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A comparison of plants regenerated from a variegated *Epipremnum aureum*

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Abstract

In order to study chloroplast biogenesis, we chose natural variegated *Epipremnum aureum* (golden pothos) and regenerated pale yellow, variegated and green plants from all three types of tissue explants. The percentage of three types of regenerated shoots from three different explants was very close. Regenerated plants have been maintained for a year and show no sign of a colour switch. By comparing their protein profiles, two major differences between pale yellow and green plants were observed at the 15 and 40 to 50 kDa proteins. Moreover, pale yellow plants had unexpected high molecular mass proteins (greater than 60 kDa). Both variegated and green plants had more chlorophyll (Chl) *a* than Chl *b*, the ratios were about 1.46 and 1.93, respectively. In contrast, the pale yellow plants not only had less total Chl, but also the reduction of Chl *a* was much greater than Chl *b*, resulting in a higher content of Chl *b* than Chl *a*. Microscopic analysis revealed that pale yellow plants contained predominantly undeveloped chloroplasts with low Chl contents, even though their mesophyll cells were similar to green and variegated plants. PCR amplification of chloroplast DNA with 14 universal chloroplast primers did not reveal any difference among these regenerated plants.

Additional key words: chlorophyll content, chloroplast morphology, PCR, protein profiles.

Introduction

Variegated plants are widely present in nature and many of them are cultivated as ornamental plants. However, not many studies of natural variegated plants have been reported. They can help in the understanding of how chloroplasts develop and are maintained (Sakamoto 2003, Aluru *et al.* 2006, Yu *et al.* 2007). Variegated leaves usually consist of a mixture of green and white or yellow sectors. The studies of an *Arabidopsis* variegated mutant *yellow variegated 2* (*var2*) demonstrated that the white sectors existing in variegated leaves were not dead cells, rather these cells contained undifferentiated chloroplasts (Chen *et al.* 2000, Kato *et al.* 2007).

Plant variegation has been used as a genetic trait that led to the discovery of non-Mendelian inheritance (Tilney-Bassett 1975), which may be caused by mutations in nuclear and/or organellar encoded genes that result in pigment-deficiency leading to white or pale yellow cells (Wetzel *et al.* 1994, Yu *et al.* 2007). Recently, studies on variegated mutants of *Arabidopsis* such as *immutans* (Aluru *et al.* 2001), *var1* and *var2* (Sakamoto 2003), and *sca3* (Hricova *et al.* 2006), and tobacco periclinal chimera and *vdl* (Bae *et al.* 2000, Wang *et al.* 2000), as well as monocot barley *albostrians* and maize *yellow stripe 1* (Curie *et al.* 2001, Yaronskaya *et al.* 2003) have provided scientists with a great deal of knowledge about chloroplast biogenesis. Since there are an estimated about 100 plastid-encoded proteins and an additional 3 500 nucleus-encoded proteins targeted to the chloroplast (Peltier *et al.* 2002), the study of a few mutants is not sufficient to understand the functions and interactions of these proteins. Many current studies focus on *Arabidopsis* variegated mutants because of the availability of both *Arabidopsis* genomic information and many mutants, including a large number of T-DNA insertion lines (Yu *et al.* 2007). The major challenge of using *Arabidopsis* mutants is their low biomass, which limits the analysis of some low abundance proteins. The other limitation of using variegated mutants for studies of

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Abbreviations: CPPU - *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea; MS - Murashige and Skoog's medium; NAA - naphthaleneacetic acid; PCR - polymerase chain reaction.

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chloroplast biogenesis is the difficulty of dissecting the green and white sectors from variegated leaves, making comparative analysis of protein profiles from different sectors nearly impossible due to a normalization problem (Kato *et al.* 2007).

Golden pothos, a natural variegated plant, is an herbaceous evergreen native to Southeast Asia and the Solomon Islands (Huxley 1994). It has been widely grown as an ornamental plant because of its tolerance of low light environments and climbing habit (Chen *et al.* 2005). Its regeneration system has been well established (Zhang *et al.* 2005) which made it possible to regenerate plants from both green and pale yellow cells. In addition, golden pothos is easily propagated and can be kept in a vegetative growth state for years. In previous reports, however, no further observation of different colours of regenerated plants has been mentioned. If green and white cells can be separated and plants can be regenerated, these materials could be unlimited sources for chloroplast biogenesis study.

