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Collection and analysis of *Arabidopsis* color mutants

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Abstract

It is highly possible that color mutants of *Arabidopsis* have abnormalities in chlorophyll biosynthesis steps or in the development of chloroplasts, where chlorophyll is accumulated. In this aspect, we are collecting and analyzing color mutants of *Arabidopsis* to study the biogenesis of chloroplasts. We screened seedlings from 35,000 ethyl methanesulfonate (EMS) mutagenized seeds of M2 generation with an average germination ratio of 47.7 %. Theoretically reached saturated mutagenesis, out of the mutant library we found 77 color mutant lines and characterized them. We found, by means of the quantification of chlorophyll content, that five lines showed a specific reduction of chlorophyll *b* content but not chlorophyll *a* content. A similar phenotype was reported with other lines carrying a mutation in the gene coding for chlorophyllide *a* oxygenase (*CAO*), which catalyzes the conversion of chlorophyll *a* to chlorophyll *b*. Complementation test was carried out by crossing all five lines with each other. Results indicate that mutations in at least two loci caused reduction of chlorophyll *b* content. One turned out to be a *CAO* mutation as determined using both chromosome mapping and complementation test with an established *CAO* mutant. The other was located near 35 cM of the 5th chromosome and was considered to be a novel mutation.

Introduction

The chloroplast is one of the most important organelles in plant cells, and it is involved in various metabolic pathways including photosynthesis and nitrogen assimilation. Almost all biological activities on earth are supported by the energy fixed through photosynthesis. With the knowledge of the structure and function of the chloroplast, photosynthesis can be analyzed at the molecular level, and the improvement of the efficiency of energy fixation can be expected. It will also lead to the un-

derstanding of the capability of starch and/or lipid storage, metabolism of substances including biosynthesis of amino acids, and biosynthesis of phytohormones.

A number of components required for the construction and development of plastids are encoded in the nuclear genome as well as in the chloroplast genome. Chloroplast genomes of higher plants have about 90 - 190 genes, depending on the plant species. In 1986, the complete nucleotide sequence of the tobacco chloroplast genome was determined, and the number of genes in the genome was estimated to be about 130 (Shinozaki et al., 1986), chloroplast genomes of *Arabidopsis*, spinach, rice, and pine were also sequenced and were found to have almost the same sizes and compositions (Sugiura and Takeda, 2000). However, these are only 2 - 10 % of the total number of genes required to develop a chloroplast. Most of the genes encoding components of the chloroplast are located in the nuclear genome. The expressions of chloroplast genes are governed and regulated by the chloroplast in cooperation with the nuclear genome (Sugita and Sugiura, 1996).

The *Arabidopsis* genome project was completed in December, 2000, and the gene information obtained is now available. The number of genes that encode products with a signal peptide which target the chloroplast was presumed to be 3,574, which is equivalent to 14.0 % of the total number of genes in the nuclear genome (The Arabidopsis Genome Initiative, 2000). However, the functions of about 50 % of these genes are not yet clear.

By increasing our understanding to the individual functions of the components of the chloroplast, and integrating them into the construction of the complex structures consisting chloroplast, our insight into the evolution of the chloroplast, into the diverged development to amyloplast, chloroplast, etioplast, and the other plastids, into the interactions between the organelle including nucleus in a cell and into the improvement of the maximum ability of photosynthesis will be broadened.

It is highly possible that color mutants of plant have abnormalities in the chlorophyll biosynthesis or in the development of chloroplasts. Mutations in chloroplast-related genes induce abnormalities in color of *Arabidopsis* (Ogren, 1994), for example, (i) deficiency in the synthetic pathway of pigments, such as chlorophyll and carotenoid (Tanaka et al., 1998; Espineda et al., 1999; Pogson et al., 1998), (ii) deficiency in the transcription / translation system in the chloroplast (Kanamaru et al., 2001), (iii) deficiency in the gene related to the transport into the chloroplasts (Pilgrim et al., 1998; Motohashi et al., 2000; Klimyuk et al., 1999), (iv) deficiency in the metabolism of components of the photosynthetic membrane (Dormann et al., 1995; Jarvis et al., 2000), (v) deficiency in the other component proteins of the chloroplast, that encoded

by the nuclear genome (Ogren, 1994).

