Characterization of the Leaf Color Mutant *hy* and Identification of the Mutated Gene in Chinese Cabbage

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ADDITIONAL INDEX WORDS. chlorophyll-descent, chlorophyll fluorescence, ethyl methanesulfonate mutant, yellow-green leaf

ABSTRACT. Leaf color mutants play an important role in our understanding of chlorophyll biosynthesis and catabolism. In this study, we obtained a yellow-green leaf mutant *hy* in an ethyl methanesulfonate mutagenized population of chinese cabbage (*Brassica rapa* ssp. *pekinensis*). The *hy* phenotype was controlled by a recessive allele at a single locus. The intrinsic photochemical activity of photosystem II (PSII) is impaired in *hy*, suggesting that absorbed light energy is not efficiently transferred from the light-harvesting complexes antenna to the PSII reaction centers and dissipated as heat or fluorescence. We measured chlorophyll content and chlorophyll precursors and analyzed the expression of key genes in the chlorophyll synthetic pathway in *hy* and wild type. The mutation phenotype was consistent with inhibited expression of chlorophyll *a* oxygenase (*CAO*) gene in the chlorophyll synthetic pathway. In mutant *hy*, *CAO* cDNA was cloned so that a C to T mutation at 1099 bp caused a conserved proline (Pro) to serine (Ser) mutation at the 367th amino acid in C-domain, which changed the secondary structure of CAO protein. We speculate that the mutation amino acid changed in the C-domain may affect the catalytic function in mutant *CAO*.

Leaf color is largely the result of photosynthetic pigments, primarily chlorophyll (Chl). Chl plays an essential role in light absorption for energy transfer (Stern et al., 2004). The Chl biosynthesis pathway is complex and involves more than 20 genes encoding 16 enzymes, and blocking at any step during Chl synthesis can cause changes in leaf color (Beale, 2005).

In higher plants, Chl has two distinct forms, chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*), that together represent the major light-harvesting pigments in plants. They are associated with Chl *a*, b-binding polypeptides in the peripheral light-harvesting antenna with Chl *b* restricted to the light-harvesting complexes (LHC) and is associated with photosystem I (PSI) or PSII (Grossman et al., 1995). Therefore, in the absence of Chl *b*, the LHC proteins are decreased, partly due to the degradation of unbound LHC apoproteins by proteases (Espineda et al., 1999; Hooper and Eggink, 1999). Chl *a* oxygenase is the critical enzyme for Chl *b* synthesis from Chl *a* through conversion of a methyl group to a formyl group to generate Chl *b* and thus affects the Chl *a/b* ratio (Espineda et al., 1999; Tanaka et al., 1998). Chl *a* oxygenase (CAO) converts chlorophyllide *a* to chlorophyllide *b*, which is a mononuclear iron-containing

protein via 7-hydroxymethyl chlorophyllide (Oster et al., 2000). CAO is on the chloroplast envelope and acts as the initial site of Chl *b* synthesis and LHC assembly (Eggink et al., 2004; Hooper and Eggink, 1999). Overexpression of *CAO* causes the stimulation of Chl *b* synthesis and increased accumulation of the LHCII (Pattanayak et al., 2005; Tanaka et al., 2001). The mRNA and protein expression of *CAO* is altered by the LHC protein (LHCB), suggesting that enhanced *CAO* mRNA level affects the size of the LHCII (Biswal et al., 2012; Bujaldon et al., 2017; Harper et al., 2004). In *Arabidopsis thaliana* and rice (*Oryza sativa*), chlorophyll synthesis is blocked in *CAO* mutants (Abe et al., 2012; Espineda et al., 1999).

Chinese cabbage is closely related to *Arabidopsis*, providing opportunities for comparative studies of gene function (Trick et al., 2009; Zhao et al., 2005). With the completion of the *Brassica rapa* genome sequencing and comparative studies with other subspecies, more gene structure information has been acquired (Cheng et al., 2013; Wang et al., 2011). Ethyl methanesulfonate (EMS) is one of the most effective chemical mutagens used to generate single variable point and high-density mutations that are stably inherited (Henikoff et al., 2004; McCallum et al., 2000). The various mutations are ideal materials for gene function discovery to identify genes involved in key traits in *Brassica* species (Liu et al., 2010; Stephenson et al., 2010).

In our previous study, an EMS-induced mutant population has been created from *B. rapa*, and several mutants with diverse phenotypes have been identified (Lu et al., 2016). In this study, we focus on one of the EMS-induced mutants, *hy*, that exhibits a yellow-green leaf phenotype with decreased levels of chlorophyll, especially Chl *b*. We identified the leaf color mutant gene through examination of Chl and its precursors combined with expression data for important genes in the chlorophyll

Received for publication 26 Mar. 2018. Accepted for publication 24 July 2018. The authors are grateful to Dr. Kathleen Greenham at Dartmouth College in Hanover, NH, for critical reading of the manuscript.

This work was supported by the National Key R&D of China (grant 2016YFD0100204-17), the National Natural Science Foundation of China (grant 31672151), the Hundred-Talents Program of Hebei Universities (grant SLRC2017040), the Science and Technology Support Program of Hebei (C2016204170), the Science and Technology Support Program of Hebei (grant 16226304D-2), the Science and Technology Research Project of Hebei Colleges and Universities (grant QN2017084), the International Cooperation project in the Science and Technology Support Program of Hebei (grant17396315D). The Graduate Student Innovation Fund Project in Hebei Province (grant CXZZBS2017067).

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synthetic pathway. To examine the impact of the variant *CAO* alleles, we compared physicochemical and functional properties, secondary structure, and chlorophyll fluorescence in different *CAO* allele plants. We found changes in the helical and fold structure of CAO, as well as impaired PSII photochemical activity in *hy*. This screening method for leaf color proved to be an effective method for narrowing down the candidate gene in a population and provides additional material for future studies of the mutated gene function and protein structure.