For this purpose, we adapted the method derived from Zhang *et al.* (2005) to induce shoots from leaf, stem and petiole explants. Herein, we report that we regenerated pale yellow, variegated and green plants, and performed comparison analysis on the Chl content, chloroplast morphology, total leaf protein and partial chloroplast genome among three types of regenerated plants. These plants will be a valuable tool to study chloroplast biogenesis.

Materials and methods

The shoot tips with young leaves and stem were excised from golden pothos [*Epipremnum aureum* (Linden and Andre) Bunt.] and surface sterilized with 70 % ethanol for one min, then rinsed twice with sterile water. They were then separated into three parts - leaves, petioles and stems, and further treated with 14 % Clorox and 1 % Tween 20 for 15 min. After rinsing with sterile water 5 times, stems and petioles were cut into 1 cm long segments and leaves were cut into a 1 cm^2 area for tissue culture.

The tissue culture method was the same as described by Zhang *et al.* (2005). The somatic embryo induction medium in their report, which contained MS basal plus vitamins (*PhytoTechnology Laboratories*, Shawnee Mission, KS, USA), 25 g dm^{-3} sucrose, 2 mg dm^{-3} *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU), 0.2 mg dm^{-3} naphthaleneacetic acid (NAA), and 7 g dm^{-3} agar (pH 5.7), was employed in the current study. Each Petri dish was inoculated with 5 explants. An average of 40 explants from a total of 8 replicates was taken for statistical analysis. They were incubated in a growth chamber under a 16-h photoperiod (irradiance of 50 μmol m^{-2} s^{-1}) and temperature of 25 °C. Regenerated shoots were observed and counted after culturing for 10 weeks. For rooting, shoots were transferred onto a hormone free MS medium.

Cross-section of mature leaves from green, variegated and pale yellow plants were observed under a light microscope (*Leica*, Houston, TX, USA). Microscopy images were collected using a *MicroPublisher 5.0* cooled RTV camera (*QImaging*, Burnaby, BC, Canada) and processed in *Adobe Photoshop*.

The Chl content was measured as described by Hanfrey *et al.* (1996). Leaf tissues from regenerated plants were harvested and ground in liquid nitrogen. Chl was extracted in acetone and quantified by *DC® 800* spectrophotometer (*Beckman Coulter*, Fullerton, CA, USA). An average of three independent experiments was taken for statistical analysis.

The leaf tissues were used for DNA preparation with the *DNeasy®* plant mini kit (*Qiagen Sciences*, Valencia, CA, USA). DNA was quantified by a spectrophotometer *ND-1000* (*NanoDrop Technologies*, Wilmington, DE, USA). PCR was performed in a thermocycler (*Biometra*, Göttingen, Germany). Each 0.025 cm^3 reaction mix contained 50 ng DNA templates, 1.25 units of Taq DNA polymerase (*Sigma*, St. Louis, MO, USA), and a final concentration of 0.2 mM dNTP, 300 nM of each primer, 2 mM $MgCl_2$. The initial denaturing step was 94 °C for 2 min, then followed by 31 amplification cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature for 40 s and elongation at 72 °C for 1 min, plus the final extended elongation at 72 °C for 10 min. Fourteen of a total of thirty-two pairs of universal chloroplast primers (Cheng *et al.* 2006), which were able to amplify chloroplast DNA are listed in Table 1. The amplified DNA was resolved on a 2 % agarose gel with 0.5 mg dm^{-3} ethidium bromide and visualized under UV by the *FluoChem™* imaging system (*Alpha Innotech Corpporation*, San Leandro, CA, USA).

For total protein analysis, leaf tissue was first ground into powder in liquid nitrogen, and then extracted in homogenizing buffer as described in Weigel and Glazebrook (2002). Protein content was determined by the Bradford method using the *Bio-Rad* protein assay reagents (*Bio-Rad*, Hercules, CA, USA). Proteins were separated by SDS-PAGE on a 4 -12 % SDS gradient gel (*Invitrogen*, Carlsbad, CA, USA) that ran with a SDS low-range running buffer (30 mM MOPS, 60 mM Tris, 0.1 % SDS, and 2.5 mM sodium bisulfite, pH 8.3). Proteins were stained with a Coomassie Blue solution for 6 h and de-stained for 16 h. Protein molecular mass was compared to the *Novex ® Sharp* pre-stained protein standard (*Invitrogen*).

Table 1. Fourteen pairs of universal chloroplast primers in golden pothos and their PCR products. [a] and [b]: PCR products of CCMP4 and ARCP6 were larger than previously reported in Cheng *et al.* (2006).

