Differential Protein Expression of Two Photosystem II Subunits, PsbO and PsbP, in an Albino Mutant of *Bambusa edulis* with Chloroplast DNA Aberration

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ABSTRACT. The chloroplast genome of an albino mutant isolated from tissue culture of the bamboo *Bambusa edulis* Munro was examined to identify aberrations. A number of the chloroplast genes encoding ATP synthases, photosystem II subunits, NADH dehydrogenase, and ribosomal proteins had been deleted, at least partially, in the albino mutant. Comparison of the two-dimensional electrophoresis profiles of albino and green bamboos revealed three spots of reduced intensity, indicating repression of these proteins in the albino mutants. Mass spectroscopic analysis subsequently revealed that two of these proteins are 33-kDa subunits of the photosystem II oxygen-evolving protein complex (PsbO) and one is a 23-kDa subunit of photosystem II oxygen-evolving protein complex (PsbP). The genes encoding these two proteins were cloned from *B. edulis*, and were denoted *BePsbO* (accession no. EF669513) and *BePsbP* (accession no. EF669512). Reverse transcription polymerase chain reaction and two-dimensional gel analyses of *BePsbO* and *BePsbP* in green and albino bamboos grown in the light or dark revealed that the albino mutant, similar to its green counterpart, sensed the light signal, resulting in the induction of *BePsbO* and *BePsbP* transcription, but it did not accumulate the protein products. We conclude that the repression of protein-expressing *BePsbO* and *BePsbP* is because of a defect in post-transcriptional regulation in the albino mutant.

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Chloroplast genomes are composed of multiple copies of circular double-stranded DNA molecules each typically about 150 kb in size, encoding more than 120 different genes (Peltier et al., 2000). The chloroplast genome replicates independently

of the cell cycle, and when errors occur during replication, resulting in chloroplast genomic aberration(s), one of the consequences may be the formation of albino plants (Day and Ellis, 1984; Dunford and Walden, 1991; Harada et al., 1991, 1992). Albino plant cells contain a reduced number of plastids, and those that are present are severely distorted and the thylakoids are poorly developed. This lack of (well-)functioning chloroplasts restricts the photosynthetic capability of these albino mutants, which become heterotrophic as a result. Such albino mutants are incapable of growing in the field (Careda et al., 2004).

Many chloroplastic genes are located in the nuclear genome (Martin et al., 1998). As a result, the chloroplast genome is unable to synthesize all of the nearly 3000 proteins required by a functional chloroplast. More than 90% of chloroplastic proteins are encoded by the nuclear genome, synthesized in precursor form on cytosolic ribosomes, and targeted post-translationally to the chloroplast. Each precursor protein (preprotein) contains an NH₂-terminal targeting signal, called the envelope transit peptide, to guide the protein to the chloroplast. Therefore, any defect(s) in the expression of a particular nuclear-encoded protein may result in the formation of albino or pale-green plants (Hsieh and Goodman, 2005; Teng et al., 2006).

The coordinated expression of chloroplastic protein synthesis and the biogenesis of chloroplasts is the result of a well-organized communication between the chloroplastic and the nuclear genomes (Leon et al., 1998; Rochaix, 2001; Surpin et al., 2002). Therefore, it is possible that albino mutants that derive from a chloroplast (cp)DNA aberration may be the result of impaired signaling between the nucleus and chloroplast. If this assumption is valid, any study of the factors involved in the determination of the albino phenotype cannot be limited to an analysis of one chloroplastic genome sequence, as this would ignore the (possible) effect of nucleus-encoded genes regulated by chloroplast-encoded genes. Two-dimensional protein electrophoresis followed by mass spectrometric analysis of the separated proteins is a powerful tool in identifying large numbers of proteins in proteome-containing nucleus- and chloroplast-encoded proteins (Friso et al., 2004). To date, no study has been published on the bamboo proteome.

The objectives of the study reported here were to identify the plastid- and nuclear-encoded genes that are deficient in gene content or expression in the albino mutant of the bamboo *Bambusa edulis*. We compared chloroplast morphology, chloroplast gene content and expression, and the results of proteomic analyses of green and albino bamboos with the aim of determining and assessing chloroplastic aberrations. To our knowledge, this is the first proteomic analysis of bamboo and has led to the discovery that the reduced levels of two subunits of photosystem II, BePsbO and BePsbP protein, observed in the albino mutant are not regulated at the mRNA level but at the protein level.

