-3' and 5'-GCACAAAATTT CGCAACTTG-3') for *porA* and *porB* Open Reading Frame (ORF), respectively. PCR was performed in a *Bio-Rad* thermocycler as follows: 2-min denaturation at 94 °C, 1 min annealing at 55 °C, and 3-min extension at 72 °C for 40 cycles. Elution of PCR fragments, subcloning into the PMB18 vector, and sequence analysis were performed according to Sambrook *et al*. (1989).

Results

Conversion of ALA into Chl *a*: In order to evaluate the potential of the enzyme system for transformation from ALA to Chl *a*, it is necessary to estimate the precursors of Chl *a*. As shown in Fig. 1, contents of porphobilinogen (PBG), uroporphyrinogen (Urogen III), coproporphyrinogen III (Coprogen III), protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-proto), and protochlorophyllide (Pchlide) were close to or higher in the mutant than in the WT (Student's *t*-test was used for comparison between *NYB* and WT; statistically significant differences at $p<0.05$). In addition, feeding of exogenous ALA (data not shown) revealed that activities of the enzymes converting ALA to Pchlide in *NYB* were almost the same as they were in the WT. However, compared to WT, the *NYB* leaf contained about one-half of Chl *a* or Chl *b*. This result at least means that some enzyme converting Pchlide to Chl *a* was inhibited in *NYB*.

Etiolated *NYB* and WT seedlings had no difference in the content of Pchlide (Fig. 2*A*). Pchlide content decreased markedly, and Chlide content increased swiftly 1 min after a flash. However, the decline of Pchlide in WT was more significant than that in *NYB*, while the content of Chlide in WT was higher than that in the mutant. Therefore, the synthesis rate of Chlide in the mutant was no more than one-half of the WT (Fig. 2*B*). We conclude that protochlorophyllide oxidoreductase

(POR) was most likely damaged. Chlide could be transformed into Chl *a* about 90 min after the first flash and then in darkness (Lay *et al*. 2000). Fig. 2*C* shows that the efficiency of converting Chlide into Chl *a* in the mutant was close to that in the WT.

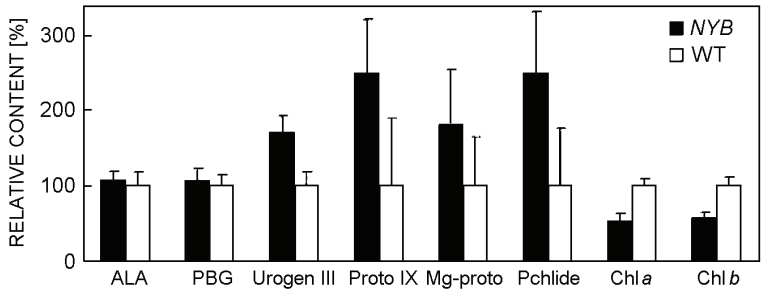

Fig. 1. Comparison of chlorophyll precursors between *NYB* and WT. Means ± standard deviations of three independent experiments.

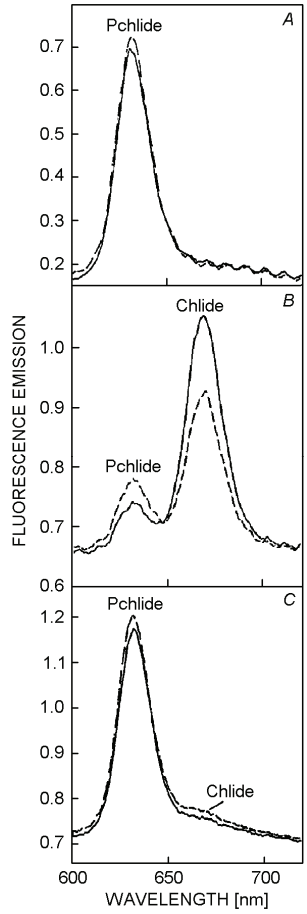

Fig. 2. Fluorescence emission spectra in acetone of Pchlide and Chlide extracted from etiolated leaves of *NYB* (*dotted lines*) and wild type, WT (*solid lines*): (*A*) etiolated seedlings grown in dark; (*B*) exposed to a flash for 1 min; (*C*) 90 min in dark after 1 min irradiation. The excitation wavelength was 440 nm.

