Characterization and Inheritance of 'Variegated-leaf' Mutant in *Petunia hybrida*

Chika Aoki, Tomikichi Wada, Takao Nishimura and Kazumi Hattori

School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan

Summary

An F₁ hybrid plant of *Petunia hybrida* 'Violet' × 'White' produced a spontaneously mutated branch with leaves displaying white or yellow variegation on the margin. Morphological observation showed that the mutant had abnormal plastids which caused the white or yellow leaf color. The polypeptide pattern of the thylakoid protein in the variegated area differed from that in normal leaf and green area of the mutant. The data relating to selfing and back-crossing of the mutant with normal 'Violet' and 'White' showed that the abnormal plastids were almost totally maternally inherited, that is, the progenies from crosses with the mutant as female parent were almost all albino. However, variegated progenies were also occasionally observed when the mutant was used as the female parent. The results of this study suggest that normal chloroplasts were transmitted exceptionally from paternal parent or from green plastids occasionally kept in the generative cells of the maternal parent.

Key Words : *Petunia hybrida*, variegated leaf, plastid, thylakoid protein, maternal inheritance.

Introduction

An F₁ hybrid plant of *Petunia hybrida* 'Violet' × 'White' produced a spontaneously mutated branch with variegated leaves. The leaf of the mutated branch showed a pale green color with white or yellow variegation of irregular width on the margin (Fig. 1). The mu-

Fig. 1. Variegated leaves of the mutant.

tated branch has been propagated by rooted cutting.

Chlorophyll variegation has been attributed to the nuclear gene system. For example, the recessive allele of *lojap* (*ij*) induces plastid mutation, resulting in the formation of white or yellow striped leaves in maize (Rhoades 1943, 1946).

Chlorophyll variegation can also be ascribed to the presence of periclinal chimeras. Based on observations of cross-sections, Burk *et al.* (1964) and Stewart and Burk (1970) reported that periclinal chimeras gave rise to variegations of leaves in tobacco.

Another cause is the biparental inheritance of plastids. In *Pelargonium*, embryos with both normal green plastids from the maternal parent and mutant white plastids from the paternal parent were produced by biparental inheritance (Tilney-Bassett and Almouslen 1989). Corriveau and Coleman (1988) reported that cytological observation with an epifluorescence microscope using the DNA-fluorochrome, 4, 6-diamidino-2-phenylindole (DAPI), enabled to screen the mode of transmission for plant species capable of biparental inheritance of plastid DNA, and 18 % of 235 angiosperm species (187 genera, 80 families) were considered to inherit the plastids from both parents. In their report, the results of cytological observation and genetic evidence for the mode of inheritance were in agreement in 42 species. Consequently, plastid DNA was detected in generative and/or sperm cells from biparentally inherited species, and, not detected in maternally inherited species. However, results based on cytological and genetic data in 5 species were conflicting. Van Went (1984) suggested that there are four types of paternal plastid inheritance during fertilization in higher plants : a) generative cells lack plastids *ab initio* ; b) generative cells contain plastids which are eliminated during subsequent cell development ; c) plastids are present in both generative and sperm cells, whereas no plastids are transmitted to the zygote ; d) plastids in sperm cells are transmitted during fertilization. The plants of the a)-c) types inherited maternally, show plastids in the sperm cells which disappeared before fertilization. In the biparental inheritance, paternal plastids were transmitted to the zygote.

In the present study, we attempted to characterize the mutant and compared it with the normal plant based on the phase of leaf surface, the plastid form and polypeptide pattern of the thylakoid protein. The morphological differences in the plastids between the normal leaf, green area and variegated area (white or yellow area) of the mutant leaf were observed by light

Received March 23, 1994. Accepted December 22, 1994.

microscope and transmission electron microscope (TEM). The mode of inheritance of the white or yellow leaf color is also described.

Materials and Methods

In the mutant plant propagated by rooted cutting, the plant type, flower shape and flower color were identical with that of the normal F₁ hybrid. The plant materials used in this study were the mutant, normal 'Violet', 'White', the reciprocal F₁ hybrids of 'Violet' and 'White', the progeny derived from selfing of the mutant and 4 reciprocal backcross strains of the mutant with normal 'Violet' and 'White'.

