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Characterization of Albino Tobaccos (*Nicotiana tabacum* L.) Derived from Leaf Blade-Segments Cultured *in vitro*

Chang-Hyu Bae*, Tomoko Abe, Hyo-Yeon Lee[1], Dong-Choul Kim[1], Kyung-Soo Min[2], Kwan-Sam Choi[3], Tomoki Matsuyama, Takeshi Nakano, Shigeo Yoshida

The Institute of Physical and Chemical Research (RIKEN), Wako-shi, 351-0198, Japan; [1]College of Agriculture, Sunchon National University, Sunchon, 540-742, Korea; [2]College of Agriculture, Chonnam National University, Kwangju, 500-757, Korea; [3]College of Agriculture, Chungnam National University, Taejon, 305-764, Korea.

Key words: Albino tobacco, chloroplast genes, DNA copy number, leaf blade-segment, shoot induction, transcription

Abstract

The leaf blade-segments of albino tobacco (*Nicotiana tabacum* L.) were cultured on MS media containing different concentrations of BAP (0, 0.4, 2.2, 4.4, 22.2 μM) with or without NAA (0, 0.5, 2.7 μM). Multiple shoots were induced on the media containing 0.4 to 2.2 μM BAP. The best condition for multiple shoot induction with root formation was MS media containing 4.4 μM BAP and 0.5 μM NAA. The regenerated albino plants showed a significant reduction in accumulation of chlorophylls and carotenoids. The drastic reduction of the pigments content was associated with the distinct alterations in gene expression in the albino plants. Firstly, the expression of plastid genes, such as *rbcL, psbA*, 16S rDNA and 23S rDNA, was reduced at the level of transcripts in the regenerated albino plants. Secondly, the alteration of structure of the plastid genes was not detected in the albino plants. However, the copy number of the plastid genes whose transcription level was reduced greatly was increased approximately two-fold, although the transcriptions of nuclear gene (25S rDNA) showed the wild-type level.

Abbreviations: BAP, 6-benzyl aminopurine; ctDNA, chloroplast DNA; NAA, α-naphthaleneacetic acid; ptDNA, plastid DNA

Introduction

Various kinds of plastid mutants including albinism have been reported and studied in relation to the functions in photosynthesis (Day and Ellis, 1984; Dunford and Waldon, 1991; Han et al., 1993; Hess et al., 1994; Mandel et al., 1996; Sundberg et al., 1997; Tsukahara et al., 1996; Zubko and Day, 1998). In contrast to many other photosynthetic mutations, the albino mutation is generally lethal, and albino plants do not survive to flower (Dunford and Waldon, 1991; Sundberg et al., 1997). The albino mutation causes seedling lethality when individuals homozygous for the mutation are grown on soil, since plants do not support the growth beyond the cotyledon stage (Day and Ellis, 1984; Zubko and Day, 1998). Thus, it is necessary to sustain the albino mutant autotrophically when the albino mutant is used to study mechanisms related to photosynthesis. Tissue and cell culture of albino plants enables one to sustain albino clones and study the mechanisms of photosynthesis. For example, the regeneration of albino plants has been examined (Tsukahara et al., 1996) perviously and the analyses of exogenous cytokinin treatment and transcription in white *pac*-2 tissues have been conducted (Grevelding et al., 1993). Also, the mutants generated by asymmetric cell fusion have been used to examine the mechanisms in photosynthesis (Toki et al., 1990).

Albino mutants show distinct alterations of chloroplasts, such as the deletion of plastid genes and the great reduction of transcripts (Day and Ellis, 1984; Dunford and Waldon, 1991; Harada et al., 1991;