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. The mutant *hy* was selected from the M_2 mutant population, which was obtained from M_1 induced from wild-type [WT (A03)] seeds by 0.4% EMS. *hy* was selfed strictly to make sure the site was homozygous. The M_4 generation mutant was used to cross to WT to obtain F_1 progeny, which were self-pollinated to obtain F_2 . F_1 were backcrossed to the mutant *hy* to obtain BC_1 . All the plants were sowed in seeding dishes in Jan. 2016 after 2 months of vernalization at low temperatures (3 to 8 °C), were grown in a greenhouse and cultivated normally on an experimental farm at Hebei Agricultural University in Baoding, China in Mar. 2016. The phenotypes of *hy* and WT were observed by visual inspection at the seedling stage (10 °C), the rosette stage (10 °C), and the bolting stage (30 °C). All the samples were collected 1 week after transplanting. We chose the third leaves from inside to outside in the seedling stage for the experiments. Samples for RNA analysis were frozen immediately in liquid nitrogen and stored at -80 °C.

MEASUREMENT OF CHL CONTENT AND PRECURSORS. Chls and carotenoids were extracted according to the protocol of Porra et al. (1989) and measured at 663, 646, and 470 nm with a spectrophotometer (ultraviolet-1800; Shimadzu, Kyoto, Japan). Determination of aminolevulinic acid (ALA), porphobilinogen (PBG), uroporphyrinogen III (Urogen III), and coproporphyrinogen III (Coprogen III) content was based on Bogorad's (1962) methods. ALA, PBG, Urogen III, and Coprogen III were measured at 663, 553, 405.5, and 399.5 nm, respectively.

Precursors, including protoporphyrin IX (Proto IX), magnesium protoporphyrin IX (Mg-Proto IX), and protochlorophyllide (Pchlide), were assayed as described by Rebeiz et al. (1975). Chl precursors were quantified with a fluorescence spectrophotometer (RF-5301PC, Shimadzu) using Ex400:Em632 for Proto IX, Ex440:Em633 for Pchlide and Ex420:Em595 for Mg-Proto IX.

The data were transformed to percentage by setting the WT values to 100%. Three biological and three technical replicates for WT and mutant *hy* were analyzed. Significance was conducted by *t* test with percent.

CHLOROPHYLL FLUORESCENCE MEASUREMENT. Photochemical efficiency

analysis was performed using a fluorometer (MINI-IMAGING-PAM; Walz, Effeltrich, Germany) from the leaves 1 week after transplanting, according to the manual. The original fluorescence (F_o) was induced by measuring light ($0.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, frequency 1 Hz) after 1 h dark adaptation. Pulses (0.8 s) of red light ($5000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were used to determine the maximum fluorescence (F_m). When finished, the pulsed light, actinic light ($156 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), served to induce fluorescence kinetics, steady-state fluorescence (F_s) was measured, and F_m' was determined after exposure to further saturating pulses (0.8 s, $5000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) every 20 s.

Photosynthetic parameters were calculated according to previous equations (Genty et al., 1989; Snel and van Kooten, 1990), maximum quantum yield of PSII (F_v/F_m) = $(F_m - F_o)/F_m$, photochemical quenching (qP) = $(F_m' - F_s)/(F_m' - F_o)$, non-photochemical quenching (NPQ) = $(F_m - F_m')/F_m'$, the effective quantum yields of PSII (Φ_{II}) = $(F_m' - F)/F_m'$, quantum yield of regulatory energy dissipation [$Y(NPQ)$] = $1 - \Phi_{II} - 1/[NPQ + 1 + qL(F_m/F_o - 1)]$, nonquantum yield of regulatory energy dissipation [$Y(NO)$] = $1/[NPQ + 1 + qL(F_m/F_o - 1)]$.

TRANSCRIPTIONAL ANALYSIS OF KEY GENES IN CHL BIOSYNTHETIC PATHWAY. Total RNA was isolated using EASY spin Plus (Thermo Fisher Scientific, Waltham, MA), and first-strand cDNA was synthesized via reverse transcription with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Nucleotide concentration was determined using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific). Nine related genes associated with Chl biosynthesis were chosen; primers for the Chl biosynthesis genes and the reference gene *Actin* (*Act*) for the quantitative real-time polymerase chain reaction (RT-qPCR) are listed in Supplemental Table 1. RT-qPCR was performed using an RT-PCR detection system (Lightcycler 96; Roche, Basel, Switzerland) following the manufacturer's instructions with specific primer sets using Thunderbird SYBR qPCR Mix (Toyobo). The $2^{-\Delta\Delta CT}$ method was used to analyze relative change in gene expression (Livak and Schmittgen, 2001). All reactions were performed with three biological and three technical replicates.

Fig. 1. Phenotype of leaf color of wild type [WT (green leaf)] and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage at different temperature and growth stages. (A) Phenotype characteristics of WT and mutant *hy* at 10 °C in the seedling stage. (B) Phenotype characteristics of WT and mutant *hy* at 10 °C in the rosette stage. (C) Phenotype characteristics of WT and mutant *hy* at 30 °C in the bolting stage.

cDNA CLONING, SEQUENCE COMPARISON, AND PREDICTION OF PROTEIN STRUCTURE OF THE *CAO* GENE. Gene annotation within the located region was obtained from the Brassica Database (Wang et al., 2011). cDNA sequences of *CAO* from the WT and the mutant *hy* were amplified with the three overlap primers (CAO1, CAO2, and CAO3). The resulting PCR amplicons were extracted and purified using the MiniBEST Agarose Gel DNA Extraction Kit version 4.0 (Takara, Kusatsu, Japan). The products were examined by electrophoresis in a 1% agarose gel and inserted into the pMD19-T cloning vector (Takara) at 16 °C overnight followed by transformation into *Escherichia coli* cells DH5alpha. Positive colonies were selected on Luria-Bertani (LB) -amp plates (0.1% ampicillin); single colonies were propagated in LB medium, and the plasmids were extracted and verified by sequencing.