Code	Chloroplast genome location	Primer 1 (5'-3')	Primer 2 (5'-3')	Tm [°C]	PCR [bp]
NTCP9	trnG/trnR intergenic region	CTTCCAAGCTAACGATGC	CTGTCCTATCCATTAGACAATG	50	210
NTCP40	rpsl2/trnH intergenic region	GATGTAGCCAAGTGGATCA	TAATTTGATTCTTCGTCGC	50	150
CCMP2	5' to trnS	GATCCCGGACGTAATCCTG	ATCGTACCGAGGGTTCGAAT	53	230
CCMP3	trnG intron	CAGACCAAAAGCTGACATAG	GTTTCATTCGGCTCCTTTAT	50	100
CCMP4	atpF intron	AATGCTGAATCGAYGACCTA	CCAAAATATTBGGAGGACTCT	50	270[a]
CCMP5	3' to rps2	TGTTCCAATATCTTCTTGTCATT	AGGTTCCATCGGAACAATTAT	50	100
CCMP6	intergenic region	CGATGCATATGTAGAAAGCC	CATTACGTGCGACTATCTCC	53	100
CCMP10	rpl2/rps19	TTTTTTTTTAGTGAACGTGTCA	TTCGTCGDCGTAGTAAATAG	50	100
ARCP4	16S rRNA/trnV	CAATTCGGGATTTTCCTTGA	GAGCGAAGGGGTACGAAATA	53	250
ARCP5	trnL/trnF intergenc region	GGCCATAGGCTGGAAAGTCT	GTTTATGCATGGCGAAAAGG	53	210
ARCP6	rpl33/rps18	GGCTCCACAATGGAATTGAC	GCACATTTCAGCGTCACAAA	53	350[b]
ARCP8	trnD/trnT intergenic region	TCATGAATTGTCGGATGACTC	TTGCTTCAACCCGTCAACTA	50	150
ARCP9	psbA/trnK intergenic region	GAAAAATGCAAGCACGGTTT	TACGATCCGTAGTGGGTTGC	50	100
ARCP11	ndhB/rps7 intergenic region	GAGCGAAGGGGTACGAAATA	CAATTCGGGATTTTCCTTGA	50	250

Results

After four to six weeks of culture on the induction medium, somatic embryos formed at the cut edges of stems, petioles or leaves (Fig. 1). Embryos were white or green in clusters or individuals as observed by Zhang *et al.* (2005). The percentages of shoot induction from explants of stems, petioles and leaves were 90, 90 and 68 %, respectively, which is very close to a previous report by Zhang *et al.* (2005), except a lower shoot induction in leaf explants (Table 2). Among these shoots, we found three different types of colours - green, pale yellow and variegation (Fig. 1). More than half of them were green shoots, and 18 and 21 % were pale yellow and variegated shoots, respectively (Table 2). This result implied that golden pothos are composed of green and pale yellow cells that can be separated by tissue culture. The percentages of green, pale yellow and variegated shoot occurrences were very similar for each type of explants used. This suggested that the composition of cells of the original golden pothos not only was a mixture of green and pale yellow cells, but also the overall

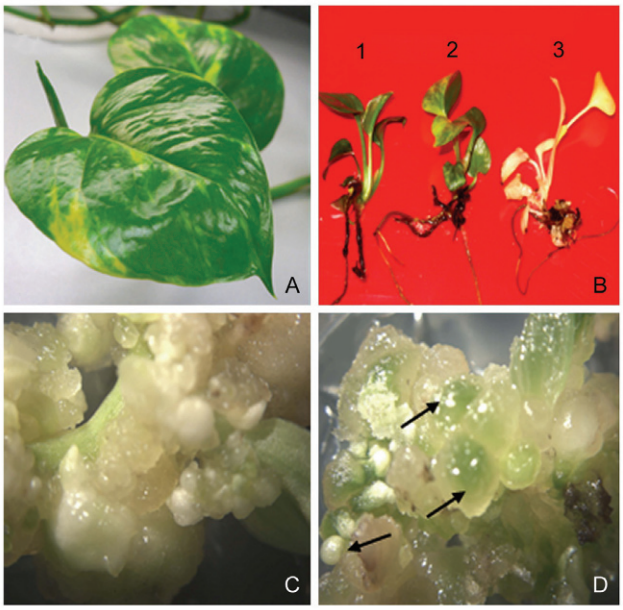

Fig. 1. Golden Pothos tissue culture and three types of regenerated plants. The house plant Golden Pothos has a characteristic of variegated leaves (*A*). Green (1), variegation (2), pale yellow (3) regenerated plants (*B*). A petiole explant was placed on the induction medium to induce somatic embryo formation (*C*). Green and white globular embryos (*D*).

