Characterization of the *Arabidopsis thaliana* Mutant *pcb2* which Accumulates Divinyl Chlorophylls

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We characterized the *pcb2* (pale-green and chlorophyll b reduced 2) mutant. We found through electron microscopic observation that chloroplasts of *pcb2* mesophyll cells lacked distinctive grana stacks. High-performance liquid chromatography (HPLC) analysis showed that the *pcb2* mutant accumulated divinyl chlorophylls, and the relative amount of divinyl chlorophyll b was remarkably less than that of divinyl chlorophyll a. The responsible gene was mapped in an area of 190 kb length at the upper arm of the 5th chromosome, and comparison of DNA sequences revealed a single nucleotide substitution causing a nonsense mutation in At5g18660. Complementation analysis confirmed that the wild-type of this gene suppressed the phenotypes of the mutation. Antisense transformants of the gene also accumulated divinyl chlorophylls. The genes homologous to At5g18660 are conserved in a broad range of species in the plant kingdom, and have similarity to reductases. Our results suggest that the *PCB2* product is divinyl protochlorophyllide 8-vinyl reductase.

**Keywords**: *Arabidopsis thaliana* — Chloroplast — Complementation — Divinyl protochlorophyllide 8-vinyl reductase — Grana stack — *pcb2* mutant.

Abbreviations: CaMV, cauliflower mosaic virus; CAO, chlorophyllide a oxygenase; Col, Columbia; dCAPS, derived cleaved amplified polymorphic sequence; EMS, ethyl methanesulfonate; ESI, electrospray ionization; EST, expressed sequence tag; LC, liquid chromatography; Ler, Landsberg erecta; MS, mass spectrometry; ODS, octadecasyl; RT–PCR, reverse transcription–polymerase chain reaction; SSLP, simple sequence length polymorphism.

**Introduction**

Chlorophyll is the main component of the photosynthetic pigments and it has the function of absorbing light energy and transferring that energy to the reaction centers to produce chemical energy. In all land plants, green algae and prochlorophytes, the photosynthetic reaction centers contain only chlorophyll *a*, and the peripheral light-harvesting antenna complexes contain chlorophyll *a* and chlorophyll *b* (Grossman et al. 1995). There are some other kinds of chlorophyll found in various photosynthetic organisms, such as bacteriochlorophylls in photosynthetic bacteria. *Prochlorococcus*, a group of marine cyanobacteria, possesses divinyl chlorophylls in place of chlorophylls (Chisholm et al. 1992). All chlorophylls are believed to be synthesized from 5-aminolevulinic acid through similar biosynthetic pathways (Willows 2003). However, these pathways are not understood completely.

Chlorophyll *a* is synthesized in 15 steps from L-glutamate in higher plants (Lange and Ghassemian 2003). Two glutamates form a porphobilinogen after the conversion to 5-aminolevulinic acids, and then four porphobilinogens make up a tetrapyrrol ring that leads to protoporphyrin IX after some modifications. Insertion of a Mg ion into protoporphyrin IX by Mg-chelatase is the specific step to chlorophyll formation at the branch point of chlorophyll and heme biosynthesis (Beale 1999). Then, Mg-protoporphyrin IX becomes chlorophyll *a* after five steps of enzymatic reactions. Chlorophyll *b* is derived from chlorophyll *a* by conversion of a methyl group to a formyl group catalyzed by chlorophyllide *a* oxygenase (CAO) (Tanaka et al. 1998). It has also been suggested that precursor molecules of chlorophyll *a* are involved in ‘plastid signal’, which is a communication system assumed to exist between the chloroplast and the nucleus (Mochizuki et al. 2001).