Materials and Methods

PLANT MATERIALS. Multiple shoots of *Bambusa edulis* were induced in a long-term tissue culture system (Lin and Chang, 1998), an albino mutant line generated spontaneously in this system in 1996. The green and albino multiple shoots were cultured in Murashige and Skoog basal medium supplemented with 0.1 mg·L⁻¹ thidiazuron (TDZ; Sigma, St. Louis) for shoot proliferation. Each explant contained about five shoots. Explants were maintained at 26 °C under a 16-h/8-h (light/dark) photoperiod with light supplied by fluorescent tubes (FL-30D/29, 40 W; China Electric Co., Taipei, Taiwan) at an intensity of 54 mol·m⁻²·s⁻¹.

TRANSMISSION ELECTRON MICROSCOPY (TEM). Leaves from in vitro-grown shoots were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, overnight at 4 °C overnight. After three rinses (20 min each) with buffer, these samples were postfixed in 1% OsO₄ in the same buffer for 4 h at room temperature and were then rinsed again three times (20 min each) with buffer. The leaves were dehydrated in an acetone series, embedded in Spurr's resin (Spurr, 1969), and sectioned with an Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany). Semithin sections (1 µm) for light microscopy were placed on slides and stained with 0.1% toluidine blue for 1 min at 60 °C on a hot plate. Thin sections on grids were stained with uranyl acetate and lead citrate (Reynolds, 1963) and were studied with a transmission electron microscope (Philips CM 100; Philips, Mahwah, NJ or JEM 1200 EX II; Jeol Co., Tokyo) at 80 KV.

DNA PURIFICATION AND GENOMIC POLYMERASE CHAIN REACTION (PCR). For the chloroplast genomic PCR analysis, total genomic DNA from green plants and albino plants was isolated using a urea extraction buffer (Sheu et al., 1996). Each gene was then amplified using primers specific for that gene (Table 1). The PCR program consisted of the following steps: 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. The sequences of the primers were designed according to rice (*Oryza sativa*)

Table 1. The primer sequences used in the analyses to characterize an albino mutant isolated from tissue culture of *B. edulis*.

Gene	Forward primer: 5'→3'	Reverse primer: 5'→3'
<i>atpB</i>	ACCAATCCTACTACTTCTCG	CTTCAATTTGTTCTCTCTTC
<i>atpH</i>	AATTGCTGCTGCTTCCGTT	TCGCAAATAAAAGCGCCAGT
<i>atpI</i>	CCGTGTTCCATTAACACTC	ACCCTCCATGGATTACCTAT
<i>BePsbO</i>	GGTGGCGTGGACTCTTTC	GACGACCCACGATAACTC
<i>BePsbP</i>	CCAACAGCAACGTCTCCGTC	GGTTCGCGATGATCAGCTGG
<i>ndhC</i>	GTTTCTGCTTCACGAATATG	ACCATTCCAAGGCTCCTTTT
<i>ndhD</i>	ATGAGTCTTTTCCTTGGTT	TTGGATAACAGAGCCTCTAC
<i>ndhE</i>	TGATGTTTGAGCATGTACTTT	TTAGATTGATTGATACGAG
<i>ndhG</i>	CCTGGGCCAATACATGAAATT	TTGCCGAGCCATAGTAATTG
<i>psbA</i>	GCAATTTTAGAGAGACGCGA	TGGAACCTTCAAGAGCAGCTA
<i>psbB</i>	ATGGGTTTGCTTGGTATC	GTTGGATCTCCACGTTTTTG
<i>psbC</i>	TATCCCTGAGGAGGTTCTA	TCATGTAAAGAACAGGCTCCA
<i>psbD</i>	TGACTATAGCCCTTGGTAGA	ACGTGGTAGAACCTCCTCAGG
<i>psbE</i>	ATGTCTGGAAGCACGGGAGAA	GGATCTACTAAATTCATCGAG
<i>rps19</i>	GACACGAAAAAAACGAATCC	GTATCCTTTCTTGCACTTTCA
<i>rps2</i>	TGACAAGAAGATATTGGAACA	ATATAGAGAGAACGGCCCTCA
<i>rps4</i>	CGTTATCGAGGACCTCGTTTT	TGACGAGAGTAATATTCTACA
<i>rps7</i>	TCACGTCGAGGTACTGCAGA	TGTGCAAGAGCTCTATTGGCC
<i>Tubulin</i>	AAATTGAATTGAGCGCCCA	TAACAACACTGGTGGAGTTGG