For the leaf surface observation, the fresh leaf of normal 'Violet' × 'White' and variegated area of the mutant were frozen in liquid nitrogen and observed by scanning electron microscope (SEM) (Hitachi, S-2300 with a cryo-stage). To observe the plastid morphology, sample preparation for TEM (Jeol, 100 CX) and light microscopy was performed according to the method of Wada and Sasakawa (1993) and Wada *et al.* (1993).

For the analysis of the thylakoid protein, leaf tissues were ground with glass beads in liquid nitrogen to crush cells completely, and the soluble components were removed by centrifugation at 17,000 xg with an equal volume of grinding buffer (0.35 M sorbitol, 50 mM Tris-PO₄ pH 7.4, 2 % 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride (PMSF)). The pellet was sus-

pended in the grinding buffer and centrifuged at 700 xg to remove high density fractions (glass beads, debris, etc.). The supernatant was centrifuged at 17,000 xg. The supernatant, which contained soluble protein, was supplemented with 8 M urea, 5 % sodium dodecyl sulfate (SDS) and 20 % sucrose at final concentration, and then, denatured by incubation for 20 min at 60 °C. The pellet was washed once in the washing buffer 1 (10 mM Na pyrophosphate pH 7.4, 2 % 2-mercaptoethanol, and 1 mM PMSF), and twice in the washing buffer 2 (35 mM Tris-HCl pH 6.7, 2 % 2-mercaptoethanol, and 1 mM PMSF). Thylakoid protein was dissolved with the extraction buffer (35 mM Tris-HCl pH 6.7, 1 mM EDTA-2Na, 5 % SDS, 5 % 2-mercaptoethanol and 8 M urea), and incubated for 20 min at 60 °C to undergo denaturation. The denatured protein was analyzed by tricine-SDS-polyacrylamide gel electrophoresis according to the method of Scheaggar and Jagow (1987). Urea concentration in the separation gel was 6 M. The separating gel consisted of 12 % T and 2 % C, while the stacking gel of 5 % T and 5 % C. Low Molecular Weight Calibration Kit (Pharmacia Fine Chemicals) was electrophoresed with the samples as a molecular weight marker. Electrophoresis was performed under a constant current of 25 mA. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

Fig. 2. Scanning electron micrographs of normal 'Violet' × 'White' (a, b) and variegated area (c, d). (a) and (c) leaf surface. (b) and (d) stomatal guard cell.

Results

The normal leaf and the variegated area of the mutant were observed by SEM (Fig. 2). The leaf surface in the variegated area was rougher than that of the normal leaf (Fig. 2a, c) and the stomatal guard cells were jutting (Fig. 2d).

By light microscopy, normal lens-shaped chloroplasts containing starch granules were observed in the periphery of normal vacuolated leaf cells (Fig. 3a). In the variegated area of the mutant, although a few chloroplasts were detected in the epidermal cells, no normal chloroplasts were found in the mesophyll cells (Fig. 3b). Green area of the mutant leaf showed small and abnormal plastids in the outer part of the palisade and spongy parenchyma cells. In the inner regions near vascular strands, normal chloroplasts were observed. However, the difference in the distribution of the cells with normal plastids and those with abnormal ones was distinct in the area adjacent to the inner region displaying plastids with a normal shape, while the outer region showed plastids with an abnormal shape (Fig. 3c).

The plastids in the variegated area by TEM differed morphologically from normal chloroplasts. Normal green leaves displayed normal spindle-shaped chloroplasts whose thylakoid membrane systems were well developed (Fig. 4a). In the variegated area, the plastids were abnormal. The plastids in the epidermal cells showed a regular shape and a membrane system to some extent, whereas the mesophyll cells exhibited plastids irregular shape in which almost no membrane system could be found (Fig. 4b).

Different polypeptide patterns were obtained between thylakoid protein and soluble protein from the leaves of the normal 'Violet' × 'White', green area and variegated area of the mutant (Fig. 5). No differences between the polypeptide pattern of the thylakoid protein derived from the normal leaves of 'Violet', 'White' and the reciprocal F_1 hybrids and that of green area of the mutant were detected. However, in the polypeptide pattern of the variegated area, some bands were missing or faint (Fig. 6, open arrows), while some bands were added or intensified (Fig. 6, arrowheads).