In a previous study, we collected the color mutants of *Arabidopsis thaliana* in order to analyze the biogenesis of the chloroplast (Nakanishi et al. 2004). It is highly likely that color mutants have defects in chlorophyll biosynthesis steps or in the development of the chloroplast, where chlorophyll is accumulated. The *pcb2* (pale-green and chlorophyll b reduced 2) mutant line isolated from the ethyl methanesulfonate (EMS) mutant library has a reduced amount of chlorophylls resulting in pale-green leaves, and the reduction of chlorophyll *b* content is especially remarkable. Allelism tests and mapping showed that *pcb2* is not an allele of any known similar mutants such as *ch1-1* (Oster et al. 2000), *ch42* or *cch* (Mochizuki et al. 2001). In this study, we describe the novel mutant *pcb2*.  

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Results

Phenotype of the pcb2 mutant

The pcb2 mutant has a pale-green phenotype (Fig. 1). In the F2 populations of a backcross with wild-type Columbia (Col), normal and mutant plants segregated in a ratio very close to 3:1 (85:32; $\chi^2 = 0.34; P = 0.56$). This result indicates that the pcb2 mutation was inherited in a single recessive manner. The growth rate of the pcb2 mutant was severely reduced compared with the wild-type.

The thylakoid membranes of the pcb2 chloroplasts were arranged in a disorderly fashion and did not develop distinct grana stacks; extensive adhesion of two to a few thylakoid membranes was observed instead (Fig. 2). There were no distinct differences in the size and the number of chloroplasts between the wild-type and the mutant. Starch granules were not found in the mutant chloroplasts, suggesting the reduction of photosynthetic activity in the mutant.

Analysis of chlorophylls

The chlorophylls in leaf extracts were separated chromatographically and the peaks that were assumed to be chlorophylls a and b from their retention time and absorption spectrum were compared. The comparative amounts of chlorophylls between wild-type and pcb2 were calculated from the peak areas at 660 nm. The amounts of chlorophylls a and b extracted from the pcb2 plants were approximately one-half and one-quarter of that from the wild-type, respectively (Fig. 3A, B).

The peaks corresponding to chlorophylls a and b of the pcb2 mutant were eluted a little earlier than those of the wild type (Fig. 3C). Peaks 1' and 2' in Fig. 3B are recovered, and their spectra in diethyl ether are compared with those of the chlorophyll a and b standards. The result showed that the peaks shifted from 430 to 435 nm (chlorophyll vs. peak 2'), from 455 to 462 nm (chlorophyll b vs. peak 1'), respectively. The profiles of these spectra are consistent with those of divinyl chlorophylls (Shedbalkar and Rebeiz 1992).

The mass spectra obtained for chlorophyll a and b standards showed major ions at m/z 912 [M + NH$_4$] and m/z 926 [M + NH$_4$], whereas the corresponding peaks (peaks 1' and 2', Fig. 3) from the pcb2 mutant showed major ions at m/z 910 [M + NH$_4$] and m/z 924 [M + NH$_4$], respectively. The mass of 2 Da shifted from the standards is equivalent to the mass changing in the reduction of a –CH=CH– double bond to a –CH$_2$–CH$_2$– single bond.

Candidate for the pcb2 gene

In a previous study, we mapped pcb2 to a locus at the upper arm of chromosome 5 (Nakanishi et al. 2004). Further analysis using 442 F$_2$ seedlings revealed that PCB2 is located

![Fig. 1 Photograph of the whole plant. (A) Wild-type, (B) pcb2 mutant and (C) pcb2 mutant bearing the PCB2 genomic copy under the control of the 35S promoter in the sense direction. (D) Wild-type plant bearing the PCB2 genomic copy under the control of the 35S promoter in the antisense direction. All plants are 14 d old. Bar = 0.5 cm](https://academic.oup.com/pcp/article-abstract/46/3/467/1817359)