chloroplast-encoded genes and on *PsbO* (accession no. BAF20777.1) and *PsbP* (accession no. BAB64069.1) based on the sequences that encoded the matched peptides. The PCR products derived from the green plant and albino mutant were sequenced. DNA sequencing was carried out with the BigDye Terminator Cycle Sequencing kit using a Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

RNA PURIFICATION AND REVERSE TRANSCRIPTASE (RT)-PCR. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Gibco-BRL, Grand Island, NY). First-strand cDNAs were synthesized by M-MLV reverse transcriptase (RNase H Minus, Point mutant; Promega, Madison, WI) and a poly (dT) primer. In the preliminary experiments, we tested a varying number of PCR cycles (20, 23, and 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) to determine the number required for optimum amplification. Based on these results (data not shown), in subsequent experiments we applied a 25-PCR cycle for primers specific for each gene to amplify each transcript. Bamboo α -tubulin (accession no. DV668278) was used as the internal control. Each experiment was repeated three times on three different RNA samples. Densitometric determination of the RT-PCR product amounts was carried out using TINA 2.09e software (Raytest, Straubenhardt, Germany).

PREPARATION OF BAMBOO PROTEINS AND TWO-DIMENSIONAL ELECTROPHORESIS. To avoid any potential interference from plant secondary metabolites, we carried out phenol extraction and methanol ammonium acetate precipitation of the bamboo proteins (Hurkman and Tanaka, 1986). Bamboo shoot tissue (1 g) was frozen in liquid nitrogen and ground with a pestle in a mortar kept on ice. The ground tissue was homogenized for 30 s in a solution containing 2.5 mL Tris (pH 8.8)-buffered phenol, 2.5 mL extraction buffer (0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, and 0.9 M sucrose), and 25 μ L protease inhibitor cocktail (Sigma). The homogenized tissue solution was transferred to a 15-mL centrifuge tube, shaken on ice for 30 min, and centrifuged for 10 min at 5000 g_n at 4 °C. The upper layer (phenol phase) was collected as the first phenol fraction. The lower layer (aqueous phase) was then mixed with 2.5 mL of Tris (pH 8.8)-buffered phenol and 2.5 mL extraction buffer, and was incubated for 5 min on ice. The mixture was centrifuged for 10 min at 5000 g_n at 4 °C, and the phenol phase was

collected and combined with the first phenol fraction. The combined phenol fractions were combined with a 5-fold volume of 0.1 M ammonium acetate in methanol, transferred to a centrifugation tube, mixed well by inverting the tube for several times, and incubated for 1 h at -20 °C. The mixed solution was then centrifuged for 10 min at 20,000 g_n at 4 °C. The precipitate was homogenized with 1 mL of ice-cold 80% acetone, incubated for 15 min at -20 °C, and centrifuged for 8 min at 12,303 g_n at 4 °C. This precipitate was then washed with 80% ice-cold acetone and dried. The dried precipitate was resuspended with 1 mL of isoelectric focusing (IEF) rehydration buffer and 50 μ L of 1 M DTT, shaken at room temperature for 1 h, and centrifuged for 10 min at 14,7000 g_n at 4 °C. The supernatant was quantified by Bradford method (Bradford, 1976). A 200- μ g volume of protein was adjusted to 250 μ L and placed into a sample tray. The IEF strip (pH 4-7), with its gel phase facing downward, was immersed into the protein

Fig. 1. Chloroplast morphology revealed by TEM. Green (A) and albino (B) leaves of *B. edulis* plantlets were incubated in 0.1 mg L-1 thidiazuron medium and subjected to morphological examination. Green and albino shoots contained normal nuclei (n), but only green shoots contained normal plastids (p). Higher magnification showed typical chloroplasts with organized thylakoids, grana, and starch grains in a green plantlet, bar = 1 μ m (C). Some of plastids in the albino leaves had starch grains (s) and plastoglobule (marked by an asterisk, bar = 1 μ m; D).