Comparisons of the *CAO* sequences in *hy* and WT was performed using DNAMAN 5.0. Homologous sequences of *CAO* in arabidopsis were identified using the arabidopsis information resource [TAIR (Huala et al., 2001)]. The transit peptide was predicted by the TargetP Server (Emanuelsson et al., 2000). Analysis of the physicochemical properties of amino acid residues and secondary structure in the mutant and WT was performed using

EXPASY (Gasteiger et al., 1999) and TMHMM (Krogh et al., 2001). Prediction of conserved domains was performed using the NCBI Conserved Domain Search (Marchler-Bauer et al., 2015).

Results

PHENOTYPIC CHARACTERIZATION OF THE *hy* MUTANT. The mutant *hy* exhibited yellow-green leaves and grew slower than WT that was light green at the seedling stage (Fig. 1A). The leaf color of *hy* mutants remained a yellow green at 10 °C during the rosette stage and at the 30 °C bolting stage (Fig. 1B and C). The new leaves were yellow green and did not recover with an increase in temperature, which suggests that *hy* was not sensitive to temperature, and the yellow-green leaves mutant phenotype remains present in different cultural condition at different stages.

GENETIC CHARACTERIZATION OF THE *hy* MUTANT. F₁ plants displayed the WT phenotype, and their F₂ population segregation ratio tended to be 3:1 [green:yellow-green plants = 164:52, $\chi^2 = 0.099$, $P > 0.05$ (Table 1)]. The BC₁ segregation ratio of crosses with *hy* was 1:1 [green:yellow-green plants = 58:51, $\chi^2 = 0.099$, $P > 0.05$ (Table 1)]. This result indicates that the yellow-green phenotype was most likely controlled by a recessive mutation at a single locus.

QUANTIFICATION OF CHLOROPHYLL AND CHL PRECURSORS IN THE *hy* MUTANT. Chl *a* level in the mutant *hy* was 67.4% lower than WT. Chl *b* level in the mutant *hy* was significantly lower at 19.5% that of WT (Fig. 2). Because of the lower Chl *b* in the mutant, there was a substantial increase in the Chl *a*:*b* ratio compared with WT.

The results showed that the levels of ALA, PBG, Urogen III, Coprogen III, and Mg-Proto IX in the *hy* mutant were similar to those of WT, the contents were between 90% and 97%, Proto IX was slightly higher (122.6%) than the WT, and Pchlide content was significantly lower (74.6%) than in the WT [$P < 0.01$ (Fig. 2)].

These results suggested that the decrease in Chl was caused by a decrease of Pchlide. Therefore, we speculated that the mutated gene in *hy* is involved in the conversion of Mg-Proto IX to Chl *b*.

CHLOROPHYLL FLUORESCENCE OF *hy* MUTANT. To further examine the impact of *hy* mutant on the photosynthetic electron flow and the assembly of PSI and PSII reaction center complexes, we analyzed their chlorophyll fluorescence using PAM techniques (Maxwell and Johnson, 2000). *Fv/Fm* represents the photoenergy conversion efficiency of PSII and initial photoenergy capture efficiency that was 0.731 in *hy* was similar compared with 0.747 in WT. The Chl fluorescence from PSII showed that Φ_{II} in *hy* was lower than WT. Moreover, the fraction of the photochemical electron transport energy from PSII represented by qP was decreased in *hy* compared with WT. The fraction of the nonphotochemical electron transport energy from PSII, NPQ

Table 1. Segregation phenotype plant of F₂ and BC₁ population in chinese cabbage with a chi-square test.^z

Population	F ₂	BC ₁
Green plants (no.)	164	58
Yellow green plants (no.)	52	51
Total plants (no.)	216	109
χ^2	0.099	0.450
<i>P</i>	0.753	0.503

^zThe phenotype of *hy* mutant is yellow-green leaf induced by ethyl methanesulfonate. The phenotype of wild type (WT) is green leaf. All plants were detected by visual inspection. $P > 0.05$ was considered significant. F₂ plants were self-pollinated F₁ derived from the cross between the M₄ generation mutant and WT. BC₁ plants were F₁ backcrossed to the mutant *hy*.

Fig. 2. The contents of chlorophyll biosynthesis precursors and chlorophylls of wild type [WT (green leaf)] and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage [** above the vertical bar indicates the differences are significant between the means ($n = 3$) at $P < 0.01$ via *t* test]. ALA = aminolevulinic acid; chla = chlorophyll *a*; chlb = chlorophyll *b*; chls = chla and chlb; Coprogen III = coproporphyrinogen III; Mg-Proto = Mg-protoporphyrin; PBG = porphobilinogen; Pchlide = protochlorophyllide; Proto IX = protoporphyrin IX; Urogen III = uroporphyrinogen III.

was increased in *hy* compared with WT, which the absorbed light energy that was not dissipated by heat. The photochemical quantum yield by PSII can be divided into two parts, quantum yield of light-induced nonphotochemical quenching $Y(NPQ)$ and quantum yield of nonregulated energy dissipation $Y(NO)$ (Kramer et al., 2004). Although ΦII was lower in *hy* than those in WT, $Y(NPQ)$ was much higher than WT (Table 2).

ΦII and qP represent the proportion of absorbed energy being used and the proportion of PSII reaction centers that are open, respectively (Genty et al., 1989). These data suggest the photochemical efficiency decrease of PSII resulted in retarded photosynthetic electron transport in *hy*. $Y(NPQ)$ and $Y(NO)$ were the most important index of PSII energy dissipation and NPQ mechanism also protects against high irradiation (Ögren, 1991). The observed levels suggest that the intrinsic photochemical activity of PSII is impaired in *hy*, and the absorbed light energy is not efficiently transferred from the LHC antenna to PSII reaction centers and dissipated as heat or fluorescence.