* Corresponding author, E-mail; chbae63@hanmail.net
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Hess et al., 1994). Previously, it has been shown that albino plants of rice, wheat and barley do not contain mature chloroplasts. They have reduced amounts of ptDNAs and extensively deleted plastid genomes, such as the 16S and 23S rDNAs (Day and Ellis, 1984; Dunford and Waldon, 1991; Harada et al., 1991). However, the amount of ctDNA of albino plants has been reported to be slightly increased in some cases (Dunford and Waldon, 1991). Transcripts of the plastid genes for photosynthesis system, such as *rbcL, psbA* and *psbD-psbC*, and those of the 16S and 23S rRNA were significantly reduced or were sometimes not detected in albino plants (Dunford and Waldon, 1991; Zubko and Day, 1998). Furthermore, the transcription of the nuclear-encoded genes (*cab, rbcS*) are markedly decreased in albino plants (Dunford and Waldon, 1991; Hess et al., 1994)

In this study, albino plants derived from the leaf blade segment were used to generate directly multiple shoots, because plants derived from direct shooting are more stable genetically than those derived from calli (Evans and Sharp, 1986). In the present work, shoot induction and molecular characteristics of albino plants are presented which were obtained from leaf blade-segment culture. Our data indicate that the regenerated albino mutants have almost the same frequency of shoot induction and greatly reduced transcription levels of the plastid genes for the photosynthesis system, *psbA, rbcL*. In addition, the possibility that the regenerated albino mutants have the increased copy number of ptDNA is discussed.

Materials and Methods

Plant material and culture procedure

Seeds that produce albino and green plants (Bae et al., 1998) were surface-sterilized by soaking in an aqueous solution of sodium hypochlorite (1% active chloride) for 15 min. After the three 3-min rinses in sterile water, the seeds were plated on half strength MS (Murashige and Skoog, 1962) medium. Fully expanded young leaves (from third to forth) of albino and wild-type tobacco (*Nicotiana tabacum* L.) were taken from the plants that were cultured *in vitro* on half strength MS medium for 12 weeks. The leaves were aseptically cut into the sections of 7~9mm in length and 3~5mm in width before being placed onto the culture medium. Explants were cultured in 94×25mm petri dishes (ten explants per dish) with the abaxial surface in contact with the medium. The shoot induction media consist of MS medium containing different concentrations of BAP (0, 0.4, 2.2, 4.4 and 22.2 μM), with or without NAA (0, 0.5, 2.7, 5.8 and 26.8 μM), and 30 gL^{-1} of sucrose. The pH of the medium was adjusted to 5.8. Growth regulators were added prior to sterilization (15 min at 121 ℃), when they were used. Cultures were kept at 25℃ under continuous fluorescence light (80 μmoL $m^{-2}s^{-1}$). The average number of shoots per explant was scored after 5 weeks of cultures.

Pigment estimation

Chlorophyll content was determined as previously described (Aron, 1949). To determine the content of chlorophyll, fresh leaves (0.3 g) from green and albino plants were homogenized in 80% acetone and centrifuged at 9,000×g for 10 min. The absorption spectra of the cleared supernatant were estimated at 645nm and 663nm.

Total carotenoid content was determined as previously described (Lichtenthaler, 1987) with slight modifications. Fresh leaf tissue (0.3 g) from green and albino plants were treated with 6% KOH in methanol (w/v) and incubated at 45℃ for 5 min. The methanol layer was then extracted with *n*-hexane:diethylether (1:1 v/v). Absorbance values at 445 were measured with a spectrophotometer.

Microscopic observation of the numbers of stomata and chloroplasts

Number of stomata on leaf surfaces was determined. Replicas of the epidermis were obtained by coating the leaf surfaces with clear nail polish (Bonnett et al., 1993), and the stomata on the replica were counted under a microscope (Olympus, IMT-2, Tokyo, Japan). In order to rule out the possibility of obtaining different stomata number, which can be caused by the difference in leaf areas of albino and wild-type plants, stomata numbers from the leaves of 5-week-old plants that have almost the same leaf areas were scored.