sample and covered with 1 mL of mineral oil. The sample tray was applied to the Bio-Rad (Hemel Hempstead, UK) IPG Cell for IEF (rehydration 50 V, 12 h/S1 250 V, 15 min/S2 250-4000 V, 2 h/S3 V h accumulated to 20,000 V h/S4 500 V hold). The IEF strip was washed with water to remove the mineral oil and then placed into 2.5 mL of Equi I and incubated for 15 to 20 min. The strip was equilibrated in 2.5 mL of Equi II for 15 to 20 min, applied to a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Mini-P III; Bio-Rad), and covered by 1% agarose containing bromophenol blue. The SDS-PAGE was performed at 100 V for 120 min, following which the gel was stained by Coomassie blue. The experiments were repeated three times. The gels were scanned by a 5000U scanner (BENQ, Taipei, Tawan) and the images were given a pseudo-color (green bamboo: green; albino mutant: red). The two images were then merged. The color of each spot was measured visually, and green spots (spots 1, 2, and 3; Fig. 3) were picked for the protein identification experiments.

MASS SPECTROMETRY (MS) ANALYSIS AND PROTEIN IDENTIFICATION. Gel digestion of the proteins and MS analysis were performed as previously described (Tsai et al., 2005; Wu et al., 2005). Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis was carried out on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). Searches were performed without constraining protein molecular weight (MW) or isoelectric point, thereby allowing for carbamidomethylation of cysteine, partial oxidation of methionine residues, and one missed trypsin cleavage. All spectra were subjected to an internal two-point calibration using two peptides derived from autodigested trypsin (m/z 842.51 and 2211.10 Da). Mass lists were subsequently used to search the NCBI database using Mascot software (Matrix Science, London, UK; Yang et al., 2004). Coomassie-stained protein spots were in-gel digested with sequencing grade-modified trypsin (Promega). The resulting peptides were extracted, lyophilized, and reconstituted in 10 μ L of water-acetonitrile (1:1, v:v; 0.1% trifluoroacetic acid) for mass analysis. These peptides were loaded onto a 150 $\times$ 10-mm C18 reversed-phase column using the CapLC system (1100 series; Agilent Technologies, Palo Alto, CA) with a gradient solution of 5% to 90% acetonitrile aqueous solution and separated over a 45-min period. Peptides were eluted at 200 nL·min⁻¹ directly into a spray source of a quadrupole TOF mass spectrometer (QTOF2; Micromass, Manchester, UK). The QTOF2 is fitted with an electrospray in an orthogonal configuration with a Z-SPRAY interface. The source block temperature was set to 80 °C, and the cone voltage was kept at 25 V. The quadrupole analyzer was used to select precursor ions for subsequent fragmentation in the hexapole collision cell. The argon was used as the collision gas, and the collision voltage was maintained at 20 to 30 V to provide optimal fragmentation. The fragmented ion products were analyzed in an orthogonal TOF analyzer fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. The mass spectrum acquisitions were recorded within the mass range of 400 to 2000 m/z , and the tandem mass spectra were recorded within 50 to 3500 m/z . The recorded spectral data were processed with MassLynx, version 4.0 software (Micromass) to give centroid MS/MS data. Database searching was performed using Mascot software (Matrix Science; Yang et al., 2004) with the following parameters: NCBI database, trypsin protease specified

with one possible missed cleavage, peptide tolerance of 2 Da, MS/MS tolerance of 0.8, and a variable carboxyamidomethyl modification.

Results

ANALYSIS OF CHLOROPLAST STRUCTURE IN THE ALBINO MUTANT AND NORMAL BAMBOO CELLS. The morphology of the chloroplasts of albino and normal green leaves were compared by TEM. The cells of green bamboo leaves contained numerous chloroplasts (Fig. 1A), whereas those of the albino bamboo leaves were structurally abnormal (Fig. 1B). Chloroplasts in normal bamboo cells were diamond shaped with an abundance of distinct thylakoids packed into grana and many starch granules (Fig. 1C). In contrast, the chloroplast of the albino mutant contained few thylakoids, and those present were poorly developed (Fig. 1D). Some of the plastids had become vacuole-like, and only a few contained a plastoglobule and starch grains.