EXPRESSION ANALYSIS OF GENES INVOLVED IN CHL BIOSYNTHESIS IN *hy* MUTANTS. To identify the mutation accurately, we performed RT-qPCR analysis of some genes associated with Chl biosynthesis in the WT and *hy* mutant (Fig. 3). The expression of glutamyl-tRNA reductase (*HEMA*), porphobilinogen synthase (*HEMB*), hydroxymethylbilane synthase (*HEMC*), and uroporphyrinogen decarboxylase (*HEME*) were slightly downregulated (80% to 92%) in *hy*, suggesting a reduction of glutamyl-tRNA (Glu-tRNA) to Coprogen III conversion in the mutant. The synthesis gene coproporphyrinogen oxidative decarboxylase (*HEMF*), which is important for conversion of Coprogen III to Proto IX, was slightly higher in *hy* (106.4%) than in WT. The expression of Mg chelatase I subunit (*CHLI*) and Mg-protoporphyrin IX methyltransferase (*CHLM*), involved in the conversion of Proto IX to Pchlide, were slightly downregulated (86.6% and 87.6%) in *hy*, which also may promote the accumulation of Proto IX. Protochlorophyllide oxidoreductase (*PORA*) is the gene in the synthesis of Pchlide to Chl *a* and was more highly expressed (108.1%) in *hy* compared with WT. Nevertheless, these slight changes of expression may not be the reason that the phenotype changed in *hy*. CAO is the critical enzyme for conversion of Chl *a* to Chl *b* and thus affects the Chl *a:b* ratio (Pattanayak et al., 2005). CAO expression was found to be lower (31%) in the *hy* mutant.

The results from the expression analysis of Chl biosynthesis-related genes support the observed differences in the levels of Chl precursors between *hy* mutants and WT. On the basis of these results, we considered CAO to be a putative inhibited gene, the expression of which was disrupted in the *hy* mutant, thereby influencing synthesis of Chl *b*. However, whether the inhibited expression of CAO was due to function loss or the

downstream Chl metabolism-related genes that were acted on remained unknown.

IDENTIFY THE CANDIDATE GENE AND FUNCTIONAL PREDICTION OF THE MUTATED PROTEIN. To understand the CAO change, three pairs of primers (CAO1, CAO2, and CAO3) were used to amplify the CAO full cDNA fragments in mutant *hy* and WT. The sequencing results showed that there was one mutation in the CAO2 fragment located at the 1099 bp of cDNA causing T to C change (Supplemental Fig. 1) that corresponds to change from a proline (Pro) to serine (Ser) amino acid in the 367th codon (Supplemental Fig. 2).

Next, the amino acid sequence of CAO in *hy* and WT were aligned and compared with those of *AtCAO* (Fig. 4). These sequences of CAO in *hy* and WT were highly homologous with the *AtCAO*, the identity was 96.89%. A sequence of 36 amino acid residues was predicted as a transit peptide using TargetP. The three successive conserved sequences, classified by research of Nagata et al. (2004), were as follows: A-domain, from 37th to 170th amino acid, 134 amino acid residues; B-domain, from 171th to 198th amino acid, 28 amino acid residues; C-domain, from 199th to 534th amino acid, 336 amino acid residues. The mutation site was in the C-domain.

We predicted the conserved domains using the National Center for Biotechnology Information Conserved Domain Search and found a conserved Rieske domain from amino acid residues 202 to 330 in the C-domain. This domain contains an iron-sulfur reaction center structure, the alpha subunits contain an electron transfer complex in the N-terminal, whereas the C-terminal plays a catalytic role. There is a predicted START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC (SRPBCC) ligand-binding domain at amino acid residues 406 to 499. The SRPBCC domain has a hydrophobic ligand-binding capsule for binding various ligands. The mutated amino acid is located between these two domains.

The secondary structure of the CAO protein consists of four motifs, including an alpha helix, extended strand, beta turn, and random coil (Supplemental Fig. 3). The number of random coils in the protein structure was predicted to be reduced from 40.45% to 40.26% in the *hy* mutant, and the number of amino acid residues was reduced from 216 to 215. The alpha helix was predicted to increase from 30.52% to 30.90%, and the number of amino acid residues was increased from 163 to 165. The proportion of extended strand was unchanged (19.85%), and the number of amino acid residues was 106. The proportion of beta turn was the smallest of the four motifs and in the mutant was predicted to be reduced from 9.18% to 8.99% with the number of amino acid residues decreasing from 49 to 48.

Table 2. Chlorophyll fluorescence parameters of wild type [WT (green leaf)] and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage.

Phenotype	F_v/F_m^z	ΦII	$Y(NPQ)$	$Y(NO)$	NPQ	qP
	(mean $\pm$ SE)					
WT	0.747 $\pm$ 0.001	0.661 $\pm$ 0.005	0.004 $\pm$ 0.002	0.346 $\pm$ 0.016	0.000 $\pm$ 0.000	0.915 $\pm$ 0.015
<i>hy</i>	0.731 $\pm$ 0.005**	0.542 $\pm$ 0.047*	0.093 $\pm$ 0.009**	0.365 $\pm$ 0.045	0.289 $\pm$ 0.022**	0.855 $\pm$ 0.011**

^z F_v/F_m = maximum quantum yield of PSII; ΦII = the effective quantum yields of PSII; qP = photochemical quenching; NPQ = nonphotochemical quenching; $Y(NPQ)$ = quantum yield of regulatory energy dissipation; $Y(NO)$ = nonquantum yield of regulatory energy dissipation.

Differences significant between the means ($n = 3$) at * $P < 0.05$ and at ** $P < 0.01$ via *t* test.

Fig. 3. Expression of chlorophyll synthesis genes of wild type [WT (green leaf)] and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage [** above the vertical bar indicates the differences extremely significant between the means ($n = 3$) at $P < 0.01$ via t test]. *HEMA* = glutamyl-tRNA reductase; *HEMB* = porphobilinogen synthase; *HEMC* = hydroxymethylbilane synthase; *HEME* = uroporphyrinogen decarboxylase; *HEMF* = coproporphyrinogen oxidative decarboxylase; *CHLI* = Mg chelatase I subunit; *CHLM* = Mg-protoporphyrin IX methyltransferase; *PORA* = Protochlorophyllide oxidoreductase; *CAO* = Chl *a* oxygenase.