In this aspect, we are collecting and analyzing color mutants of *Arabidopsis* from a large-scale mutant library, generated from 100,000 seeds, which were subjected to ethyl methanesulfonate (EMS) mutagenesis. EMS is the alkylating agent that adds an ethyl group, and changes a GC pair into an AT pair. EMS mutagenesis has the advantage over other mutagenesis in that it is able to acquire many kinds of mutants easily. Out of the mutant library we found 77 lines and characterized them.

Materials and Methods

All the *Arabidopsis* plants were grown on rock wool (NICHIAS, Tokyo, Japan) at 22 °C, a humidity of 40 % and an illumination of 3000 lux. Seeds of wild type (WT) Colombia were washed with 0.05 % Triton X-100, and treated with EMS at 0.2 % v/v for 16 hours at room temperature. The bud of the maternal line was dissected and stamens were removed before blooming. The stamen of the paternal line was pollinated to the stigma of a pistil. F1 seeds were collected from matured brown pods, two weeks after crossing. Two-week-old seedlings that germinated from M2 seeds derived from mutagenesis were screened based on color and shape. All putative mutants were individually transferred to new rock wools and grown to obtain M3 seeds. The mutant lines were classified into experimental groups systematically. Chlorophyll was extracted by soaking approximately 10 mg of fresh 3 ~ 5-week-old seedlings in 96 % ethanol for overnight at 4 °C in complete darkness. The extract was subjected to spectrophotometric measurements at 649, 665, and 750 nm. Specific chlorophyll content was calculated according to the following formulas (Wintermans et al., 1965) and normalized to the fresh weight (FW).

$$\text{Chlorophyll } a \text{ (μg / ml)} = 13.70(A_{665} - A_{750}) - 5.76(A_{649} - A_{750})$$

$$\text{Chlorophyll } b \text{ (μg / ml)} = 25.80(A_{649} - A_{750}) - 7.60(A_{665} - A_{750})$$

Two pale-green mutants (4-15 and 4-43) were crossed with L*er* plants. The pale-green seedlings from the F2 population were subjected to PCR-based SSLP analysis (Bell and Ecker, 1994) with commercially available primers (Research Genetics, Huntsville, USA). Leaves of the seedlings were collected and macerated at room temperature in a sterilized micro tube with 200 μl of extraction buffer [200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA and 0.5 % SDS], and left at room temperature for 10 minutes. The extracts were centrifuged at 15,000 rpm for 2 minutes and the supernatant was transferred to a fresh tube. Isopropanol (200 μl) was added to the supernatant and the mixture was left at room temperature for 2 - 5 minutes, followed by centrifugation at 15,000 rpm for 10 minutes. The pellet was dissolved in 50 μl of TE buffer (pH 8.0), and 1.0 μl of the solution was used for PCR

reaction.

Results

Isolation and collection of putative color mutants: The number of genes in the *Arabidopsis* genome is estimated to be about 25,000 genes (The Arabidopsis Genome Initiative, 2000). Thus, 100,000 seeds were treated with EMS, so that at least one mutant for all genes could be obtained (Jurgens et al., 1991). The candidate color mutants were isolated from 35,000 M2 seeds derived from 20,000 EMS-treated seeds until now. These M2 seeds were sown on rock wool as four times, followed by screening for abnormality of visual color, for example, pale-green, sectored and dark-green, and the putative color mutants were isolated individually (Table 1).

Table1: Screening profile of EMS-treated mutants based on color

Screening experiment	Parental group	M2 seeds sown	Germination ratio	Calculated average No. of lethal mutations	Putative mutants with alterd color phenotype		
					Total	Fertile	Heritable
1	1-1	20,000	0.43	3.27	73	32	19
4	2-2	5,000	0.78	0.99	33	21	20
5	3-1	5,000	0.41	3.57	49	21	19
6	3-2	5,000	0.43	3.27	75	38	19
total	20,000*	35,000	0.48**	2.99***	230	112	77

* Total number of EMS-treated parental seeds. 5,000 seeds were treated with EMS for each parental group.
** Average germination ratio of the mutant library tested in this study.
*** Average number of lethal mutations of the mutant library tested in this study.

Many of the isolated candidates showed poor growth and/or sterile. The phenotype of the M3 generation of the fertile line was determined in order to check whether they are inherited. Consequently, 77 lines of color mutants were obtained and classified based on their phenotype. Of these lines, 26 showed variegation and several mutants were found to have chloroplasts with abnormal shapes.