ABSENCE OF SEVERAL CHLOROPLAST-ENCODED GENES IN THE ALBINO BAMBOO. Because plastid-encoded genes are highly conserved, we used the conserved regions of selected chloroplast-encoded genes to design primers that would reveal whether specific chloroplast-encoded genes were absent in the albino mutant. Four groups of chloroplast-encoded genes that encode ATP synthases, ribosomal proteins, photosystem II, and NADH dehydrogenase, respectively, were analyzed by genomic PCR and RT-PCR (Fig. 2). Of the 16 genes tested, eight were found to be deleted (*atp H*, DQ908947) or present at reduced copy numbers in the albino mutant, including ATP synthases genes (*atpB*, DQ908948; *atpI*, DQ908946;), photosystem II genes (*psbB*, DQ908938; *psbE*, DQ908935), NADH dehydrogenase (*ndhC*, DQ908945), and ribosomal proteins

Fig. 2. Comparison of genomic PCR and expression level of selected chloroplast-encoded genes between green and albino *B. edulis*. The presence and level of expression of four major groups of chloroplast-encoded genes were determined. The nucleus-encoded tubulin gene served as the internal control. All experiments were repeated three times.

Fig. 3. Two-dimensional electrophoresis analysis of green and albino *B. edulis* proteins. Proteins were separated by gel electrophoresis followed by Coomassie blue staining. The pH and molecular weight standards are labeled. Spots 1, 2, and 3 are circled. All experiments were repeated three times. pI = isoelectric points.

genes (*rps2*, DQ908933; *rps4*, DQ908932). As expected, products of RT-PCR for these genes were below the level of detection, indicating that defects in the genome of the chloroplast may possibly be the causal factor determining the formation of albinos.

COMPARISON OF THE PROTEOME OF GREEN BAMBOO AND ALBINO BAMBOOS. To identify the proteins that may be absent in the albino mutant, we conducted a comparative proteomic analysis. Total proteins were isolated from shoots and leaves of albino and green bamboos under light conditions and resolved by two-dimensional-gel electrophoresis. About 250 protein spots were detected on two-dimensional-gels within the pH range of 4 to 7 and a MW range of 17 to 76 kDa (Fig. 3). Three protein spots, with pI values and MW of 4.7/33 kDa (spot 1), 4.8/33 kDa (spot 2), and 5.4/25 kDa (spot 3), respectively, were found to have a decreased intensity in albinos in all three independent experiments. MALDI-TOF MS analysis followed by peptide mass fingerprinting analysis revealed that the peptides from spots 1 and 2 matched seven peptides of rice (*O. sativa*) PsbO (PsbO, NP_918587 pI: 5.1; MW: 34 kDa), but no significant hits were obtained for peptides from spot 3. Therefore, these were further analyzed by Q-TOF to obtain sequence information. The results of an MS/MS ion search confirmed that peptides from spots 1 and 2 matched five peptides of rice PsbO (22.5%, score: 322, accession no. BAB64069, Fig. 4A). In addition, peptides from spot 3 could be matched to three peptides of wheat (*Triticum aestivum* L.) oxygen-evolving protein complex 2 (PsbP, Q00434; coverage: 11%, score: 169). PsbO and PsbP have theoretical MWs of about 36 and 27 kDa, respectively, which are larger than the observed MWs of spots 1 and 2 (Fig. 3; ~33 kDa for PsbO and 25 kDa for PsbP). Therefore, we analyzed the protein sequence of PsbO and PsbP by PSORT and identified the transit peptide of their N-terminus (Fig. 4). The size of the processed forms of PsbO and PsbP were reduced to 33 and 25 kDa, respectively, which correlated with the observed MWs of spots 1 and 2 (Fig. 3).

CLONING OF BEPSBO AND BEPSBP. Because the bamboo peptides were highly similar to rice *PsbO* and *PsbP*, we used nucleotide sequences of rice *PsbO* and *PsbP* to design specific primers to clone gene fragments of bamboo *PsbO* and *PsbP* by RT-PCR (Fig. 5, A and C). A 482-bp fragment of bamboo *PsbO* (*BePsbO*, accession no. EF669513) and a 393-bp fragment of

bamboo *PsbP* (*BePsbP*, accession no. EF669512) were subsequently cloned and sequenced (Fig. 5, B and D). The deduced amino acid sequence of the *BePsbO* gene showed a 96% (153/160) similarity and a 91% (146/160) identity to rice *PsbO*, whereas the deduced *BePsbP* protein sequence fragment had a 97% (125/129) similarity and 90% identity (117/129) to rice *PsbP* (Fig. 5, B and D).

