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Development of a highly responsive leaf-based regeneration system for Peperomia species

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Abstract: Several species in the genus *Peperomia* Ruiz & Pav. have giant chloroplasts in the palisade parenchyma of their leaves. Because of this unique feature, *Peperomia metallica* L. Linden & Rodigas has become a valuable model plant for studying plastid biology. However, the use of *Peperomia* for molecular studies has been limited by the lack of efficient regeneration and transformation protocols. In this work, we aimed to develop a reliable regeneration system for *Peperomia* from leaf explants. Using MS medium complemented with different combinations of phytohormones, a highly efficient in vitro regeneration protocol was developed for *P. metallica* and *P. peduncularis*, another species with giant chloroplasts. Regeneration occurs through direct organogenesis from leaf discs at a very high frequency. Our protocol can provide a reliable tissue culture system for future *Peperomia* genetic transformation experiments.

Key words: *Peperomia*, tissue culture, giant chloroplast, regeneration, gibberellic acid

Introduction

The genus *Peperomia* belongs to the family *Piperaceae* and comprises approximately 1500-1700 mostly tropical species, making it one of the largest genera of basal angiosperms (Wanke et al., 2006). Several species of *Peperomia* grow quickly and vary considerably in appearance, making them a fine choice for gardeners. A few species are also popular as house plants. One well-known species in the genus is *Peperomia metallica*, which was reported to contain giant chloroplasts in its palisade parenchyma cells (Schürhoff, 1908) (Figure 1A). The palisade parenchyma of *P. metallica* largely consists of a single cell layer with chloroplasts as large as 20-25 µm in diameter (Neumann, 1973) (Figures 1B and 1C). The large size of the chloroplasts is compensated for by a much smaller chloroplast number, which is in the range of only 2-6 per palisade cell (Bartels, 1965). Because of this unique feature, *P. metallica* chloroplasts have been a preferred model object of electrophysiological studies (Bulychev et al., 1972). In addition, the large size of the chloroplasts might make chloroplast transformation more efficient than in species with normal chloroplasts, which are nearly 10 times smaller. Thus, a reproducible tissue culture system facilitating the efficient genetic engineering of this model plant will be of particular

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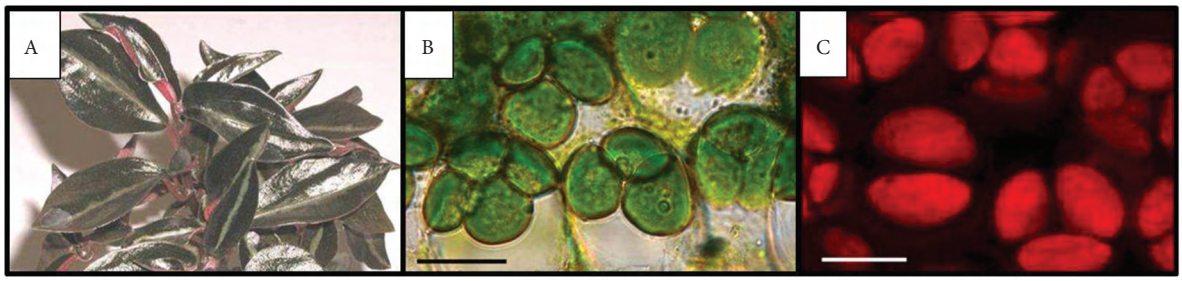

Figure 1. Plant morphology (A) and chloroplasts (B, C) in palisade mesophyll cells of *Peperomia metallica*. Image (B) was taken from thin leaf cross sections using high-resolution light microscopy and image (C) shows visualisation of chloroplasts by confocal laser-scanning microscopy based on chlorophyll fluorescence in leaf palisade cells. Scale bars: 20 μm.

interest for studying plastid biology. Development of a highly efficient regeneration system will provide an alternative propagation system for existing methods, including cuttings and propagation by seeds. In this work, we aimed to develop a tissue culture system for *Peperomia* that can be established from leaf material with high efficiency.