Most of the seedlings of the self-propagated mutant were albino, and soon died prior to the expansion of the foliage leaf. However, variegated seedlings were obtained at low frequencies (<1 %) by self-propagation of the mutant. In about 1,600 progenies of the mutant × 'Violet' and mutant × 'White' (i.e. mutant × normal), all of the seedlings were albino, except for five variegated seedlings derived from the cross of mutant × 'Violet'. Therefore, it was considered that if the mutant is used as a female parent, seedlings of the progenies will be mostly albino. The variegated seedlings grew to normal plants and flowered. In some plants, all the leaves were variegated. In other plants, branches with green, variegated and white leaves grew separately. The cross-

Fig. 3. Light micrographs of cross sections of normal 'Violet' × 'White' (a), variegated area (b) and green area (c). Arrows point to normal plastids and arrowheads to abnormal plastids. EP : epidermis, N : nucleus. Bar represents 50 μ m.

sing of a normal green plant ('Violet' and 'White') as a female parent with the mutant as the male parent yielded green progenies only.

Discussion

The morphological characteristics of the variegated leaf were observed by SEM, light microscope and TEM. In the mutant, the leaf surface showed a different appearance from that of normal leaf, and, both normal and abnormal plastids existed. In the abnormal plastids, the membrane organization was destroyed and the polypeptide components of the thylakoid protein were altered. Only abnormal plastids were observed in the variegated area. In the green area, the distribution of abnormal plastids in the epidermis and subepidermal region resulted in the pale color. Abnormal chloroplast

Fig. 4. Transmission electron micrographs of a normal chloroplast in the leaf of normal 'Violet' x 'White' (a) and an abnormal plastid in variegated area of the mutant (b). Bar represents 1 μm.

Fig. 5. Polypeptide patterns of thylakoid protein and soluble protein. The lanes of 'Violet' x 'White' show polypeptide patterns from normal leaves. The lanes of 'Green' and 'White variegation' show patterns from green and variegated area of the mutant, respectively. T and S indicates thylakoid and soluble protein. LS and SS indicate the large and small subunits of ribulose biphosphate carboxylase.

ultrastructure and changes in the polypeptide composition the thylakoid protein were also detected in the chlorophyll deficient mutant of pea (Stummann and Henningsen 1980).

The genetic analysis by selfing and crossing of the

Fig. 6. Polypeptide patterns of thylakoid protein. The lanes of 'Violet', 'White', 'Violet' x 'White' and 'White' x 'Violet' show polypeptide patterns from normal leaves. The lanes of 'Green' and 'White variegation' show patterns from green and variegated area of the mutant, respectively. LS and SS indicate the large and small subunits of ribulose biphosphate carboxylase. In 'White variegation', arrowheads point to added or intensified bands, and open arrows point to missing or faint bands compared to other patterns.

mutant indicated that the abnormal plastids were almost always maternally inherited, suggesting that the egg cell of the mutant had only abnormal plastids. However, variegated progeny with normal plastids were occasionally observed when the mutant was used as the female parent.

In the case of maternal inheritance, the disappearance of organelle DNA in the male parent was observed. The stages of the disappearance differ with species. In *Chlamydomonas reinhardtii*, maternal inheritance of chloroplast genes occurred by preferential degradation of chloroplast DNA of the male after mating based on examination by phase-contrast microscopy of DAPI-stained DNA (Kuroiwa *et al.* 1982). In contrast, male derived chloroplast DNA of *Bryopsis* and *Chara* disappeared during male-gametogenesis (Kuroiwa and Hori 1986, Ogawa 1988, Sun *et al.* 1988). In angiosperm, the stages of the disappearance of organelle DNA during pollen maturation were investigated under a fluorescence microscope by DAPI staining. It was ascertained that the disappearance of organelle DNA occurred at various stages in every species (Miyamura *et al.* 1987). While, paternal inheritance of chloroplast DNA was observed in gymnosperm (Szmidt *et al.* 1987, Neale and Sederoff 1989). Organelle DNA is inherited maternally in many higher plants. In a Yellow-virescent mutant (Yv) of *Petunia hybrida*, transmission of plastid-DNA from the paternal parent was detected (Cornu and Dulieu 1988).