LOSS OF BEPSBO AND BEPSBP PROTEINS WERE REGULATED AT TRANSLATIONAL/POST-TRANSLATIONAL LEVELS. It is known that *PsbO* and *PsbP* are induced by light in *Arabidopsis thaliana* (L.) Heynh. (Yamamoto et al., 1998), which led

us to investigate whether their reduced protein levels in the albino mutants were from their incapability of sensing light. The RT-PCR analyses of *BePsbO* and *BePsbP* and two-dimensional analysis of green and albino bamboo seedlings grown under light or dark conditions, respectively, revealed that *BePsbO* and *BePsbP* transcripts were present at similar levels in normal bamboos and the albino mutants (*BePsbO*:

A	
1	MAASLQAAAT LMQPAKLGG ASSAALPSRP SSHVARAFGV DTGAAGRITC
51	SLQSDIREVA MKCADAAKLA GFALATSALL VSGASAEGVP RRLTFDEIQS
101	KTYMEVKGTG TANQCPTVEG GVDSFAPKAG KYNMKKFCLE PTSFTVKAEG
151	VAKNAPPEFQ KTKLMTRLTY TLDEIEGPLE VSSDGTVKFE EKDGIDYARV
201	TVQLPGGERV PFLFTIKNLV ATGKPESFGG PFLVPSYRGS SFLDPKGRGG
251	STGYDNAVAL PAGGRGDEEE LAKENVKNAS SSTGNITLSV TKSKPETGEV
301	IGVFESVQPS DTDLGAKVVK DVKIQGVWYA QLE
B	
1	MAASLQAAAT LMQPAKLGG ASSAALPSRP SSHVARAFGV DTGAAGRITC
51	SLQSDIREVA MKCADAAKLA GFALATSALL VSGASAEGVP RRLTFDEIQS
101	KTYMEVKGTG TANQCPTVEG GVDSFAPKAG KYNMKKFCLE PTSFTVKAEG
151	VAKNAPPEFQ KTKLMTRLTY TLDEIEGPLE VSSDGTVKFE EKDGIDYARV
201	TVQLPGGERV PFLFTIKNLV ATGKPESFGG PFLVPSYRGS SFLDPKGRGG
251	STGYDNAVAL PAGGRGDEEE LAKENVKNAS SSTGNITLSV TKSKPETGEV
301	IGVFESVQPS DTDLGAKVVK DVKIQGVWYA QLE
C	
1	MASTSCFLHQ STARLAASAR PAPAAGRTQL FVCKAQKND E AASDAAVVTS
51	RRAALSLLAG AAAIAVKVSP AAAAYGEAAN VFGKAKKNTD FVAYSGEQFK
101	LMIPAKWNPS KEREFPGQVL RYEDNFDATS NLSVIINPTT KKTITDYGSP
151	EEFLSQVGF LQQSYGGKT DSEGGFESDA VATANVLESS APVVDGKQYY
201	SITVLTRTAD GDEGGKHQLI TATVADGKLY VCKAQQRDM FKGAKKFVEN
251	AAGSFSVA

Fig. 4. The quadrupole-TOF MS/MS spectrum of spots 1, 2, and 3. The MS/MS spectrum of each identified peptide and matched peptide sequence are shown in A (spot 1: *O. sativa* PsbO, accession no. BAB64069), B (spot 2: *O. sativa* PsbO, accession no. BAB64069), and C (spot 3: *Triticum aestivum* PsbP, accession no. Q00434). The mapped sequences of peptides are shown in black and bold. The underlined sequences are chloroplast transit sequences.

Fig. 5. Nucleotide and protein sequences of *B. edulis* photosystem II subunit PsbO (BePsbO) and PsbP (BePsbP). A and C are the partial DNA sequences of BePsbO (accession no. EF669513) and BePsbP (accession no. EF669512), respectively. B and D are the deduced protein sequences of BePsbO and BePsbP compared with *O. sativa* PsbP (accession no. BAB64069.1) and PsbO (accession no. BAF20777.1). The underlines indicate identical sequences to the sequences of tryptic peptides analyzed by quadrupole-time of flight MS. Gray letters indicate the positions of the primers.