![Fig. 2 Electron microscoic analysis of the pcb2 mutant. Electron micrographs of chloroplasts in mesophyll cells from wild-type (A) and pcb2 (B). Portions of (A) and (B) are shown in (C) and (D), respectively, with higher magnification to reveal the detailed structure of thylakoid membranes. C, chloroplast; S, starch; V, vacuole; IS, intercellular space; M, mitochondria.](https://academic.oup.com/pcp/article-abstract/46/3/467/1817359)
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between PAT1.2 [simple sequence length polymorphism (SSLP) marker, AGI position; 5,397 kb] and dPCB2 [derived cleaved amplified polymorphic sequence (dCAPS) marker, AGI position; 6,335 kb]. Three recombinations were observed in 884 chromosomes between PAT1.2 and the pcb2 locus, one in 884 chromosomes between the pcb2 locus and dPCB2. Calculating the recombination ratio, the presumed area was narrowed to a range of about 190 kb where 49 genes are predicted (Fig. 4A, B). According to TargetP (Emanuelsson et al. 2000) and the PSORT program (Nakai and Horton 1999), products of four genes are suggested to localize in the chloroplast. By means of DNA sequence analysis of these four genes, one of them, At5g18660, had a single nucleotide substitution at the 85th residue (cytosine to thymine) resulting in a stop codon in the pcb2 mutant, instead of glutamine in the wild-type (Fig. 4C).

Gene cloning and complementation analysis

In order to confirm that At5g18660 is the gene causing the pcb2 mutation, we carried out complementation and antisense analysis. A genomic copy of At5g18660 under the control of the cauliflower mosaic virus (CaMV) 35S promoter was introduced into the pcb2 mutant (sense) and wild-type (antisense) by the floral dipping method (Fig. 5A). The resultant transgenic plants bearing the 35S-At5g18660 sense gene had green leaves (Fig. 1C) and normal chlorophylls (Fig. 5B). On the other hand, the transgenic plants suppressed with antisense DNA turned out to show the same phenotype as the pcb2 mutant (Fig. 1D, 5B). The reverse transcription–polymerase chain reaction (RT–PCR) analysis of At5g18660 sense gene had green leaves (Fig. 1C) and normal chlorophylls (Fig. 5B). On the other hand, the transgenic plants suppressed with antisense DNA turned out to show the same phenotype as the pcb2 mutant (Fig. 1D, 5B). The reverse transcription–polymerase chain reaction (RT–PCR) analysis of At5g18660 mRNA in leaves showed that PCB2 was expressed in the wild-type and the pcb2 complemented plants, but not in the pcb2 mutant, nor in antisense suppressed plants (Fig. 5C, D). Thus, a mutation in At5g18660 seems to be responsible for the pcb2 phenotype.

Expression pattern and localization of PCB2

We examined the tissue-specific expression pattern of PCB2 in various organs by RT–PCR. PCB2 was expressed highly in leaves, stems and flower buds, and slightly in roots (Fig. 6A). To determine the subcellular localization of the PCB2 protein, the sGFP gene was fused to the 3′ end of the PCB2 gene, generating the PCB2-sGFP chimeric gene under the control of the CaMV 35S promoter (Fig. 6B). Confocal laser scanning microscopic observation revealed that the PCB2-sGFP protein localized in chloroplasts in mesophyll cells (Fig. 6C).
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Comparison with PCB2 homologs

The PCB2 gene encodes an uncharacterized protein with a transit peptide to chloroplast (Fig. 7). A BLAST search revealed that there are no other genes significantly similar to PCB2 in the genome of A. thaliana. Referring to the DDBJ database, expressed sequence tags (ESTs) that encode proteins highly homologous (>55% identities) to PCB2 were found in various species of dicots (Solanum tuberosum, Medicago truncatula, Lycopersicon esculentum, etc.), monocots (Oryza sativa, Triticum turgidum, etc.), a moss (Physcomitrella patens), a cyanobacterium (Synechococcus sp. WH8102) and a photosynthetic bacterium (Chlorobium tepidum TLS), but not in yeast and animals. None of these homologous proteins have been characterized yet. The search also yielded the noteworthy information that PCB2 has similarity to some reductases such as NADH dehydrogenase/oxidoreductase and isoflavone reductase (27 and 26% identities, respectively). Another feature of the PCB2 protein is the presence of a stretch of hydrophobic amino acid residues that constitute a putative transmembrane domain near its C-terminus (Fig. 7, asterisks).