Fig. 4. Alignment of the amino acid sequence of Chl *a* oxygenase (*CAO*) with three domains of wild type [WT (green leaf)] and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage and Arabidopsis. The sequences in blue box represent three domains (A-, B-, and C-domain). The sequence before the A-domain is predicted transit peptides. The sequences in the red box represent conserved domains (Rieseke domain, SRPCC ligand-binding domain). The location in the green circle represents the mutational amino acid between WT and *hy*. The protein sequence of AtCAO and BrCAO has the 96.89% identity.

Discussion

THE METHOD OF SCREENING FOR LEAF COLOR MUTANTS. In our study, we found that the contents of Chl and the Chl precursors

ALA, PBG, Urogen III, Coprogen III, Mg-ProtoIX, and Pchlide were slightly lower in *hy* than WT, whereas Proto IX was slightly higher in *hy* than WT. Consistent with these findings, the expression of *HEMF*, the gene responsible for Coprogen III to Proto IX conversion, was increased, and the expression of other genes in the Chl biosynthesis pathway were slightly reduced in *hy*. We speculated that the increased expression of *HEMF* and the slightly decreased expression of *CHLM* and *CHLI* led to increased levels of Proto IX. These results suggest that the levels of Chl precursors are affected by expression differences in these key genes in the synthesis process. However, the expression of *PORA* that is required for synthesizing Chl *a* was only marginally increased in *hy* (108.1%), although the content of Chl *a* in *hy* was 67.4% that of WT. The expression of *CAO* was significantly lower at 31.1% of WT, and Chl *b* was also significantly lower than the WT at 19.5%. Chl *b* is produced from Chl *a* by the chlorophyll oxygenase encoded by the *CAO* gene (Espinosa et al., 1999; Tanaka et al., 1998). On the basis of these results, we hypothesized that *CAO* function was impaired in *hy* mutant. Sequencing of the gene confirmed a T to C mutation corresponding to a change from Pro to Ser amino acid at codon 367 in *hy*. The mutation agrees with typical EMS mutagenesis patterns (McCallum et al., 2000).

At the same time, we speculated that the decrease of Chl *a* was not caused by Chl *a* synthesis but by the effects of altered *CAO* gene function on the chlorophyll cycle. LHC apolipoprotein can combine with chlorophyllide *a* under the action of *CAO* to synthesize chlorophyllide *b* in the thylakoid membrane, and fold after assembly to synthesize Chl *a* and Chl *b*, but *CAO* participating in the transportation of LHC apolipoprotein has not been a subject of controversy (Hooper and Eggink, 1999; Tanaka and Tanaka, 2007). In this study, we

found that the *CAO* gene expressed was impaired and the content of Chl *a* was reduced, suggesting that *CAO* may influence transport during Chl *a* and Chl *b* synthesis. Our results also suggested the decrease in photochemical efficiency

of PSII resulted in retarded photosynthetic electron transport in *hy*. These results suggest that the intrinsic photochemical activity of PSII is impaired in *hy*, and the absorbed light energy is not efficiently transferred from the LHC antenna to PSII reaction centers and dissipated as heat or fluorescence.

ΦII and qP are measures of the light energy absorbed by PSII that can be used in photosynthetic electron transport for excitation capability and transfer ability of the reaction center of PSII (Genty et al., 1989). Biswal et al. (2012) suggested that overexpression of *CAO* in plants leads to an increase in light capture and enhanced (40% to 80%) electron transport rates of PSI and PSII. Consistent with previous research, our data suggest that the photochemical efficiency decrease of PSII resulted in retarded photosynthetic electron transport in *hy*. *Y* (NPQ) and *Y*(NO) were the most important indices of PSII activity to protect the photosynthetic mechanism. NPQ is a self-protection mechanism (Ögren, 1991).

These results demonstrate that quantification of chlorophyll and its precursors, combined with the expression of key biosynthetic genes, can effectively reduce the putative genes and be used as a screening method for leaf color mutants.

SUSPECT PREDICTION OF CAO MUTANT PROTEIN FUNCTION IN *hy*. In addition to regulation on the transcriptional level, Chl *b* synthesis is also regulated by protein stability (Nakagawara et al., 2007). The *CAO* gene is divided into three domains (ABC) in arabidopsis: the A-domain (134 amino acid residues) and B-domain (30 amino acid) were located at the C-terminal, and the C-domain (337 to 344 amino acid residues) was located at the N-terminal (Nagata et al., 2004). Compared with AtCAO, the sequence of BrCAO protein had a high identity [96.89% (Fig. 4)], so the classification rule of domains was consistent with arabidopsis in *hy* and WT.

A-domain controls Chl biosynthesis, especially the process of de-etiolation in seedlings (Yamasato et al., 2008). Currently, the function of the B-domain has not been well defined but it has been speculated to be used as the connection of the A- and C-domains (Sakuraba et al., 2007). The C-domain plays a catalytic role as a unique structure of CAO; when the accumulation of the C-domain by removed A- and B- domains, Chl *a/b* is decreased from 3.6 to 2.2 (Nagata et al., 2004; Yamasato and Tanaka, 2005). In our study, the Chl *a* level in the mutant *hy* was 67.4% lower than that of WT. Chl *b* levels in the mutant *hy* were significantly lower at 19.5% that of WT. By predicting the conserved domains, we found that there were two conserved domains (Rieske domain, SRPBCC ligand-binding domain) related to catalytic function in the C-domain. The mutation site was in the C-domain, not in one of the conserved sequences, but the mutation amino acid was conserved residue in the C-domain (Nagata et al., 2004). Analysis of the secondary structure models of the mutated protein revealed multiple helical and folding structure effects (Supplemental Fig. 3). To eliminate the transcription effects of the *BrCAO* promoter, the 1500-bp upstream sequence of *BrCAO* was cloned; no difference between *hy* and WT was seen (Supplemental Fig. 4). Therefore, we speculated that the mutation amino acid change affected the catalytic function in the C-domain. The functional deficiency of *CAO* expression may be due to the change in amino acid in the domain that influences the structure of the C-domain, but further work is needed to verify this prediction.