1.46:1.69; BePsbP: 1.31:1.74) grown in the presence of light and that the transcripts of both genes were still present in normal bamboo and albino bamboo grown under dark conditions—but at lower levels (Fig. 6). The two-dimensional analysis revealed the presence of these proteins in the normal bamboo cultured under light conditions but not in those cultured in the dark. In contrast, the BePsbO and BePsbP proteins spots were absent in albino bamboos grown under both light and dark conditions (Fig. 7). As expected, the BePsbO and BePsbP proteins were induced by light in normal bamboo tissue.

Discussion

ABERRATION IN CHLOROPLASTS: A POSSIBLE CAUSE OF THE ALBINO MUTANT IN BAMBOO? Albino mutants have been observed to spontaneously appear during bamboo somatic embryogenesis (Rout and Das, 1994) and during micropropagation (Lin and Chang, 1998). After 9 years of subculturing bamboo *Bambusa edulis* Murno, we obtained three albino mutant lines. Compared with the anther culture system of Day and Ellis (1984), the frequency of albino mutants was low in our multiple shoot culture system. Chloroplast DNA aberration is one of major

Fig. 6. RT-PCR of *B. edulis* photosystem II subunit PsbO (BePsbO) and PsbP (BePsbP) in green and albino plants grown under dark or light conditions. The specific primers were designed based on sequences of BePsbO and BePsbP and were used in the RT-PCR analyses to determine the mRNA level of these two genes in green and albino bamboos, respectively. The tubulin gene served as an internal control. All of the experiments were repeated three times. Densitometric determination of the RT-PCR product amounts was carried out using TINA 2.09e software (Raytest, Straubenhardt, Germany). The density was normalized using the internal control, tubulin. The number indicates the ratio of gene and internal control.

mechanisms of albino formation in tissue culture (Day and Ellis, 1984). One proposal for the mechanism of albinism is that the plastid DNA is degraded in the vegetative cell during mitosis in the formation of pollen-tube growth, leading to the disappearance of plastids in the pollen grain after fertilization (Careda et al., 2004). However, in our study, the albino mutant of *B. edulis* was derived from somaclonal variation during micropropagation and not from anther culture. We speculate that rapid mitosis and plastid division in vitro resulted in the production of abnormal plastids and, consequently, albino plants.

The deletion or reduced copy number of several chloroplast-encoded genes, as revealed by the genomic PCR analysis of selected chloroplast-encoded genes, suggests that deletion of the chloroplast genome and heterogeneity occurred in the albino bamboo, a phenomenon that has also been observed in other albino mutants of bamboo (Liu et al., 2007). Accordingly, the plastids in this bamboo albino plant were considered to be a mixture, a concept supported by the wide spectrum of structures and sizes shown by the plastids in our albino mutants. Such heteroplasmy has also been observed in other albino plants (Day and Ellis, 1984; Dunford and Walden, 1991; Harada et al., 1991, 1992).

Similar to the bamboo albino mutants reported here, other albino plants derived from another culture have been shown to lack gene function in ATP synthesis, photosynthesis, and ribosome biogenesis, but still to show *trnE*, plastid-encoded tRNA (Harada et al., 1992). In *B. edulis*, only one gene, *atpB*, has been found to be deleted in all three albino mutants (Liu et al., 2007). The lack of these gene functions results in impairment in photosynthesis, chlorophyll-binding, and energy utilization from the light reaction. Low photosynthetic activity

Fig. 7. Proteome analysis of green and albino *B. edulis* grown under dark or light conditions. Proteins isolated from green and albino bamboo proteins grown in the dark or light were separated by two-dimensional gel electrophoresis. Spots 1 and 2 bamboo PsbO (BePsbO) and spot 3 bamboo PsbP (BePsbP) were detected only in green bamboo grown in the light but not in albino bamboo grown in the light or dark. All experiments were repeated three times. pI = isoelectric points.

has also been reported to reduce the redox ratio and repress the expression of nucleus-encoded chloroplastic proteins (Pfannschmidt, 2003). However, the question of how many gene mutations result in the albino mutant remains unanswered. Future research directed at sequencing the chloroplast genome of *B. edulis* and using different albino mutants to investigate the candidate genes causing albino bamboos will provide interesting answers to this question.