Quantitative analysis of chlorophyll content: In order to quantitatively evaluate their color, the chlorophyll contents of 58 mutants were measured. Figure 1 shows the results of the quantitative analysis of chlorophyll content. We identified 24 lines of mutants whose chlorophyll content is decreasing (< 1 μg / mg fresh weight) compared with that of the WT. The chlorophyll content correlated with visual color, generally. However, reduction of chlorophyll content was seen in a few lines, although the degree of their color change is less than that of the WT.

The chlorophyll *a*/*b* ratio showed a nearly constant value in almost all lines of the color mutant. However, five mutant lines showed a relatively lower chlorophyll *b* content than chlorophyll *a* content (Figure 1). These mutants are pale-green color,

and can easily be distinguished from the WT visually, these mutants were analyzed further.

Isolation and genetic analysis of chlorophyll-b-reduced mutants: Figures 2 and 3 show the photographs of the pale-green and chlorophyll-*b*-reduced mutants and the measured chlorophyll contents, respectively. In all five mutants, 1-52, 4-14, 4-15, 4-28, and 4-43, chlorophyll *a* content decreased to about 60 - 70 % of the WT, and chlorophyll *b* content decreased to 15 - 35 % of the WT. Normally, the chlorophyll *a*/*b* ratios of the WT and the other color mutants ranged from 2 to 3, but that of the chlorophyll-*b*-reduced mutants was larger than 4.5.

All of these five mutants carry a recessive mutation. To determine whether these mutants are caused by the mutation in the same locus, mutant lines were crossed mutually. As a result of complementation analysis, F1 plants of all crosses between 4-14, 4-15, 4-28, and 1-52 showed a phenotype of reduced chlorophyll *b* content, indicating that these plants carry a mutation in the same locus (Figure 4). Only the pro-

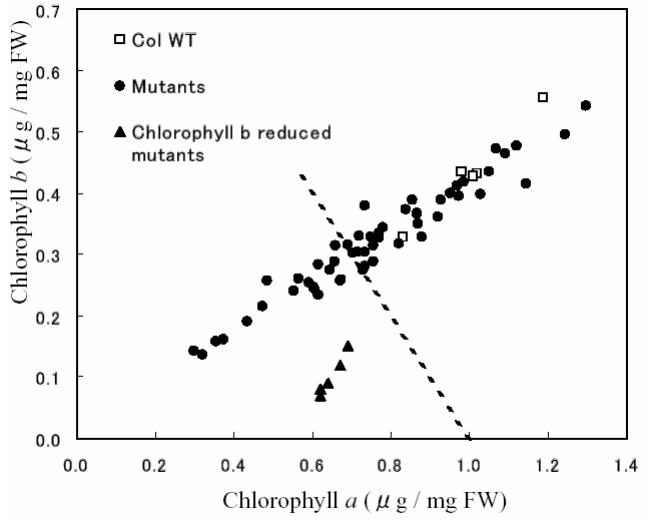

Figure 1: Quantitative analysis of chlorophyll content. The chlorophyll *a* and chlorophyll *b* contents are plotted. Symbols are explained in the figure. Mutants that inherited color phenotypes were subjected to quantification of the contents of chlorophyll *a* and *b*. One symbol represents one mutant line. Five Col WT plants were represented to refer to variation in chlorophyll content. The diagonal dashed line indicates 1 μg / mg FW of total chlorophyll content. (FW: fresh weight)

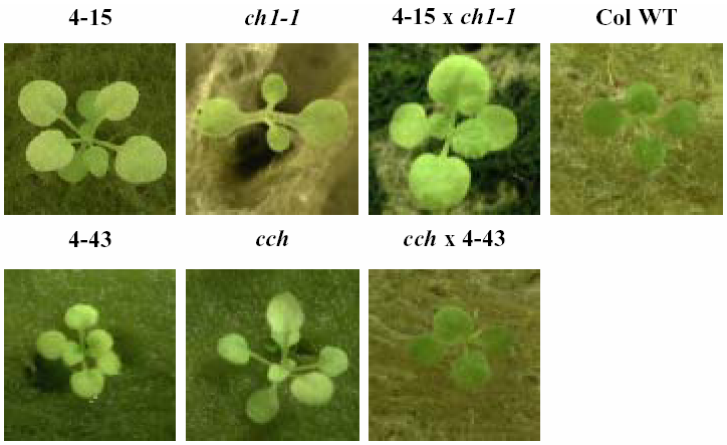

Figure 2: Photographs of chlorophyll-*b*-reduced mutants. Seedlings are 2 -3 weeks old.

geny of 4-43 showed a phenotype that same as WT, suggesting that 4-43 belongs to another group. 4-15 and 4-43 were chosen for chromosome mapping to determine their mutation loci; *pcb1, pcb2* (*p*ale-green and *c*hlorophyll-*b*-reduced).