Conclusions

Yellow-green leaf EMS-mutated *hy* was controlled by a recessive allele at a single locus. In mutant *hy*, pchl_{ide}, Chl *a*, and Chl *b* were significantly decreased, and the intrinsic photochemical activity of PSII was impaired. This resulted in retarded photosynthetic electron transport, suggesting that absorbed light energy is not efficiently transferred from the LHC antenna to the PSII reaction centers and dissipated as heat or fluorescence. Analysis of the expression of key genes in the Chl synthetic pathway indicated that *CAO* expression was inhibited in *hy*. The *CAO* gene was cloned, showing that a C to T mutation at 1099 bp into cDNA causes a conserved Pro to Ser mutation in the C-domain of CAO protein. We speculate that the mutation amino acid changed in the C-domain may affect catalytic function.

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WT-1	ATGAACGCCGCGTGTACTTCTCTGCTTTATCTCTACCCATATCTCTGTAAGACTAGATCATCTCAACTACCAGAAAGAGGAGTGAAGGAGAGTTACGGGTTTTTCTGTGTTTGGGGAAGATAGTGGATTAGTGAGAA	150
WT-2	ATGAACGCCGCGTGTACTTCTCTGCTTTATCTCTACCCATATCTCTGTAAGACTAGATCATCTCAACTACCAGAAAGAGGAGTGAAGGAGAGTTACGGGTTTTTCTGTGTTTGGGGAAGATAGTGGATTAGTGAGAA	150
WT-3	ATGAACGCCGCGTGTACTTCTCTGCTTTATCTCTACCCATATCTCTGTAAGACTAGATCATCTCAACTACCAGAAAGAGGAGTGAAGGAGAGTTACGGGTTTTTCTGTGTTTGGGGAAGATAGTGGATTAGTGAGAA	150
hy-1	ATGAACGCCGCGTGTACTTCTCTGCTTTATCTCTACCCATATCTCTGTAAGACTAGATCATCTCAACTACCAGAAAGAGGAGTGAAGGAGAGTTACGGGTTTTTCTGTGTTTGGGGAAGATAGTGGATTAGTGAGAA	150
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WT-1	GTGGAACACGGGTTGCTACTAGATAATCTCTGGATCTTGGCATGCTGCTCACTCACTCACACATCTACTTTTGCAAAAGGCTTGAAGTGTCCCAAGCTGGTAAAGTTCTTAAACACATCTTCAGGTCTTCAGGATACTGGGATCCGAT	1200
WT-2	GTGGAACACGGGTTGCTACTAGATAATCTCTGGATCTTGGCATGCTGCTCACTCACTCACACATCTACTTTTGCAAAAGGCTTGAAGTGTCCCAAGCTGGTAAAGTTCTTAAACACATCTTCAGGTCTTCAGGATACTGGGATCCGAT	1200
WT-3	GTGGAACACGGGTTGCTACTAGATAATCTCTGGATCTTGGCATGCTGCTCACTCACTCACACATCTACTTTTGCAAAAGGCTTGAAGTGTCCCAAGCTGGTAAAGTTCTTAAACACATCTTCAGGTCTTCAGGATACTGGGATCCGAT	1200
hy-1	GTGGAACACGGGTTGCTACTAGATAATCTCTGGATCTTGGCATGCTGCTCACTCACTCACACATCTACTTTTGCAAAAGGCTTGAAGTGTCCCAAGCTGGTAAAGTTCTTAAACACATCTTCAGGTCTTCAGGATACTGGGATCCGAT	1200
hy-2	GTGGAACACGGGTTGCTACTAGATAATCTCTGGATCTTGGCATGCTGCTCACTCACTCACACATCTACTTTTGCAAAAGGCTTGAAGTGTCCCAAGCTGGTAAAGTTCTTAAACACATCTTCAGGTCTTCAGGATACTGGGATCCGAT	1200
hy-3	GTGGAACACGGGTTGCTACTAGATAATCTCTGGATCTTGGCATGCTGCTCACTCACTCACACATCTACTTTTGCAAAAGGCTTGAAGTGTCCCAAGCTGGTAAAGTTCTTAAACACATCTTCAGGTCTTCAGGATACTGGGATCCGAT	1200
Consensgtggaacacaggtgtgctactagataatctctggatcttgcgcatgctcattcaactcacacatctacttttgcaaaaggctggaggtgtcccaagcctggttaagttcttaacacatcttcagggtcttcaggatactgggataccggtat		
WT-1	CCAATCGATATGGAGTTTAAACCACTTTGATCGTGTATCGACATAGGAATCTCAAACCTGGGAAGCTAGAAGGAAGAGCACTGAGCAGTGTGCAACACATCTTCATCAACTCCATGTTTGTATACCTTCTCAAGAAACAGGACA	1350
WT-2	CCAATCGATATGGAGTTTAAACCACTTTGATCGTGTATCGACATAGGAATCTCAAACCTGGGAAGCTAGAAGGAAGAGCACTGAGCAGTGTGCAACACATCTTCATCAACTCCATGTTTGTATACCTTCTCAAGAAACAGGACA	1350
WT-3	CCAATCGATATGGAGTTTAAACCACTTTGATCGTGTATCGACATAGGAATCTCAAACCTGGGAAGCTAGAAGGAAGAGCACTGAGCAGTGTGCAACACATCTTCATCAACTCCATGTTTGTATACCTTCTCAAGAAACAGGACA	1350
hy-1	CCAATCGATATGGAGTTTAAACCACTTTGATCGTGTATCGACATAGGAATCTCAAACCTGGGAAGCTAGAAGGAAGAGCACTGAGCAGTGTGCAACACATCTTCATCAACTCCATGTTTGTATACCTTCTCAAGAAACAGGACA	1350
hy-2	CCAATCGATATGGAGTTTAAACCACTTTGATCGTGTATCGACATAGGAATCTCAAACCTGGGAAGCTAGAAGGAAGAGCACTGAGCAGTGTGCAACACATCTTCATCAACTCCATGTTTGTATACCTTCTCAAGAAACAGGACA	1350
hy-3	CCAATCGATATGGAGTTTAAACCACTTTGATCGTGTATCGACATAGGAATCTCAAACCTGGGAAGCTAGAAGGAAGAGCACTGAGCAGTGTGCAACACATCTTCATCAACTCCATGTTTGTATACCTTCTCAAGAAACAGGACA	1350
Consensccaatcgatagaggtttaaacacactctgtatcgtgttatcgacaataggaatctcaaacactgggaagctagaaggaagagcactgagcaggtgtgcaacacatcttcacactccatggtttgttactctctcaagaacaagaca		
WT-1	AGACTTCTATACCGAATGCTACTAGACTTTGCTCCAAATATTGAAGAATCTCCATTCTATGGAACATCTCTGGAGACATTTTCGCTGAGCAGGCTTAAATGAAGATCTACGGTTGGTTTTGGGACAGCAAGAAGGATGTTGAATGGTGCA	1500
WT-2	AGACTTCTATACCGAATGCTACTAGACTTTGCTCCAAATATTGAAGAATCTCCATTCTATGGAACATCTCTGGAGACATTTTCGCTGAGCAGGCTTAAATGAAGATCTACGGTTGGTTTTGGGACAGCAAGAAGGATGTTGAATGGTGCA	1500
WT-3	AGACTTCTATACCGAATGCTACTAGACTTTGCTCCAAATATTGAAGAATCTCCATTCTATGGAACATCTCTGGAGACATTTTCGCTGAGCAGGCTTAAATGAAGATCTACGGTTGGTTTTGGGACAGCAAGAAGGATGTTGAATGGTGCA	1500
hy-1	AGACTTCTATACCGAATGCTACTAGACTTTGCTCCAAATATTGAAGAATCTCCATTCTATGGAACATCTCTGGAGACATTTTCGCTGAGCAGGCTTAAATGAAGATCTACGGTTGGTTTTGGGACAGCAAGAAGGATGTTGAATGGTGCA	1500
hy-2	AGACTTCTATACCGAATGCTACTAGACTTTGCTCCAAATATTGAAGAATCTCCATTCTATGGAACATCTCTGGAGACATTTTCGCTGAGCAGGCTTAAATGAAGATCTACGGTTGGTTTTGGGACAGCAAGAAGGATGTTGAATGGTGCA	1500
hy-3	AGACTTCTATACCGAATGCTACTAGACTTTGCTCCAAATATTGAAGAATCTCCATTCTATGGAACATCTCTGGAGACATTTTCGCTGAGCAGGCTTAAATGAAGATCTACGGTTGGTTTTGGGACAGCAAGAAGGATGTTGAATGGTGCA	1500
Consensagacttctataccgaatgctactagactttgctccaaattatgaagaatctccattctggaacatctctggagacatttcgctgagcaggtcttaaatgaagatctacggttgggtttgggacagcagaagaagatgttgaatggtgca		
WT-1	AATATATGGAACCTTACCTGTTGCTTATGACAAGCTTGGAGTTCGGTATAGACTATGAGGAAGACCTGTAGATCGTGGTGATATAAATTAACCTTCTCAGGCTA	1604
WT-2	AATATATGGAACCTTACCTGTTGCTTATGACAAGCTTGGAGTTCGGTATAGACTATGAGGAAGACCTGTAGATCGTGGTGATATAAATTAACCTTCTCAGGCTA	1604
WT-3	AATATATGGAACCTTACCTGTTGCTTATGACAAGCTTGGAGTTCGGTATAGACTATGAGGAAGACCTGTAGATCGTGGTGATATAAATTAACCTTCTCAGGCTA	1604
hy-1	AATATATGGAACCTTACCTGTTGCTTATGACAAGCTTGGAGTTCGGTATAGACTATGAGGAAGACCTGTAGATCGTGGTGATATAAATTAACCTTCTCAGGCTA	1604
hy-2	AATATATGGAACCTTACCTGTTGCTTATGACAAGCTTGGAGTTCGGTATAGACTATGAGGAAGACCTGTAGATCGTGGTGATATAAATTAACCTTCTCAGGCTA	1604
hy-3	AATATATGGAACCTTACCTGTTGCTTATGACAAGCTTGGAGTTCGGTATAGACTATGAGGAAGACCTGTAGATCGTGGTGATATAAATTAACCTTCTCAGGCTA	1604
Consensaatataggaacttaccgttctcttgaacagcttggagtcggttactagactacagcttcggtgatgtggtgatgataaattacattctcaggcta		