Discussion

In this report, we demonstrated the replacement of chlorophylls $a$ and $b$ by other pigments in the pcb2 mutant (Fig. 3), together with a severe reduction of grana stacks in chloroplasts (Fig. 2). We found a single nucleotide substitution in At5g18660 in the pcb2 mutant (Fig. 4), which is probably responsible for the pcb2 phenotype. This hypothesis is strongly supported by transformation experiments using sense and antisense constructs of At5g18660 (Fig. 1, 5). At5g18660 encodes...
a chloroplast-localized protein that has low homology to a certain category of reductases, and that seems to be conserved in a broad range of species of photosynthetic organisms.

Chlorophyll a is synthesized in 15 steps from L-glutamate. The genes coding for the proteins which catalyze 14 of these steps have been cloned in Arabidopsis (Lange and Ghassemian 2003, Tottey et al. 2003). Divinyl protochlorophyllide 8-vinyl reductase, which catalyzes the conversion of divinyl protochlorophyllide a to monovinyl protoclorophyllide a (Parham and Rebeiz 1992, Parham and Rebeiz 1995), is the last one to be identified. Electrospray ionization (ESI) mass spectrometry analysis of the pcb2 mutant detected the chlorophyll-like substances with masses of 892 and 906 Da, corresponding to divinyl chlorophylls a and b respectively. The absorption spectra in diethyl ether and the elution time of HPLC also indicated that the pcb2 mutant accumulates divinyl chlorophylls instead of monovinyl (normal) chlorophylls (Fig. 3, Shedbalkar and Rebeiz 1992, Zapata et al. 2000). Considering this together with the similarity of the PCB2 protein to some reductases, divinyl protochlorophyllide 8-vinyl reductase is a potential candidate to be the PCB2 product. This is supported by the absence of a homologous gene in the completely sequenced genome of Prochlorococcus, a photosynthetic prokaryote using divinyl chlorophylls as photosynthetic pigments (Chisholm et al. 1992). Enzymatic study should be able to confirm this aspect.

We isolated the pcb2 mutant as a chlorophyll b reduced mutant at first. The result of HPLC analysis in this study showed that divinyl chlorophyll b was accumulated at a very low level compared with divinyl chlorophyll a. The answer to the question as to why the disruption of divinyl protochlorophyllide 8-vinyl reductase caused a reduction of divinyl chlorophyll b may lie in their chemical structure. In divinyl chlorophyll a, an ethyl group is replaced by a vinyl group at the C8 site of chlorophyll a. CAO catalyzes a reaction that converts a methyl group to a formyl group at the C7 site of chlorophyll a. It is thought that the replacement of an ethyl group with a vinyl group at the C8 site of divinyl chlorophyll a probably interferes with the CAO reaction. Prochlorococcus which accumulates the divinyl chlorophyll b carries no CAO homologous gene in the genome (CyanoBase; http://www.kazusa.or.jp/cyano/, Nakamura et al. 2000), suggesting that another enzyme(s) which carries out the reaction of divinyl chlorophyll a to divinyl chlorophyll b may exist in the bacteria. The amount of divinyl chlorophyll b is also reduced to almost half of that of monovinyl chlorophyll a in wild-type. This fact may support the idea that the divinyl chlorophyllide a is not exactly suited as a substrate to fill the enzymatic pathway for chlorophyll a synthesis in Arabidopsis. We cannot deny another possibility that the trouble in assembly or stability of divinyl chlorophylls in the thylakoid membrane are brought in the pcb2 mutant.