ratio of green and pale yellow cells was the same throughout the whole plant body. Green and variegated shoots rooted well on rooting medium whereas pale yellow shoots rooted slowly with fewer roots. All three types of regenerated plants grew well in MS medium and have been maintained for a year. No colour switch has been observed indicating that individual green or pale yellow regenerated plants were genetically different from each other.

Table 2. The rate of shoot regeneration from different types of explants. A total of 40 shoot tips were used to obtain three types of explants. Each explant was separated from individual shoot tips.

Explants	Numbers of explants with shoots	Total induced shoots	Pale yellow shoots	Variegated shoots	Green shoots
Stems	36	193	35	47	111
Petioles	36	170	27	28	115
Leaves	27	82	19	20	43

The Chl contents of three types of regenerated pothos were compared. The content of Chl *a* and *b* was very different among them (Table 3). On average, the green plants contained 1.2 mg(Chl *a*) and 0.62 mg(Chl *b*) g^{-1}(f.m.) with the Chl *a/b* ratio of 1.93. In contrast, in the pale yellow plants, the Chl content was greatly reduced with about an 80 % reduction in Chl *a* and a 50 % reduction in Chl *b*. Interestingly, both Chl *a* and *b* levels in the variegated pothos were the average of those in green and pale yellow, strongly suggesting that the regenerated plants are from the separation of two types of cells.

Table 3. Chl *a* and *b* contents in three types of regenerated plants [mg g^{-1}(f.m.)]. Data are the average of three independent experiments ± standard deviation. In each experiment, harvested tissues were from newly developed leaves from the same cluster of regenerated shoots.

	Green	Pale yellow	Variegated
Chl *a*	1.20 ± 0.20	0.21 ± 0.10	0.77 ± 0.03
Chl *b*	0.62 ± 0.14	0.32 ± 0.14	0.53 ± 0.15
Chl *a/b*	1.93	0.66	1.46

In green leaves, most of the chloroplasts were present in mesophyll cells which consisted of one layer of palisade mesophyll cells and 3 layers of spongy mesophyll cells (Fig. 2*A*). In variegated and pale yellow leaves, the structure of mesophylls were similar to the