The number of chloroplasts (or plastids) in wild-type and albino plants was compared with the number obtained by the DAPI staining (Nemoto et al., 1990) that helps in counting albino plastids. Mesophyll protoplasts of wild-type and albino plants were prepared as described (Nakada and Takebe, 1971). The number of chloroplasts per protoplast was counted after fixing with glutaraldehyde and staining with an equal volume (2 μL) of 4', 6-diamidine-2-phenylindole (DAPI) in TAN buffer [17% (w/w) sucrose, 20 mM Tris-HCl (pH 7.6), 0.5 mM EDTA, 1.2 mM spermidine, 7 mM 2-mercaptoethanol]. Five μL of the protoplast suspension was placed on a glass slide and covered with a glass cover slip. Gentle pressure flattened the protoplasts and produced a monolayer of easily countable

chloroplasts. Observations were made with an Olympus IX70 fluorescence microscope.

Southern gel blot analysis

Total DNA was isolated from green and white leaves using the method described by Martin et al. (1985). DNA was digested with *Bam*HI and *Hin*dIII, and hybridized with full-length fragments of *rbcL*, *psbA*, 16S rDNA, 23S rDNA and 25S rDNA. *rbcL* encodes the large subunit of ribulose-1,5-bisphophate carboxylase; *psbA* encodes the D1 subunit of the photosystem II reaction center; 16S rDNA encodes the plastid 16S rRNA; and 23S rDNA encodes the plastid 23S rRNA. A genomic DNA fragment (cytoplasmic 25S rDNA) was used as a control probe. Full-length fragments of *psbA*, *rbcL*, 16S rDNA, 23S rDNA, and 25S rDNA were cloned from a cDNA library of *Nicotiana tabacum* cv. Samsun NN and the sequence were analyzed (Nakano et al., unpublished data). The clones were labeled with ^{32}P (6,000 Ci/mmole) and used as hybridization probes. After hybridization for 24 hrs at 68℃, the filters were washed in 1×SSPE[0.15 M NaCl, 0.015 M sodium citrate and 0.1% (w/v) SDS] twice at room temperature and three times at 68℃, and then exposed to X-ray films for autoradiography. The radioactivity of the bands was measured using the BAS 2000 system (Fuji Film Co. Ltd., Tokyo, Japan). In addition, DNA gel hybridization of the probes was performed using the ECL labeling and detection system (Amersham Life Science, Buckinghamshire, England) and then detected with X-ray film (Fuji Film Co. Ltd., Tokyo, Japan).

Northern gel blot analysis

Leaves of seedlings were frozen in liquid nitrogen and the total RNA was prepared according to the method by Sambrook et al. (1989) with minor modification. Total RNA (3 μg) was electrophoresed on a formaldehyde denaturing 1.5% agarose gel in 1× MOPS buffer (20 mM MOPS-KOH, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA) and then blotted onto a GeneScreen Plus Membrane (Du Pont) according to the standard protocols (Sambrook et al., 1989). Full-length fragments of the *psbA*, *rbcL*, 16S rDNA, 23S rDNA and 25S rDNA clones were labeled with ^{32}P (6,000 Ci/mmole) and used as hybridization probes. After hybridization for 24 hrs at 68℃, the filters were washed in 1×SSPE [0.15 M NaCl, 0.015 M sodium citrate and 0.1% (w/v) SDS] twice at room temperature and three times at 68℃, and then exposed to X-ray films for autoradiography. The radioactivity of the bands was measured using the BAS 2000 system (Fuji Film Co. Ltd., Tokyo, Japan).

Results and Discussion

Influence of growth regulators on shoot induction

As shown in Figure 1 and Table 1, multiple shoots were induced from leaf blade-segments cultured on the medium containing 0.4 to 22.2 μM BAP. Approximately the same number of shoots was induced from both wild-type and albino plants. These results showed that the high concentration of NAA inhibited shoot induction (Table 1, Figure 1). The best result of shoot induction with root formation was obtained from the medium containing 4.4 μM BAP and 0.5 μM NAA, showing multiple shoots with 4.4±0.4 roots per explant after 6 week culture. The albino plants derived from the medium containing 4.4 μM BAP and 0.5 μM NAA were used for the next experiments. The regeneration frequency of the albino as well as the green plants is governed by many factors, such as culture method, media, phytohormones, and genotype and physiological status of albino plants (Grevelding et al., 1996; Tsukahara et al., 1996). Previous experiments of rice seed-derived albino callus showed that the regeneration frequency of the albino plants was reduced in liquid medium (Tsukahara et al., 1996). In the present work, no significant difference in the frequency of shoot induction was detected in the solid medium culture. It may be due to the physical factors associated with