Based on the results of this study the following assumptions could be made: normal chloroplasts were exceptionally transmitted from the paternal parent or

from normal organelles occasionally kept in generative cells of the maternal parent. The fertilized egg cell displayed both normal and abnormal plastids, and during growth, the plastids were somatically segregated.

Literature Cited

- Burk, L. G., R. N. Stewart and H. Dermen (1964) Histogenesis and genetics of a plastid-controlled chlorophyll variegation in tobacco. *Amer. J. Bot.* 51 : 713-724.
- Corriveau, J. L. and A. W. Coleman (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *Amer. J. Bot.* 75 : 1443-1458.
- Cornu, A. and H. Dulieu (1988) Pollen transmission of plastid-DNA under genotypic control in *Petunia hybrida* Hort. *J. Heredity* 79 : 40-44.
- Kuroiwa, T., S. Kawano, S. Nishibayashi and C. Sato (1982) Epifluorescent microscopic evidence for maternal inheritance of chloroplast DNA. *Nature* 298 : 481-483.
- Kuroiwa, T. and T. Hori (1986) Preferential digestion of male chloroplast nuclei and mitochondrial nuclei during gametogenesis of *Bryopsis maxima* Okamura. *Protoplasma* 133 : 85-87.
- Miyamura, S., T. Kuroiwa and T. Nagata (1987) Disappearance of plastid and mitochondrial nucleoid during the formation of generative cells of higher plants revealed by fluorescence microscopy. *Protoplasma* 141 : 149-159.
- Neale, D. B. and R. R. Sederoff (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theor. Appl. Genet.* 77 : 212-216.
- Ogawa, S. (1988) Disappearance of chloroplast nucleoids during male gamete formation in *Bryopsis plumosa* (Hudson) C. Ag. (Chlorophyceae). *Bot. Gaz.* 149 : 25-29.
- Rhoades, M. M. (1943) Genic induction of an inherited cytoplasmic difference. *Proc. Nat. Acad. Sci.* 11 : 327-329.
- (1946) Plastids mutation. *Cold Spr. Harb. Symp. Quant. Biol.* 11 : 202-207.
- Scheagger, H. and G. von Jagow (1987) Tricine-sodium dodecyl sulfate-polyacrylamide electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166 : 368-379.
- Stewart, R. N. and L. G. Burk (1970) Independence of tissues derived from apical layers in ontogeny of the tobacco leaf and ovary. *Amer. J. Bot.* 57 : 1010-1016.
- Stummann, B. M. and K. W. Henningsen (1980) Characterization of chlorophyll deficient mutants of pea. *Heredity* 93 : 261-275.
- Sun, G.-H., T. Q. P. Uyeda and T. Kuroiwa (1988) Destruction of organelle nuclei during spermatogenesis in *Chara corallina* examined by staining with DAPI and anti-DNA antibody. *Protoplasma* 144 : 185-188.
- Szmidt, A. E., T. Alden and J. Hällgren (1987) Paternal inheritance of chloroplast DNA in *Larix*. *Plant Mol. Biol.* 9 : 59-64.
- Tilney-Bassett, R. A. E. and A. B. Almouslem (1989) Variation in plastid inheritance between *Pelargonium* cultivars and their hybrids. *Heredity* 63 : 145-153.
- Went, J. L. van (1984) Unequal distribution of plastids during generative cell formation in *Impatiens*. *Theor. Appl. Genet.* 68 : 305-309.
- Wada, T., H. Miyake, H. Suzuki, K. Suzuki and Y. Takeoka (1993) An improved method for light microscopy using extraction techniques of Spurr's resin sections. *Jpn. J. Crop. Sci.* 62 : 128-129 (In Japanese).
- and H. Sasakawa (1993) Light and electron microscopy of stem nodule development in *Aeschynomene indica* under induced condition. *Proceeding of the 7 th International Congress of SABRAO* (in press).