A nonsense mutation close to the N-terminus of PCB2 provided a null phenotype, the pcb2 mutant (Fig. 4). It is unlikely that other proteins compensate the lack of PCB2, because there are no homologs of PCB2 in the genome of A. thaliana and because the monovinyl chlorophylls were replaced by divinyl chlorophylls in the pcb2 mutant. Why could the pcb2 mutant survive? The answer may be related to the accumulation of divinyl chlorophylls. The pcb2 mutant may survive by using divinyl chlorophylls as photosynthetic pigments as Prochlorococcus does. Additionally, it was reported that a Zea mays mutant accumulating only divinyl chlorophyll instead of monovinyl chlorophyll was also capable of photosynthetic growth with divinyl chlorophylls (Bazzaz and Breereton 1982).

The pcb2-related genes are found in higher plants, Chlorobium tepidum TLS and Synechococcus sp. WH8102, but not in other sequenced genomes of photosynthetic prokaryotes. In Rhodobacter capsulate and C. tepidum TLS, bchJ was identified as divinyl protochlorophyllide 8-vinyl reductase by genetic methods (Suzuki and Bauer 1995), but the bchJ homologous gene was not found in higher plants. As there is no homology...
between pcb2 and bchJ, they seem to be distantly related to each other or evolutionarily coming from different origins. There are some other organisms that utilize monovinyl chlorophylls but have neither pcb2 nor $bchJ$, suggesting the existence of a third gene(s) that encodes enzyme(s) acting as divinyl protochlorophyllide 8-vinyl reductase. Further analysis of PCB2 and mono/divinyl chlorophylls from the standpoint of evolution of photosynthetic efficiency may be worthwhile.

Electron microscopic observation of the chloroplast in the pcb2 mutant showed a remarkable decrease in the number of grana stacks. There would seem to be three possibilities. First, the thylakoid membrane may have become immature because of poor accumulation of chlorophylls, since the total amount of chlorophyll in the pcb2 mutant is only 40% of that of the wild type. The second possibility is that accumulations of divinyl chlorophylls disturb the formation of grana stacks, and that perhaps divinyl chlorophylls are not available for the composition of grana stacks. The third possibility focuses on the (divinyl) chlorophyll a/chlorophyll b ratio. It is possible that the balance of (divinyl) chlorophyll a vs. b contents is important for the composition of the grana stack as discussed in Murray and Kohorn (1991). Further research is required to understand the morphogenesis of thylakoid membranes including grana stacks, and the pcb2 mutant analyzed here should provide good material for this research.

Materials and Methods

Plant materials and growth condition

Arabidopsis thaliana ecotype Columbia (Col) and Landsberg erecta (Ler) were used. The pcb2 mutant was isolated from the EMS mutant library of Col as a pale-green mutant (Nakanishi et al. 2004). Seeds were sterilized in 0.2% sodium hypochlorite with 0.05% Tween-20, then kept at 4°C in the dark for 2 days. The seeds were germinated on a 1% sucrose agar plate containing 1/1,000 volume of liquid fertilizer (Hanakoujo; Sumika-Takeda-Engei, Tokyo, Japan) at 40% humidity and in a 14 h light/10 h dark cycle at 22°C. Seedlings 2 to 4 weeks old were transferred to rock wool (NICHIAS, Tokyo, Japan) and grown under the same conditions.

Mapping and DNA sequencing of the pcb2 gene

To fine map PCB2, an F2 population was generated from a cross between pcb2 and Ler wild-type. Markers used for PCR-based mapping were nga106 (TAIR Accession No. Genetic Marker: TAC145512), cwi8 (TAIR Accession No. Genetic Marker: 19455439) and PAT1.2 (TAIR Accession No. Genetic Marker: 1945631). These are SSLP markers (Bell and Ecker 1994). DPCB2 was designed to distinguish Ler from Col by the unique restriction site of BamHI as a dCAPS marker (Neff et al. 1998). Primers used for creating dPCB2 on PCR were dPCB2B-F (5′-aagttgatctgctgctgat-3′) and dPCB2B-R (5′-aagttgatcagagacgacacca-3′).