Accumulation of LHC apoproteins: Thylakoid membranes of 6-d-old etiolated *NYB* and WT seedlings were continuously irradiated for 0–48 h. Thylakoids were isolated and fractionated by SDS-PAGE. Appearance of the PS proteins started after 6 h of irradiation, and their content increased dramatically both in *NYB* and WT as the irradiation time passed by (Fig. 3). However, the content of PS proteins in *NYB* was much lower than that in WT. Western blots with LHC2b1 and LHC2b2 antibodies showed a similar trend (Fig. 4). It seemed that no PS protein was lost in *NYB*, and the low content of PS proteins in the mutant was largely due to the decreased Chl contents and the subsequently declined stabilities of pigment-binding proteins. To make further confirmation, major PS2 proteins from mature leaves were also compared. Fig. 5 shows that all PS2 proteins that we detected were expressed in thylakoid extracts of both *NYB* and WT, although the contents in *NYB* were much lower. *In vivo* ^{35}S labelling of PS proteins (data not shown) also suggested that most PS proteins were synthesized in the mutant. Here, the capability of PS protein synthesis was probably fully preserved in the mutant, and the yellowish appearance of *NYB* could not be attributed to deficiency in PS protein synthesis.

***por* genes amplified by RT-PCR**: PolyA-containing RNAs were isolated from 6-d-old etiolated barley seedlings under darkness. PCR fragments about 1.3 kb could be amplified from both *NYB* and WT. Then the sequences were identified to *porA* and *porB* cDNAs. No difference of *porA* was found between *NYB* and WT. However, the ORF of *porB* in the mutant was inserted with a 95 bp fragment, which contained a stop codon (TAA) at the beginning (Fig. 6). Therefore, we assume that the PORB protein in *NYB* was truncated and partly disfunctioned.

Discussion

We first studied the mutation principle of the important barley mutant *NYB*. Considering that the yellowish leaves of *NYB* are controlled by a recessive nuclear gene, we deduce that the mutation must happen either in PS protein synthesis or in Chl synthesis. In spite of the low contents of major PS proteins in *NYB*, the capability of their synthesis in the mutant is probably fully preserved. The mutant exhibits a normal content of the respective PS mRNAs and is capable of forming the protein precursors and importing them into chloroplasts (Lin *et al.* 1999). Alternatively, the mutant *NYB* should have a defect in Chl biosynthesis, while PS polypeptides do not accumulate to the normal levels because of the absence of Chls that stabilizes them (Rudoi and Shcherbakov 1998,

Hoober and Eggink 1999).

In etiolated seedlings irradiated for 1 h, a lag phase of Chl accumulation is not completed yet and all Chl is formed from initial Pchlide of etiolated leaves. In this period the complexes of reaction centres of both photosystems only begin to form, while LHCs do not appear yet. Therefore, Chl contents in this time point represent the initial Chl synthesis rates (Rudoi and Shcherbakov 1998). After greening for 1 h, both Chl *a* and Chl *b* were synthesized in *NYB*, but both at about half the levels in WT (data not shown), also suggesting that Chl synthesis was inhibited in the mutant.