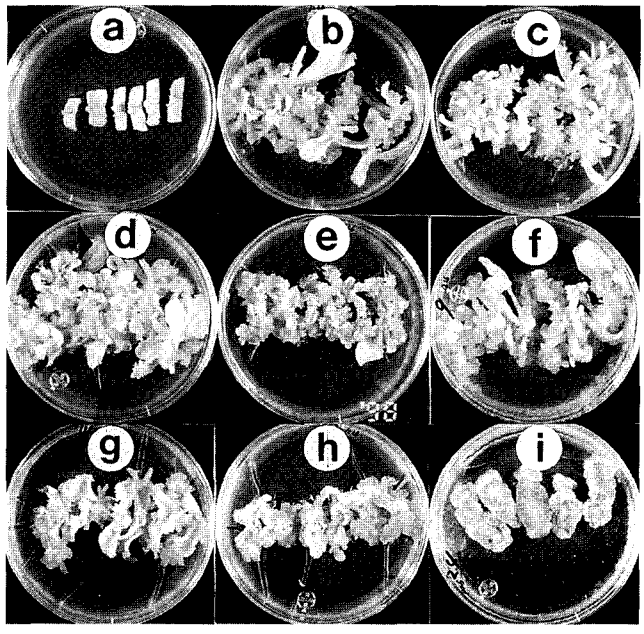

Figure 1. Organogenesis from leaf blade-segments of albino tobacco (*Nicotiana tabacum* L. cv. BY-4) cultured for 4 weeks on Murashige and Skoog media (1962) containing different concentrations of BAP (0, 0.4, 2.2, 4.4 and 22.2 μM) with or without NAA (0.5, 2.7, 5.8, 26.8 μM) described in Table 1.

Table 1. Effect of different hormonal combinations on shoot induction from leaf explants of wild-type and albino tobaccos (*Nicotiana tabacum* L. cv. BY-4). Results were scored after 5 weeks of culture.

Media	BAP (μM)	NAA (μM)	No. of explants[a]		No. of shoot per explant[b]	
			W[c]	A[d]	W	A
a	0.0	0.0	20	20	0.0±0.0	0.0±0.0
b	0.4	0.0	20	20	6.8±0.4	7.0±0.4
c	2.2	0.0	20	20	10.1±0.6	9.1±0.5
d	4.4	0.0	20	20	13.5±0.6	13.4±0.6
e	22.2	0.0	20	20	16.2±1.0	15.1±0.9
f	4.4	0.5	20	20	5.2±0.6	4.0±0.4
g	4.4	2.7	20	20	5.1±0.4	3.1±0.3
h	4.4	5.4	20	20	3.9±0.3	2.1±0.2
i	4.4	26.8	20	20	0.9±0.2	0.8±0.2

[a]The size of inoculum was about 4 mm×8 mm. [b]Numbers of shoots were counted over 2 mm in height. [c]wild-type. [d]albino mutant.

the characteristics of the liquid culture rather than nutrient components of the medium. Further investigations are needed to compare the frequency of shoot induction in different media, such as semi-solid medium and liquid medium.

Morphological characterization of albino plants derived from leaf explants

The foliage of the regenerated albino plants was yellowish-white in color. As shown in Table 2, the amount of chlorophylls and carotenoids in the albino plants were 0.3% and 2.0% of those of the wild-type plants grown under similar conditions, respectively. The chlorophyll in protoplasts of wild-type plants were observed by light and fluorescence microscopy (Figure 2-a, b). However, the light and fluorescence microscopy of albino protoplasts revealed chlorophyll-deficient cells (Figure 2-c, d). It has been reported that the transfer of bleached *Nicotiana tabacum* material, obtained from spectinomycin treatment, to spectinomycin-free medium allowed complete regreening (Zubko and Day, 1998). In this experiment, the leaves of albino plants cultured *in vitro* remained yellowish-white throughout the development (data not shown). This result suggests that the albino mutant has at least a defect in signal transduction pathway leading to the chlorophyll and terpenoid biosynthesis (Lichtenthaler, 1987; Mandel et al., 1996; Wetzel et al., 1994).