Supplemental Fig. 1. The full cDNA sequence alignment of *Chl a oxygenase* (*CAO*) of wild type (WT green leaf) and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage.

WT MNAAVFTSSALSIPISFCRTRSSQLTRKKGVKGEFRVFAVFGEDSGLVEKKSQWGHLEDFVEDFRSSTPPYKGFMDVNQALEVARFDIQYLDWRARQDILL 100
hy MNAAVFTSSALSIPISFCRTRSSQLTRKKGVKGEFRVFAVFGEDSGLVEKKSQWGHLEDFVEDFRSSTPPYKGFMDVNQALEVARFDIQYLDWRARQDILL 100
ConseMnaavftssalslpsifcctrssqltrkkgvkgefrvfavfgedsglvekksqwghlfdvedprsktppykkgfmdvnqalevarfdiqyldwrarqdill

WT TIMLLHDKVVDVNLPLAREYKSIGTVKKELAGLQEELAKAHQQVHISEARVSTALEKLAHMEELVNDRLLPDRVATESDRPSYSTSVQDLDREKTNIGGK 200
hy TIMLLHDKVVDVNLPLAREYKSIGTVKKELAGLQEELAKAHQQVHISEARVSTALEKLAHMEELVNDRLLPDRVATESDRPSYSTSVQDLDREKTNIGGK 200
Consetimllhdkvvdvnlplareyksigtvkkelaglqeelakahqqvhisearvstalekalahmeelvndrllpdrvatesdrpsystsvqddldreknigggk