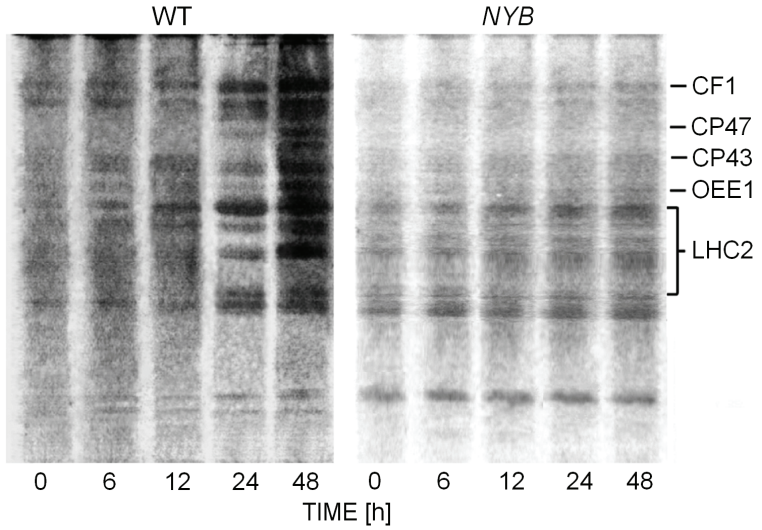

Fig. 3. SDS-PAGE of solubilized thylakoid membranes in etiolated seedlings after irradiation for 0–48 h. Gels were stained with Coomassie blue. The locations of PS proteins are marked. OEE1 – oxygen-enhancer protein 1.

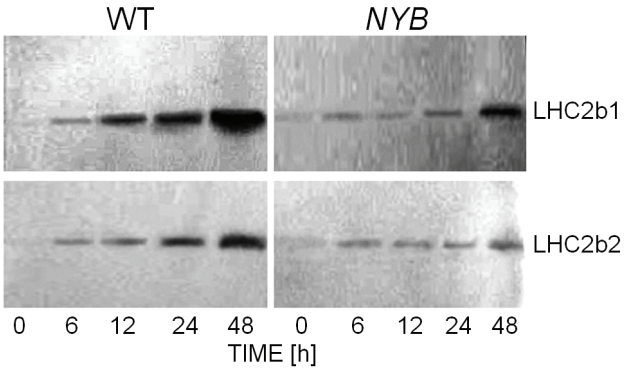

Fig. 4. Western blot of LHC2b1 and LHC2b2 in *NYB* and wild type (WT) during 48 h of greening.

NYB was less sensitive to water stress than WT. Although the amounts of Chls and PS2 proteins were less in the mutant, the remaining Chls in *NYB* bound apoproteins more effectively, and PS2 in the mutant adjusted its formation to contain less LHCs (Yuan *et al.* 2007). Tan *et al.* (1997) demonstrated that the *NYB* had a higher PS2 photochemical efficiency (F_v/F_m), which may be due to the lesser excitation energy transfer from PS2 to PS1. Therefore, the precise structures of PS proteins in *NYB* require further studies.

Chl is assembled in chloroplast from eight molecules of ALA, and the mainly next-to-last precursors in angiosperms are PBG, Urogen III, Coprogen III, Proto IX, Mg-proto IX, Pchlide, and Chlide (Wettstein *et al.* 1995). If one step of Chl biosynthesis is blocked, the former precursors should accumulate, and the latter precursors should decrease. According to our experiments about Chl precursors, the mutation can be attributed to a blockage in conversion from Pchlide to Chl *a*. The investigation of transformation from Pchlide to Chlide provided detailed information that the enzymes catalyzing Pchlide were most likely damaged.

PORA and PORB form a stable light-harvesting POR-Pchlide complex in a ratio of 5 : 1, named LHPP (Reinbothe *et al.* 1999). Functionally, LHPP may play major role in establishing the photosynthetic apparatus and in protecting against photo-oxidative damage during greening (Masuda and Takamiya 2004, Reinbothe *et al.* 2004). PORB protein in our mutant was truncated before the evolutionarily conserved residues Cys276 and Cys303, which constitute Pchlide binding sites (Reinbothe *et al.* 2006). PORB protein may be truncated in our mutant *NYB.* Consequently, configuration of LHPP in the mutant may be altered, and it may be exposed to oxidative stress during greening, which needs further investigation.