Tobacco leaves normally have more stomata on lower epidermis than upper one. Cultured albino plants have more stomata on both lower (100±10 mm^{-2}) and upper epidermis (60±5 mm^{-2}) than the wild-type plant which has 55±10 mm^{-2} and 45±6 mm^{-2} of stomata on lower and upper epidermis, respectively. Figure 2-e, f shows that the number of stomata on lower epidermis of albino plants was higher than that of wild-type plants. This result is consistent with the finding obtained from the spontaneously derived albino mutants (Sekiguchi, 1997).

Table 2. Pigment content of leaf blade-segment derived albino plants[a].

Sample	chl *a*	chl *b*	chl total	Carotenoids
Wild-type	1,525.0±288.5	640.3±22.9	2,172.3±265.4	180.2±19.9
Albino	3.0±0.8	4.1±1.3	7.1±2.4	3.6± 0.3

[a]Pigments were extracted from leaves of plants grown in Murashige and Skoog (1962) medium supplemented with 3% sucrose and analyzed as described in Materials and Methods. Pigment concentration is expressed as μg of pigment per gram of fresh weight.

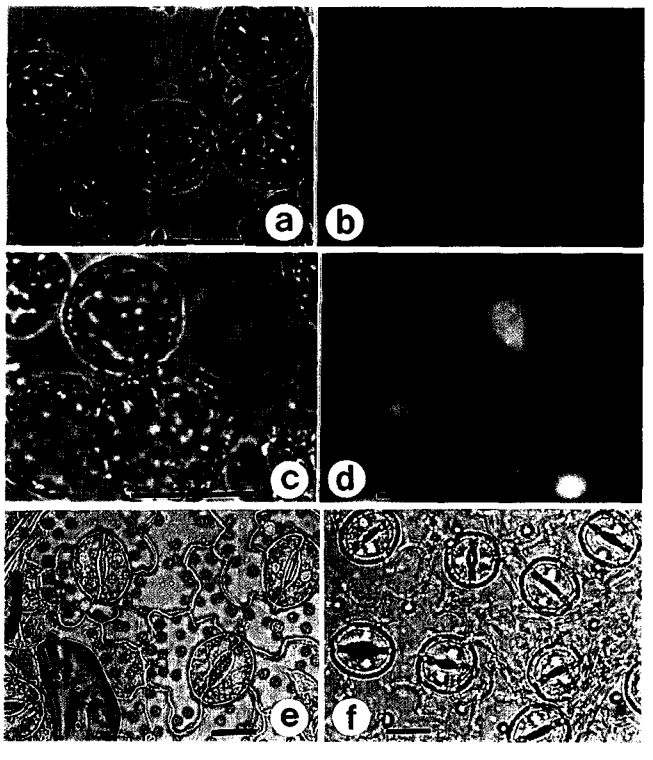

Figure 2. Chlorophyll-deficient plants of *Nicotiana tabacum* L. cv. BY-4 induced by *in vitro* culture of leaf blade-segments. (a) Protoplasts from leaves of wild-type (b) and albino plant. Scale bar, 30 μm. Chlorophyll fluorescence levels in protoplasts from wild-type (c) and albino (d) leaves. Albino plants are chlorophyll-less and do not fluorescence. Stomata at lower-epidermis of wild-type (e) and albino (f) plants.