The PCR-amplified fragments of the four genes which had a transit peptide into the chloroplast from the pcb2 genome were sequenced directly with a DSSQ-2000L (Shimadzu, Kyoto, Japan) and an ABI PRISM 310 (Applied Biosystems, CA, U.S.A.). Primers used for PCR were A5g18570-F1 (5′-ttatatgcagaaatggctttc-3′), A5g18570-R1 (5′-eggtatcccatagcaacg-3′), A5g18660-F1 (5′-agttgattccttcctcagc-3′), A5g18660-R1 (5′-ctcatccacattcttgcag-3′), A5g18910-F1 (5′-ctctctctctctctcctcc-3′) and A5g18910-R1 (5′-tctctctctctctcctcc-3′). The genomic DNA of wild-type (Col) was used as a template. The fragment was amplified again with ‘adapter primers’ and then introduced into pDONR201 (Invitrogen) using ‘BP recombination reaction’ to generate the ‘entry clone’ of A5g18660. The binary vector was constructed using an ‘LR recombination reaction’ between the entry clone and the destination vector pGWB5. The vector pGWB5 was a gift of Dr. T. Nakagawa, Shimane University.

Suppression and expression of PCB2 in transgenic plants

All plants were transformed into the Agrobacterium tumefaciens C58 strain by electroporation (GENE PULSER II; Bio-Rad, Tokyo, Japan). The pKA-PCB2 and pGWB5-PCB2 plasmids were introduced into wild-type (Col) by floral dipping as described by Clough and Bent (1998). The pKS-PCB2 plasmid was introduced into PCB2/pcb2 heterozygous plants. Seeds from transformed plants were selected on 1% sucrose agar plates containing 1/1,000 liquid fertilizer and 20 μg ml−1 kanamycin. To select the pcb2/pcb2 homozygous plants from the (T1) plants, the PCR-amplified genomic DNAs were sequenced directly. Primers used for PCR and sequencing were A5g18660-F3 (5′-ggatctttctctctctccgac-3′) and A5g18660-R2 (5′-taattgccttctctccg-3′), corresponding to positions outside of the introduced sense A5g18660 fragment (Fig. 4C). T1 and T2 progeny were used for phenotype analysis.

RT–PCR analysis

Total RNA was extracted from leaves using a PURESCRIPT Cell and Tissue RNA Isolation Kit (Gentra System, MN, U.S.A.). After DNase I treatment, complementary DNA was synthesized from 500 ng of total RNA using an oligo(dT) primer with ReverTraAce (TOYOBO, Osaka, Japan). The cDNA synthesized from 10 ng of total RNA was used for PCRs with Ex-Taq polymerase (TaKaRa, Tokyo, Japan). A5g18660-F2 (5′-gagaagagtgtaagttgggag-3′) and A5g18660-F3 (5′-ggatctttctctctctccgac-3′) were released from each plasmid and subcloned into the Smal–SacI site of the pSMASK251 vector generating pKS-PCB2 and pKA-PCB2. pKS-PCB2 encodes sense mRNA under the control of the CaMV 35S promoter and pKA-PCB2 encodes antisense mRNA (Fig. 5A). The vector pSMASK251 was a gift of Dr. H. Ichikawa, National Institute of Agrobiological Sciences, Tsukuba, Japan.

Construction of expression vectors

Full-length PCB2 genomic DNA was amplified by PCR with a set of primers, A5g18660-F1 and A5g18660-R1. The fragment was cloned into a T-vector pXcmKna12 by TA-cloning (Cha et al. 1993) to create the plasmids having the insert in both orientations. Two kinds of HindII–SacI fragments containing PCB2 were released from each plasmid and subcloned into the Smal–SacI site of the pSMASK251 vector generating pKS-PCB2 and pKA-PCB2. pKS-PCB2 encodes sense mRNA under the control of the CaMV 35S promoter and pKA-PCB2 encodes antisense mRNA (Fig. 5A). The vector pSMASK251 was a gift of Dr. H. Ichikawa, National Institute of Agrobiological Sciences, Tsukuba, Japan.