WT SLNVSGFPQYSPHKLNFYVAFATDLKHD TMVPIDCFEQFWVIFRGEDGKPGCVRNTCAHRACPLDLGSVNNGRIQCPYHGWEYSTDGKCTKMFSTKL 300
hy SLNVSGFPQYSPHKLNFYVAFATDLKHD TMVPIDCFEQFWVIFRGEDGKPGCVRNTCAHRACPLDLGSVNNGRIQCPYHGWEYSTDGKCTKMFSTKL 300
Conseslnvsgfpqysphklnfypvafatdlkhdtmvpidcfeqfwvifrgedgkpgcvrntcahracpldlgsvnnngriqcpypyhgweystdgkctkmpstkl

WT LNVKIKSIPCLQDGMVWVWPGDEPPSPTLPCLQPPSGFVIAELVMDLPVEHGLLLDNLDLAHSFTHTSTFAKGWSVPSLVKFLTSSSGLQGYWDPY 400
hy LNVKIKSIPCLQDGMVWVWPGDEPPSPTLPCLQPPSGFVIAELVMDLPVEHGLLLDNLDLAHSFTHTSTFAKGWSVPSLVKFLTSSSGLQGYWDPY 400
Conselnvkiksipclqdgmvwvwpdgdeppsptlpclqppsgfvihaelvmdlpvehgllldnldlaha fthtstfakgswpslvkfltsssglqgywdpy

WT PIDMEFKPPCIVLSTIGISKPGKLEGKSTEQCATHLHQLHVCLPSSRNKTRLLYRMSLDFAPILKNLPFMEHLWRHFAEQVLNEDRLRLVLGQGERMLNGA 500
hy PIDMEFKPPCIVLSTIGISKPGKLEGKSTEQCATHLHQLHVCLPSSRNKTRLLYRMSLDFAPILKNLPFMEHLWRHFAEQVLNEDRLRLVLGQGERMLNGA 500
Consepidmekppcivlstigiskpgklegksteqcathlhqlhvcplssrnktrlllyrmsldfapilknlpfmehlwrhfaeqvlnedrlrlvlgqgermlnga

WT NIWNLFVAYDKLGVRVRLWRNAVDRGDDKLFFS 533
hy NIWNLFVAYDKLGVRVRLWRNAVDRGDDKLFFS 533
Conseniwnlpvaydklgvrvrlwrnavdrgddklffs

Supplemental Fig. 2. The amino acid sequence of *Chl a* oxygenase (CAO) of wild type (WT green leaf) and *hy* (yellow-green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage.

Supplemental Fig. 3. The prediction of *Chl a* oxygenase (CAO) secondary structure of wild type (WT green leaf) and *hy* (yellow green leaf mutant induced by ethyl methanesulfonate) in chinese cabbage. The protein of CAO was composed of alpha helix (blue), extended strand (red), beta turn (green), and random coil (yellow). The number and the proportion of components are presented in right of each picture.

Supplemental Table 1. Primers of genes in chlorophyll biosynthesis pathway used for quantitative real-time polymerase chain reaction (RT-qPCR) and amplification.

Gene	Gene annotated	Gene ID	Primer sequence(5'-3')	Synthetic pathway	Purpose
HEMA	Glutanyl-tRNA reductase	Bra035447	F:ATTGGTGTTTCGTGCTCTT R:ATGTTTGCGGCATTAGGT	Glu-tRNA → ALA	RT-qPCR
HEMB	Porphobilinogen synthase	Bra036945	F:AGCCTGAACGCAGCATCGTAT R:GGCTCGGCTGGTTTACTCTTT	ALA → PBG	RT-qPCR
HEMC	Hydroxymethylbilane synthase	Bra005980	F:TGTCGCATCGGTCTTG R:TGTCTCCTCGTGGTTCA	PBG → Urogen III	RT-qPCR
HEME	Uroporphyrinogen decarboxylase	Bra016989	F:GACTGGCTAGGACTGGTG R:CCTTGAACCGTTATGTCTC	Urogen III → Coprogen III	RT-qPCR
HEMF	Coproporphyrinogen oxidative decarboxylase	Bra033402	F:CGTTCCTTGCTGCGGGAGTTA R:GGAACATCCTTTGGAGCATCAGT	Coprogen III → Proto IX	RT-qPCR
CHLI	Mg chelatase I subunit	Bra013314	F:TGTTCAGATTGATAGGGAGT R:TCGCTGCTCTGTTAGTCA	Proto IX → Mg-Proto IX	RT-qPCR
CHLM	Mg-protoporphyrinIX methyltransferase	Bra019192	F:AAAGCAGCAGCTAGGAGG R:TTTGTCTGAGGGTAATGT	Mg-Proto IX → Pchlde	RT-qPCR
PORA	Protochlorophyllide oxidoreductase	Bra003004	F:CAAGACCTCGGCTTCATT R:CTCGCTAACTTCCCAAAC	Pchlde → Chla	RT-qPCR
CAO	Chlorophyllide a oxygenase	Bra036948	F:TCCAGGACTTAGACAGGG R:AAAGCAACGGGATACCAA	Chla → Chlb	RT-qPCR
CAO1	Chlorophyllide a oxygenase	Bra036948	F:ATGAACGCCGCCGTGTTTACTT R:TTCCCTGTCTAAGTCCTG		PCR
CAO2	Chlorophyllide a oxygenase	Bra036948	F:TTACCAGATAGAGTTGCGACAG R:TGGGACACTCCAGCCTTT		PCR
CAO3	Chlorophyllide a oxygenase	Bra036948	F:GAACACGGGTTGCTACTA R:TTAGCCTGAGAAAGGTAAT		PCR
ACTIN	Reference gene	Bra028615	F:GCTTCCCTCAGCACTT R:GACCCGACTCATCGTAC		RT-qPCR
PCAO	Upstream of CAO		F:ATCATAAGGAAATGGTCT R:GCTGTCGTACCGGAAGGA		PCR

ALA = aminolevulinic acid; Chla = chlorophyll *a*; Chlb = chlorophyll *b*; Coprogen III = coproporphyrinogen III; F = forward; Glu-tRNA = glutamyl-tRNA; Mg-Proto = Mg-protoporphyrin; PBG = porphobilinogen; Pchlde = protochlorophyllide; Proto IX = protoporphyrin IX; R = reverse; Urogen III = uroporphyrinogen III.