Transcription of Photosynthetic Genes

In order to determine the transcription of genes related to chloroplast function, RNA blot analysis was performed using total RNA from leaves of wild-type and albino plants (Figure 3). Transcript accumulation of the albino plants was different for the two classes of genes. Transcript levels of the photosynthetic genes, *rbcL* and *psbA*, encoded by the chloroplast genome was drastically reduced in albino plants. In contrast, transcription levels of 23S and 16S rDNA in albino plants were at about half of those of wild-type plants. Thus, transcript levels of the genetic system genes, 23S and 16S rDNA were much less affected in albino plants. Transcripts for the nuclear-encoded chloroplast genes, cab and *rbcS*, were at the same level in both wild-type and albino plants (data not shown). Many of albino mutants showed significantly reduced transcriptions of the plastid genes for the photosynthesis *rbcL*, *psbA* as well as for the genetic system genes 16S rDNA, 23S rDNA (Han et al., 1993; Mandel et al., 1996; Sundberg et al., 1997; Zubko and Day, 1998). However, the transcription of those genes in this albino tobacco showed a rather similar pattern to that of *rpoB* deleted transgenic tobacco (Allison et al., 1996), indicating that our albino plants are different from the previous spontaneous mutants.

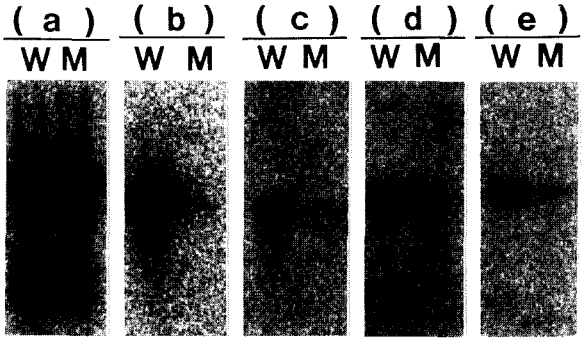

Figure 3. RNA gel blot analysis of leaf blade-segment derived albino plants. Total RNA was isolated from leaves of wild-type and albino plants. Total RNA (3 μg) was separated by electrophoresis, blotted onto a nylon membrane, and hybridized with 25S rDNA (a), *rbcL* (b), *psbA* (c), 16S rDNA (d) or 23S rDNA (e) probes. Transcript levels for photosynthetic genes are greatly reduced (b, c), but transcript levels for genetic system genes are much less affected (d, e).

Characterization of chloroplast genome and numbers of chloroplasts

DNA gel blot analysis was performed using the *rbcL* and *psbA*, and 16S rDNA as probes. The signal intensity of the plastid genes obtained by ECL detection system and RI labelling detection system showed the same manner (Figure 4, Table 3). There were no differences in number or size of bands in either wild-type or albino plants (Figure 4). Although equal amounts of total DNA from the leaves of albino and wild-type plants gave similar hybridization signals when nuclear DNA probes (25S rDNA) were used, the signals were approximately doubled in the albino than in the wild-type when plastid DNAs were used as probes (Table 3). Many of albino mutants showed deletion or great reduction of plastid genes (Day and Ellis, 1984; Dunford and Waldon, 1991; Harada et al., 1991; Hess et al., 1994), however, a few mutants showed an increase of plastid gene (Dunford and Waldon, 1991). It indicates that this albino mutant has different patterns of alteration of plastid DNAs. It is suggested that the

Table 3. Signal intensity of leaf blade-segment derived albino plants.

Genes	Signal intensity (PSL/A)[a]		Relative signal intensity	
	ALB	WT	ALB/WT	% ALB/WT
rbcL	115.4	47.4	2.4	216
psbA	342.5	144.4	2.3	211
16S rDNA	610.9	174.2	3.5	312
23S rDNA	184.2	85.3	2.1	192
25S rDNA	79.9	71.3	1.1	100

[a]Signal intensity (PSL/A) was detected by BAS 2000 system. Signal intensity of the plastid genes is approximately twice the intensity of nucleoid gene (25S rDNA).