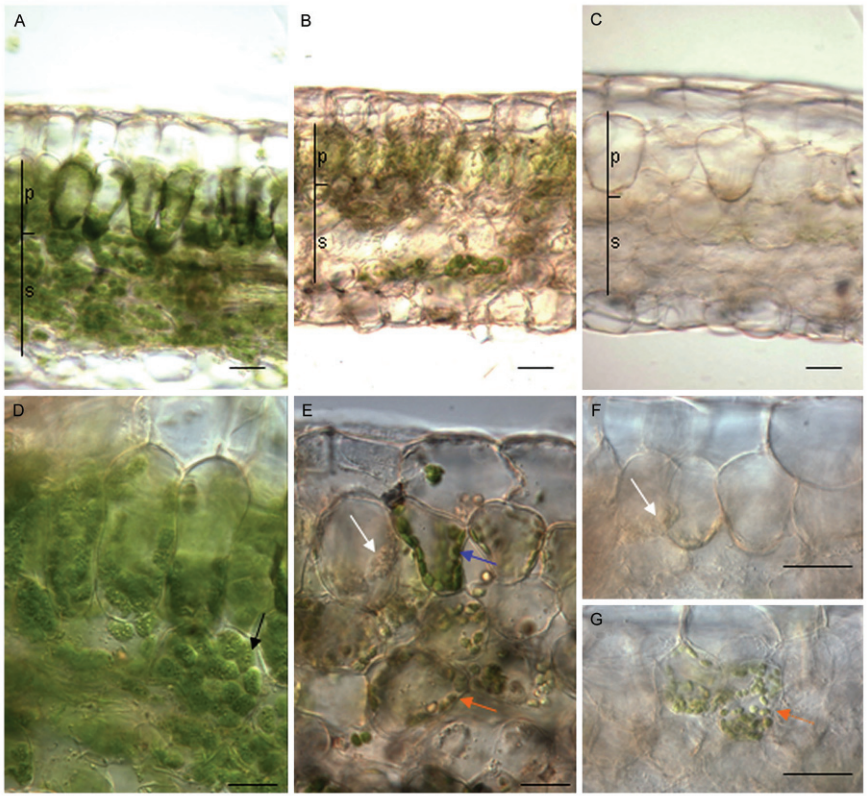

Fig. 2. Cross-sections of leaves from three types of regenerated plants green (*A*), variegated (*B*) and pale yellow (*C*) showed a similar distribution of the palisade (p) and spongy (s) mesophyll cells. The *black arrows* point to the normal chloroplasts in green plants (*D*). In variegated plants (*E*), three types of different developmental stages of chloroplasts were observed - green chloroplasts (*blue arrow*), brown chloroplasts (*orange arrow*), and plastids (*white arrow*). Most parts of pale yellow leaf (*F*) have undeveloped plastids (*white arrows*). Occasionally, in pale yellow leaves (*G*) brown chloroplasts could be found (*orange arrow*). *Bars* = 50 µm.

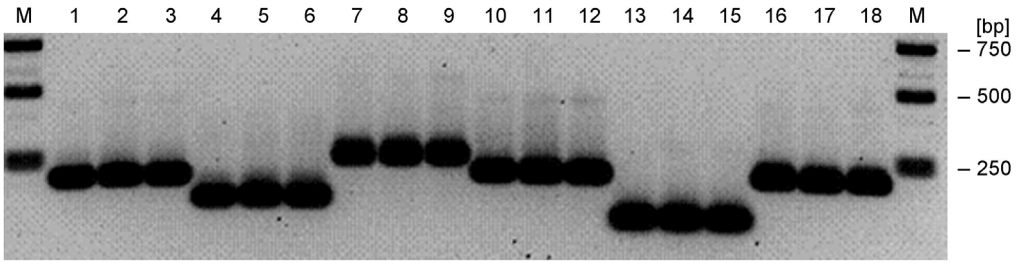

Fig. 3. PCR of golden pothos chloroplast genome. DNA isolated from green (*lanes* 1, 4, 7, 10, 13 and 16), pale yellow (*lanes* 2, 5, 8, 11, 14 and 17) and variegated (*lanes* 3, 6, 9, 12, 15 and 18) shoots were amplified by PCR using specific primers that targeted the chloroplast genome, which were NTCP9 (*lane*s 1, 2 and 3) , NTCP40 (*lanes* 4, 5 and 6), CCMP4 (*lanes* 7, 8 and 9), CCMP2 (*lanes* 10, 11 and 12), CCPM6 (*lanes* 13, 14 and 15), ARCP5 (*lanes* 16, 17 and 18). The reference marker is 0.5 μg of 1 kb DNA ladder.

green leaves (Fig. 2*B*,*C*), but their chloroplasts were strikingly different in both colour and size. Most of the variegated chloroplasts were light green or brownish and some were colourless which could be undeveloped plastids. Their sizes were smaller compared to normal chloroplasts (Fig. 2*D*,*E*). In pale yellow leaves, most of the cells contained colourless, undeveloped plastids (Fig. 2*F*). However, occasionally one or two spongy cells with light green chloroplasts were observed (Fig. 2*G*). The results were consistent with the measurements of Chl contents.

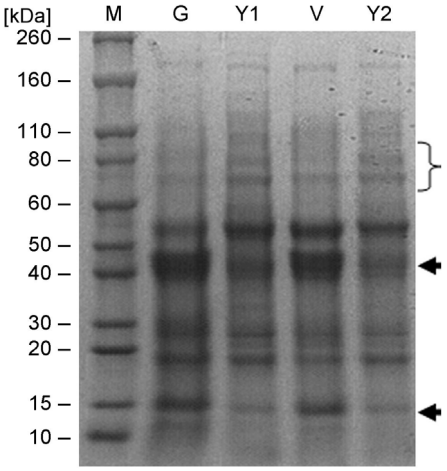

Fig. 4. SDS-PAGE (4 - 12 %) of protein patterns of green (G), variegated (V) and two pale yellow sibling plants (Y1 and Y2). Equal amounts of protein (45 μg) were used. The marker and its molecular masses were indicated. Two *arrows* point to the regions that show the greatest differences among these samples. The bracket points to proteins with molecular mass greater than 60 kDa in pale yellow leaves.