Furthermore, the mutant *NYB* is a useful material for plastid signal researches. Plant cells store genetic information in the genomes of three organelles: the nucleus, plastid, and mitochondrion. In turn, organelles send signals to the nucleus to control nuclear gene expression, such as chloroplast redox signals (Pfannschmidt 2003) and tetrapyrrole-mediated signals (Nott *et al.* 2006). Mg-proto, the Chl biosynthetic precursor, is a key inducer of tetrapyrroles' mediated plastid signals (Kropat *et al.* 2000, Strand *et al.* 2003). Our experiments showed that the content of Mg-proto was much higher in the mutant than in the WT.

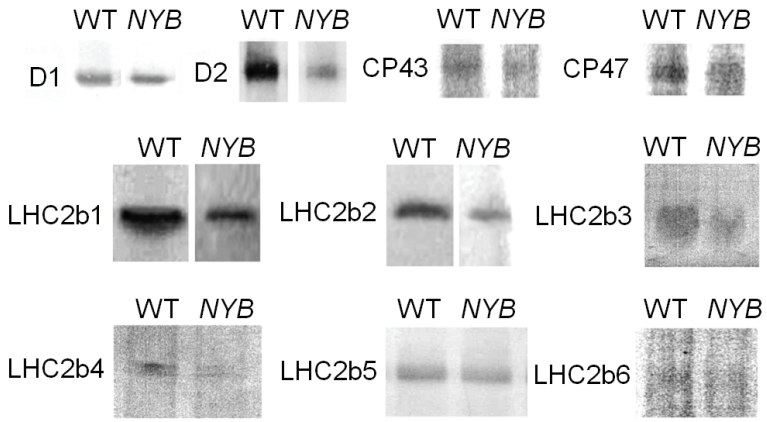

Fig. 5. Western blot analysis of PS2 proteins in mature *NYB* and WT seedlings. Antibodies for D1, D2, CP43, CP47, and LHC2b1-b6 were used.

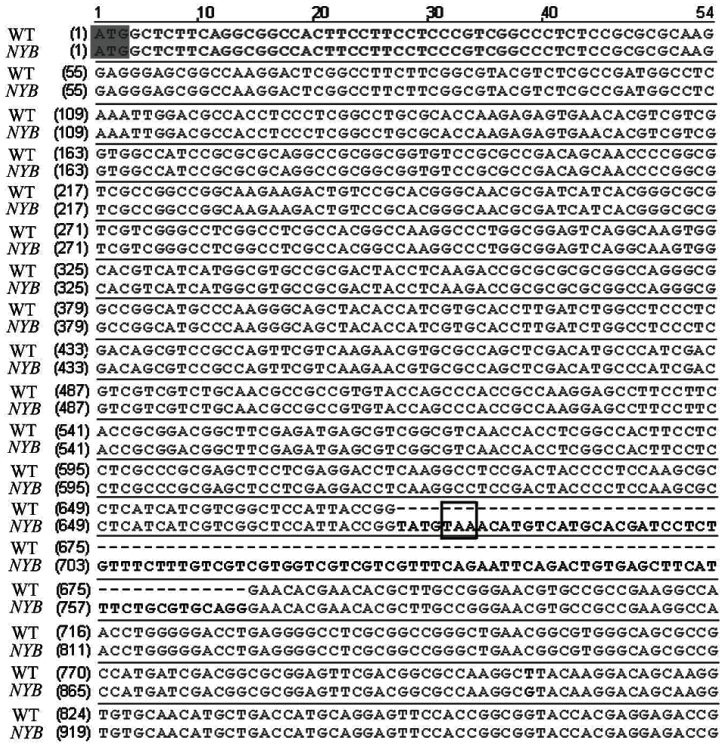

Fig. 6. Nucleotide sequences of *porB* ORFs in *NYB* and WT. The start codon is marked by "pane" and the stop codon is marked by a square frame.

Moreover, we found that *NYB* is more sensitive to norflurazon (a plastid signal inducer) than the WT (unpublished data), but it contains similar levels of *cab* mRNA compared with normal seedlings (Lin *et al*. 1998, 1999). The most likely explanation is that the amount of Mg-proto is higher in the mutant, but lower than the threshold for repression of *cab* gene. When *NYB* was treated with norflurazon, it was easier to produce plastid signals.

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