To create pGWB5-PCB2 (Fig. 6B), we used GATEWAY™ Technology (Invitrogen, CA, U.S.A.) according to the manufacturer’s instruction. The entire coding region of PCB2 was amplified by PCR using attB1-A5g18660-Fw (5′-aaaaagccggccctggatcctctcctcc-3′) and attB2-A5g18660-Rv (5′-gagaagagtgtaagttgggag-3′) as primers. The genomic DNA of wild-type (Col) was used as a template. The fragment was amplified again with ‘adapter primers’ and then introduced into pDONR201 (Invitrogen) using ‘BP recombination reaction’ to generate the ‘entry clone’ of A5g18660. The binary vector was constructed using an ‘LR recombination reaction’ between the entry clone and the destination vector pGWB5. The vector pGWB5 was a gift of Dr. T. Nakagawa, Shimane University.

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HPLC analysis

Chlorophylls were extracted by soaking approximately 5 mg of fresh 16-day-old whole plants in 100% acetone at 4°C in complete darkness. The extract was centrifuged at 15,000 rpm for 15 min, and the supernatants were separated on an octadecylsilyle (ODS) column [4.6 mm i.d.×150 mm: LUNA 5u C18 (2), Phenomenex, Torrance,
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CA, U.S.A.) with solvent (methanol : acetonitrile : acetone = 1 : 3 : 1) at a flow-rate of 1.0 ml min⁻¹ at 40°C (Zapata et al. 2000) using an LC10Avp system (Shimadzu). Elution profiles were monitored by absorbance at 660 nm using a diode array detector (SPD-M10Avp, Shimadzu). The spectrum graph of eluted peaks was developed from records in the detector. Two fractions corresponding to divinyl chlorophylls a and b were separated, then the eluate was blown dry using nitrogen gas and dissolved in diethyl ether. Their absorption spectra were measured using a spectrophotometer (DU7400, BECKMAN) between 350 and 700 nm.

**LC/ESI-MS analysis**

Liquid chromatography (LC)-mass spectrometry (MS) was carried out using an LCMS-2010A system (Shimadzu) equipped with a STR ODS II column (15 cm length) and an ESI source. Materials extracted by acetone were separated, then the eluate was blown dry using an LC10Avp system (Shimadzu) and an ESI source. Materials extracted by acetone were separated, then the eluate was blown dry using an LC10Avp system (Shimadzu) and an ESI source. Materials extracted by acetone were separated, then the eluate was blown dry using an LC10Avp system (Shimadzu) and an ESI source. Materials extracted by acetone were separated, then the eluate was blown dry using an LC10Avp system (Shimadzu) and an ESI source.

**Electron microscopy**

Leaf segments were fixed with 2% glutaraldehyde in 50 mM potassium phosphate (pH 7.0) for 2 h at room temperature, and washed with the same buffer for 10 min at room temperature. The washing step was repeated six times. They were post-fixed with 2% osmium tetroxide in 50 mM potassium phosphate (pH 7.0) for 2 h at room temperature. They were post-fixed with 2% osmium tetroxide in 50 mM potassium phosphate (pH 7.0) for 2 h at room temperature. They were post-fixed with 2% osmium tetroxide in 50 mM potassium phosphate (pH 7.0) for 2 h at room temperature. They were post-fixed with 2% osmium tetroxide in 50 mM potassium phosphate (pH 7.0) for 2 h at room temperature.

**Monitoring of sGFP expression by microscopy**

Tₐ progeny of the pGBW5-PCB2 transformant were subjected to confocal microscopic analysis (Radiance 2000, Bio-Rad). The non-fused sGFP transformant nA5-4 was used as a control (Niwa et al. 1999). Green fluorescence and red autofluorescence from chlorophyll were monitored by Kr/Ar laser excitation as described (Niwa et al. 1999).

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**References**


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