Among the universal chloroplast primer pairs applied, 14 of them amplified PCR products from golden pothos total genomic DNA. All of these amplified products have similar sizes as reported by Cheng *et al.* (2006), except the two products derived from primers CCMP4 and ARCP6 (Table 1), which had sizes of 270 bp and 350 bp. Their reported sizes were much smaller at 125 bp for CCMP4 and 200 bp for ARCP6 (Cheng *et al.* 2006). No difference in band intensity or size among the three types of plants was observed in PCR products amplified by the 14 pairs of primers (Fig. 3). The results indicate that the cause of the pale yellow colour is unlikely to be due to a major loss of chloroplast genome, but they cannot rule out the possibility of point mutations or short deletions.

In general, the green plants had a similar protein band pattern as the variegated ones (Fig. 4). The two siblings of yellow pothos (Y1 and Y2) were similar in band pattern, but differed from the green and variegated plants with major differences at the 15 kDa and the 40 to 50 kDa regions. The yellow plants had a greatly reduced level of protein in these two regions. When examined further, pale yellow plants may have a wider spread of high molecular mass proteins, greater than 60 kDa.

Discussion

Unlike albino mutants, which are normally lethal or severely stunted in growth, the variegated mutants are widely present in nature. They have been considered as a special genetic material for non-Mendelian inheritance study (Tilney-Bassett 1975) and recently, for the investigation of chloroplast biogenesis by comparing green and white or pale yellow cells in the same leaf (Sakamoto 2003). Finding molecules caused variegation has become a popular route for studying chloroplast biogenesis and chloroplast-nuclear interaction. Comparing the morphology, chloroplast development, organellar and nuclear genomic DNA, and the transcriptional and translational products of the green and white sectors have become a way to find the important clues for above purpose.

Previous study with *Arabidopsis var2* mutant indicated that its white sectors were formed by viable cells with undifferentiated plastids, and their phenotypes were different from those of cell death mutants (Kato *et al.* 2007). We hypothesized that both green and pale yellow cells in golden pothos leaves are alive, and can be regenerated into fully-developed plants. After adapting the method developed by Zhang *et al.* (2005), we regenerated pale yellow, variegated and green plants. Regenerated plants have been maintained for a year

without any sign of color switch implying that the leaf variegation of golden pothos was not caused by transposable elements. It has been reported that the comparative analysis of protein profiles from green and white sectors failed because of a dissection problem (Kato *et al.* 2007), thereby, regenerated green and pale yellow plants provide a good source of experimental material for subsequent biochemical and genetic analyses.

Using regenerated plants, their protein profiles have been compared. The major differences were at the regions of 15 and 40 - 50 kDa. We could not tell whether those proteins around 40 - 50 kDa were encoded by chloroplast or nuclear genes, however, the 15 kDa proteins that were less intense in pale yellow plants were likely the nuclear encoded small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Rodermel 1999). However, the 55 kDa band equivalent to the large subunit did not exhibit much difference in all three types of plants. Surprisingly, pale yellow plants had a wider spread of high molecular mass proteins greater than 60 kDa. The cause of the accumulation of large proteins in pale yellow plants has not been previously reported. It could be due to either less efficient proteolysis or greater synthesis of large proteins. Identifying these proteins may facilitate the elucidation of the mechanism of golden pothos variegation.

In pale yellow plants, Chl *a* and Chl *b* were reduced about 80 and 50 %, respectively, which was great enough to produce the pale yellow colour. Additionally, the Chl *a*/*b* ratio was changed. The cause of this higher Chl *b* than Chl *a* phenomenon is not clear, but keeping the Chl *a*/*b* ratio stable is thought to be very important in various physiological conditions (Rudiger 2002). The huge reduction of Chl *a* with an unbalanced Chl *a*/*b* ratio led us to believe that the photosynthetic complexes are probably not well-assembled in pale yellow plants. The much lower Chl *a* also implies that the variegation may be caused by the impairment of genes in the Chl synthetic pathway. Leaf variegation can arise *via* nuclear or organellar gene mutations (Aluru *et al.* 2001, Sakamoto 2003, Hricova *et al.* 2006, Wetzel *et al.* 1994). In the current case, variegated golden pothos may be caused by a mutation in a gene which affects a small group of key genes in chloroplast biogenesis, resulting in the accumulation of some unexpected high molecular mass proteins and an unbalanced Chl *a*/*b* ratio in pale yellow cells. Further characterization of differentially expressed genes in regenerated plants may uncover the cause.

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