4-15 was crossed with L*er* plants, and the resulting pale-green seedlings were selected from the F2 population for PCR-based SSLP analysis. The chlorophyll *a*/*b* ratios of the PCR-analyzed plants were measured to confirm the phenotype of reduced chlorophyll *b* content. Consequently, *pcb1* was found to be located close to the SSLP marker, ciw1, which is located near the center of the 1st chromosome (data not shown).

The *ch1* mutation responsible for the pale-green phenotype accompanied by specific reduction in chlorophyll *b* content was reported to be located near ciw1. The *ch1* mutant has a mutation in the gene cording for chlorophyll *a* oxygenase (*CAO*), which catalyzes the conversion of chlorophyll *a* to chlorophyll *b* (Espineda et al., 1999). The F1 progeny from the cross between 4-15 and *ch1-1*, showed a pale-green color (Figure 2) and reduced chlorophyll *b* content (Figure 5), indicating that *pcb1*s in 4-14, 4-15, 4-28, 1-52 and *ch1-1* are allelic mutations of the *CAO* gene, because all *pcb1*s and *ch1* are recessive gene.

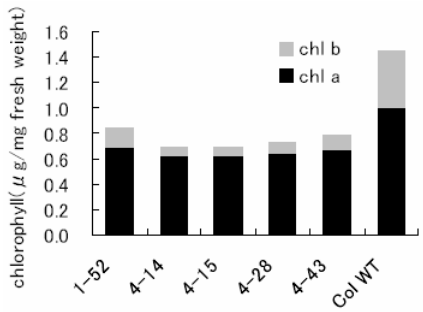

Figure 3: Chlorophyll content of chlorophyll-*b*-reduced mutants. 3 ~ 5-week-old plants are used for experiment. The averages of three measurements are represented. Chlorophyll *a* content is presented as a black bar and chlorophyll *b* content as a gray bar.

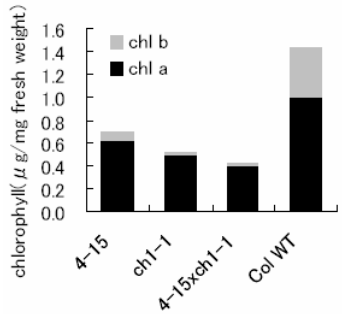

Figure 4: Complementation analysis of chlorophyll-*b* -reduced mutants. Average chlorophyll content of 3 ~ 5-week-old F1 plants of all crosses between five mutants are compared. n = 2. Bars are same as those in Figure 3.

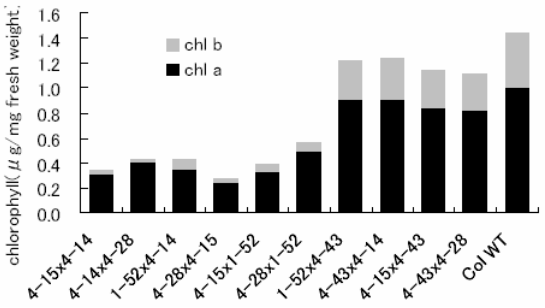

Figure 5: Complementation analysis of *CAO* mutants. Average chlorophyll content of two 4-week-old samples. Bars are same as those in Figure 3.

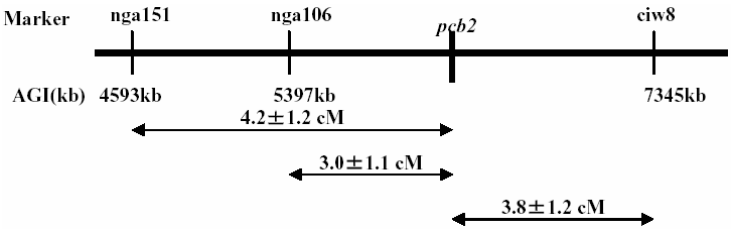

Figure 6: Chromosomal position of *pcb2*. The *pcb2* locus was mapped to chromosome 5 of the *Arabidopsis* genome. Only a small region of the upper arm of chromosome 5 is shown. The right-hand of the diagram is toward the centromere, and the left-hand is toward the telomere. The observed recombination crossovers are 11 in 264 chromosomes between nga151 and *pcb2*; 8 in 266 chromosomes between nga106 and *pcb2*; and 10 in 264 chromosomes between *pcb2* and ciw8. The positions of the markers are represented under the solid bar as kilobase pair of the *Arabidopsis* Genome Initiative (AGI) sequence.