The smaller plastids and accumulations of numerous plastoglobules that we observed in the albino bamboo have also been observed in albino barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), and *A. thaliana* (Bauer et al., 2000; Caredda et al., 2004; Gutiérrez-Nava et al., 2004; Kubis et al., 2003; Liu et al., 2007). Some of these mutants showed impairments in nuclear-encoded genes, and various proteins involved in RNA processing, protein translation, protein import, and plastidic isoprenoid biosynthesis had been affected (Bauer et al., 2000; Gutiérrez-Nava et al., 2004; Kubis et al., 2003). Hence, global analyses of gene analysis and protein expression profiles are needed to gain an understanding of factors that cause chloroplastic aberrations in albino plants.

Because deletions and transcriptional alterations of chloroplast genes may not be the only factors causing the albino phenotype, we expressly chose the proteomic approach in this study to examine changes at the protein level. This approach has been successfully used for selecting functional genes in *A. thaliana*, discovering new functions of chloroplast proteins, and

for studying the biogenesis of chloroplasts in maize (Friso et al., 2004; Lee et al., 2004; Lonosky et al., 2004). In our study, we identified two proteins, BePsbO and BePsbP, that were repressed in the albino mutant, but whose down-regulation was not regulated at the transcriptional level but at the translational or post-translational level. PsbO and PsbP were also found to be repressed in six bamboo albino mutants (three from *B. edulis*, three from *B. oldhamii* Munro; data not shown). PsbO is an extrinsic subunit of photosystem II, playing a central role in the stabilization of the catalytic manganese cluster (Yamamoto, 2001), and its deficiency affects the accumulation of PsbP (Yamamoto et al., 1998). Lower levels of PsbO and PsbP have also been observed in other mutants, such as a yellow-green chloroplast import mutant (*ppl1*; Kubis et al., 2003), indicating that PsbO and PsbP are not only affected in chloroplast-encoded gene mutants but also in nucleus-encoded gene mutants.

GENE REGULATION OF BEPSBO AND BEPSBP. PsbO was absent in the chloroplast proteome of etiolated maize plants. Lonosky et al. (2004) reported that during greening, PsbO protein could be recovered

within 4 h and that it remained at a sustainable level up to 48 h after exposure to light. Yamamoto et al. (1998) demonstrated that *PsbO* and *PsbP* were induced by light in *A. thaliana*. These data do not enable us to determine whether upregulation of the translation of PsbO and PsbP caused the upregulation of transcription under light conditions. We were also unable to precisely determine why BePsbO and BePsbP proteins could not be detected in albino bamboo even though these were transcribed in the normal green and albino bamboos grown in light. The absence of the BePsbO and BePsbP proteins in the albino bamboos may possibly be because of a deficiency of translation or defects at the post-translational level. The translational regulation of photosynthetic genes is controlled by the redox state of plant cells (see review in Pfannschmidt, 2003). The expression of some photosynthetic proteins in the albino mutant may have resulted in the change of redox state, thereby causing the impaired translation of BePsbO and BePsbP.

Conclusion

The results of this study demonstrate how gene expression of two nucleus-encoded photosystem II subunits is suppressed in an albino bamboo with the loss of a number of chloroplast-encoded genes. Two nucleus-encoded proteins, BePsbO and BePsbP, identified by two-dimensional electrophoresis and mass spectroscopy, were found to be absent in the albino mutant. The

transcription of these genes was light dependent, and transcriptional regulation in the albino bamboos was normal, as in the green bamboos, indicating that translational or post-translational regulation of these two genes was impaired in albino bamboos, thereby resulting in lower protein levels despite normal transcription. This mutant with an impaired chloroplast genome could be useful for investigating the role of nucleus-encoded chloroplast genes regulated by chloroplast-encoded genes. Given the ≈ 3000 nucleus-encoded chloroplast proteins, proteomic tools may be a valuable approach for globally screening chloroplast-encoded genes involved in the regulation of nucleus-encoded gene expression.

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