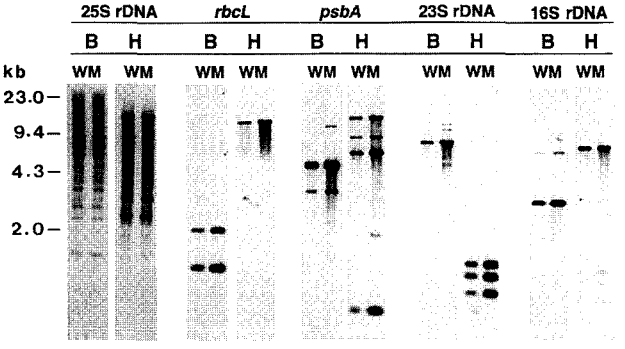

Figure 4. DNA gel blot analysis of chloroplast DNA from leaves of leaf blade-segment derived albino plants (*Nicotiana tabacum* L. cv. BY-4). Two micrograms of total DNA from the two tissues were digested with the enzymes indicated above each line and the digestion products were separated on a 0.8% agarose gel before being transferred onto nitrocellulose and hybridized. The sizes of major bands were estimated from restriction fragment markers. B: *Bam*HI, H: *Hin*dIII, W: wild-type plant, A: albino plant.

increase of amount of the plastid genes may resulted from either an increase of chloroplast number per cell due to the abnormal plastid division or an increase of chloroplast DNA copy number due to alterations of DNA replication in plastids (Koldner and Tewari, 1975; Wan et al., 1988).

In order to examine the reasons for the increase of the plastid genes, the number of chloroplasts (or plastids) per mesophyll cell were estimated by DAPI staining of nuclei per protoplast. The number of chloroplasts (plastids) per mesophyll cell in the albino plants was nearly the same as in wild-type plant (Figure 5a). Although cell sizes of albino plants were smaller than those of wild-type (Figure 5b), no significant differences in the numbers of plastids were detected. This result suggests that the albinism of the regenerated albino plant do not disrupt the normal pattern of plastid division, but disrupt subsequently the normal chloroplast development by blocking the pigmentation, and transcription. In addition, the increase in the amount of the plastid genes was not resulted from the increase in number of plastids per cell of the regenerated albino plants.

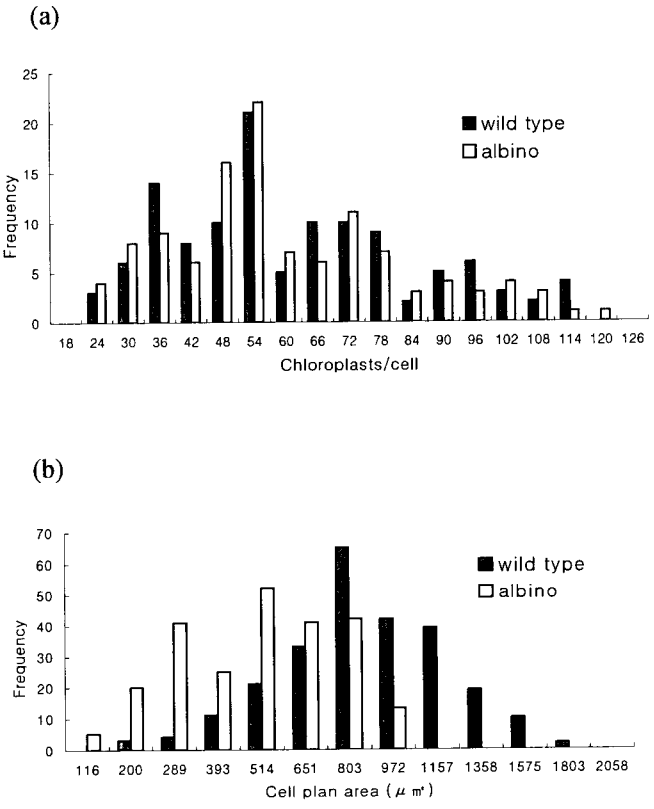

Figure 5. Distribution of chloroplast number per cell (a) and cell plan area (b) from leaf mesophyll of tobacco (*Nicotiana tabacum* L. cv. BY-4) plants. Approximately 250 protoplasts from 20 individual leaves and 100 protoplasts from 20 individual leaves were analyzed to get cell plan area (μm^2) and to compare chloroplast number, respectively.

In the present study, we demonstrated that albino plants have a direct multiple shooting system, although pigment contents and transcriptions of plastid genes were greatly reduced. The increase of the amount of plastid genes may resulted from an increase of ctDNA copy number in albino plants, making this mutant useful for analysis of pothosynthesis functions.

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