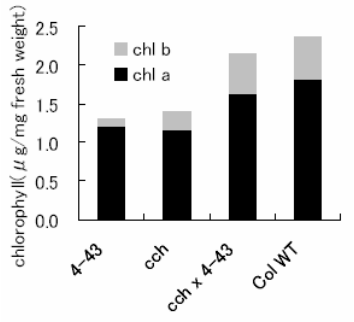

Figure 7: Complementation analysis of 4-43 mutant. Average chlorophyll content of three 4-week-old samples. Bars are same as those in Figure 3.

Similarly, the position of *pcb2* in the *Arabidopsis* chromosome map was determined. *pcb2* was located in the upper arm of the 5th chromosome (Figure 6). *cch*, the mutation of which is responsible for the pale-green phenotype with a specific reduction in chlorophyll *b* content, was mapped near this locus (Mochizuki et al., 2000). The *cch* mutant and 4-43 bearing *pcb2* are crossed, but the F1 progeny from this cross had green color and their chlorophyll content is comparable to that of the WT (Figure 7).

Discussion

In this study, a theoretically saturated mutant library was constructed and screened for visual color mutants. The theoretical saturation was calculated as described below. The majority of lethal mutations are assumed to be recessive. When one recessive lethal mutation exists in a genome, 3/4 of the M2 generation will survive. Similarly, when N recessive lethal mutations exist in a genome, $(3/4)^N$ of the M2 generation will survive.

The mutant library, generated by EMS, is thought to be a mixture of genomes containing various number of lethal mutations. The probability of the existence of the line that contains N lethal mutations is defined as R_N. For example, R_0 denotes the probability of a genome lacking lethal mutations. The probability of lethal mutations is thought to follow the Poisson distribution as $R_N = \frac{e^{-\lambda} \lambda^N}{N!}$,

where λ is the average number of lethal mutations.

Since the total germination ratio (G) of the library is considered to be the summing of multiplications [the probability of lines containing lethal mutations (R_N) x the survival rate of progeny of N lethal mutations $(3/4)^N$] as $G = \sum_N \left[\frac{e^{-\lambda} \lambda^N}{N!} \times \left(\frac{3}{4} \right)^N \right]$

Using the practical germination ratio, the average number of lethal mutations of parental groups was calculated and the results are shown in Table 1. Then, the average number of lethal mutations of the total was estimated to be 2.99. If it is assumed that one fifth of all genes are essential genes whose disruption leads to lethality (Jurgens et al., 1991), then the average number of all (not only lethal) mutations per line is 15. In addition, according to Jurgens et al., the number of lines required for saturation is calculated to be about 8,500 in this study. Here, at least 5 alleles per gene would be isolated on the average to achieve statistical saturation.

Based on the *Arabidopsis* genome sequence reported, only 4,000 of the total 25,498 genes are unique (The Arabidopsis Genome Initiative, 2000). The other genes consist of family genes capable of compensating each other's functions even when one of the family members is disrupted. Being defined essential genes as ones required for survival, that means the disruption of which cause lethal, and ruled out the possibility of simultaneous mutations in family genes, only part of 4,000 unique genes should be counted in "essential". Thus, we consider the number of essential genes is much fewer than Jurgens's estimate of 5,000. This idea is supported by some results from the tagging project. Generally, in the tagged lines, only a few genes are disrupted simultaneously in individual lines. It shows that few of the tagged lines are lethal, in-

dicating that it is hard to hit essential genes. When we calculate the number of essential genes less, "the average number of mutations per genome" will increase, and causing to decrease of "the number of lines required for saturation". Therefore, it is thought that the number of lines screened in this experiment has reached the level of statistically saturated mutagenesis. Furthermore, the lines should be backcrossed several times to remove a mutation unrelated to plastid biogenesis.

The number of mutant lines in which the chlorophyll content decreased was unexpectedly small. Because chlorophyll contents of the WT is generally unstable, we strictly selected the mutants which revealed clear diminution of chlorophyll contents, which might result in produce fewer mutants than expected. Also, we selected mutant candidates who can grow on rock wool in open air and fertile for next generation, that might neglect plants with severe albino phenotype.

Five color mutants that exhibited specific reduction of chlorophyll *b* content were analyzed. Chlorophyll *b* is synthesized from chlorophyll *a* and is found solely in the peripheral part of the antenna complex. On the other hand, chlorophyll *a* is found in every part of photosystems, the central part and the peripheral part. The size of the antenna in the central part is usually constant even the changes in light intensity. On the contrary, the antenna size and the components of the peripheral part change in response to light environments. Plants usually control the chlorophyll *a*/*b* ratio in order to adjust their light-capturing capacities in their light environments. Such adaptation is widely studied by plant ecologists and physiologists (Tanaka et al., 2001). However, the mechanism of the regulation of the antenna size and the chlorophyll *a*/*b* ratio is not yet elucidated at the molecular level.

Only three mutants that exhibited specific reduction of chlorophyll *b* content were reported. One has a mutation in the gene coding for chlorophyll *a* oxygenase (*CAO*), which catalyzes the conversion of chlorophyll *a* to chlorophyll *b* (Espineda et al., 1999). The other two have a mutation in the genes coding for the Mg chelatase H subunit (*Chl H*) and Mg chelatase I subunit (*Chl I*), respectively. Mg chelatase catalyzes the addition of Mg^{2+} to protoporphyrin forming Mg-protoporphyrin (Mochizuki et al., 2000; Rissler et al., 2002).

The *cch*, that has a mutation in the gene coding for *Chl H*, also exhibits *gun* (*g*enomes *un*coupled) phenotype, and expresses nuclear-encoded *Lhcb* and *RbcS* transcripts in the absence of chloroplast development. *gun* mutants are studied in order to understand the mechanisms of the plastid-to-nucleus signal transduction pathways. It is reported that the *Chl H* subunit of Mg chelatase is necessary for plastid signal transduction but the *Chl I* subunit is not, because *chl I* mutants do not exhibit a *gun*

phenotype (Mochizuki et al., 2000).

Because *CAO* is directly involved in chlorophyll *b* biosynthesis, it is reasonable that the mutation reduces chlorophyll *b* content. However, why is chlorophyll *a* content also reduced during *CAO* mutation? Similar question arises in the case of *Chl H*, *Chl I* mutations. Why dose not the reduction of chlorophyll *b* content paralled with that of chlorophyll *a*? It is apparent that the control mechanisms of the total chlorophyll content and that of chlorophyll *a*/*b* ratio are not same, because many mutants with reduced chlorophyll content didn't change in chlorophyll *a*/*b* ratio (Figure 1).

Interestingly, chlorophyll biosynthesis, the regulation of chlorophyll *a*/*b* ratio, and the plastid-to-nucleus signal seem to be related each other. By studying a new chlorophyll-*b*-reduced mutant 4-43, a new insight into the relationship of chlorophyll biosynthesis and control mechanisms under plastid signal transduction would be obtained.

Although variegation phenomenon was observed widely in the plant kingdom, only four lines of variegated mutants of *Arabidopsis* have been reported so far (Aluru et al., 2001; Chen et al., 2000; Takechi et al., 2000; Martinez-Zapater, 1992). A large number of variegated lines were obtained in this experiment by employment of the large-scale screening. In one variegated plant, cells display different fates of chloroplast development, in spite of their genetic information in common. The difference in sensitivity to the signals in the cells might determine the cell fate. Analyzing the gene of these variegated mutants, the mechanisms of cooperation between the nucleus and chloroplast are expected to be elucidated.

One of the morphological abnormalities of chloroplast we observed is the expansion of chloroplasts. It is considered to be a problem in the division mechanism of the chloroplast. The accumulation and replication of chloroplast (*arc*) mutants were reported as the expansion mutations of the chloroplast (Pyke and Leech, 1992; Pyke et al., 1994; Robertson et al., 1995; Robertson et al., 1996; Marrison et al., 1999). However, the phenotype of our mutants does not correspond to that of these mutants. In this study, only about 1/10 of color mutant lines are screened based on the chloroplast shape. It's expected to find more mutants, such as those with abnormalities in the chloroplast structure, which will provide variable information on the mechanism of chloroplast and plastid formation.

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