Materials and methods

Two *Peperomia* species, *P. metallica* and *P. peduncularis*, were used for tissue culture experiments. Plants were obtained from the botanical gardens of Freiburg and Berlin. For surface sterilisation, shoot tips of 1-2 cm long were incubated in 70% ethanol for 30 s. The plant segments were then washed with sterile water under aseptic conditions and transferred to Magenta boxes containing 50 mL of MS medium (Murashige & Skoog, 1962). Leaves of young sterile plants were cut into approximately 3 × 3 mm pieces. Leaf segments were then cultured on MS medium complemented with 20 g L^{-1} sucrose and different combinations of hormones, as stated in Table 1. The pH level was adjusted to 5.8 and the media were solidified by adding 7.4 g L^{-1} agar (Duchefa Biochemie B.V., the Netherlands). After 5 weeks without subculture, regeneration was evaluated and data were noted. The adopted experimental design was completely randomised with 3 replicas. The statistical comparison was performed by one-way ANOVA using SPSS software, and means were compared using Duncan's test ($P < 0.05$).

Table 1. Hormones, and their concentrations, that were tested for their effects on the in vitro regeneration response of *Peperomia* from leaf segments.

Medium	IAA (mg L^{-1})	BAP (mg L^{-1})	Zeatin (mg L^{-1})	GA_3 (mg L^{-1})
1	1	-	2	-
2	0.5	-	2	-
3	0.3	-	2	-
4	0.2	-	2	-
5	1	3	-	-
6	2	1	-	-
7	0.5	3	-	-
8	0.3	3	-	-
9	0.1	1	-	-
10	0.2	3	-	-
11	0.15	3	-	0.007
12	0.15	3	-	0.014

Gibberellic acid (GA_3) was filter-sterilised (0.2 µm, Whatman, UK) and added to the medium after autoclaving. For regeneration, cultured plates were incubated in Percival chambers (CLF Plant Climatics, Emersacker, Germany) under a diurnal cycle of 16 h light of 25 mE m^{-2} s^{-1} at 25 °C, followed by an 8 h dark period at 20 °C.

In order to study chloroplasts in the leaf cells of *Peperomia* species, thin cross sections of

50-100 µm were prepared from young leaves using a Leica vibratome (Leica Microsystems, Wetzlar, Germany). Samples were analysed by light microscopy with an Olympus BX41 high resolution light microscope (Olympus America Inc., Melville, NY, USA) connected to an Olympus U-CMAD3 microscopy camera. The cell^P imaging software (Olympus) was used to capture light microscopy images. A Leica TCS SP2 spectral laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to analyse chlorophyll autofluorescence (excitation: 543 nm, emission: 590 nm) in leaf cells.

Results

In this work, we attempted to develop an efficient leaf-based regeneration protocol for *Peperomia* species. First, MS medium supplemented with various hormone combinations at different concentrations (Table 1, Figure 2) was tested. Leaves from plants grown under aseptic conditions were cut into 3 × 3 mm pieces and placed onto the medium on their adaxial sides. When IAA and zeatin (Zea) were added to the medium in different concentrations, no regeneration was observed (Table 2, Figure 2). Replacing zeatin with BAP resulted in a very poor in vitro regeneration response (Figure 2). When low concentrations of GA_3 were applied in addition to IAA and BAP, all leaf segments responded well to in vitro regeneration, resulting in 100% regeneration efficiency from the cultured leaf explants (Table 2, Figure 2). These results suggest that gibberellic acid plays a significant role in the response of *Peperomia* somatic cells to in vitro regeneration via organogenesis. However, the effects of other concentrations of the tested hormones, especially zeatin and gibberellic acid, as well as of untested hormones such as thidiazuron (TDZ), which has been shown to have a significant effect on shoot induction, remain to be investigated (Lincy & Sasikumar, 2010; Sahai et al., 2010).

Note that the plantlets could readily form adventitious roots at a very high efficiency (100%) when transferred to growth regulator-free half-strength MS medium. Rooted plants were transferred to soil and acclimated successfully (Figure 3).

Having established an efficient regeneration system for *P. metallica*, we were interested to test whether the system is also applicable for other *Peperomia* species containing giant chloroplasts. We therefore analysed several *Peperomia* species and identified *P. peduncularis* (Figure 4A) as another species in the genus that contains giant chloroplasts in the leaf palisade cells (Figures 4B and 4C). We then established a sterile culture of *P. peduncularis* and tested plant regeneration from leaf segments on our optimised tissue culture medium. Interestingly, regeneration of *P. peduncularis* was similarly as efficient as regeneration of *P. metallica*, indicating that our tissue culture protocol is not only suitable for *P. metallica*, but can also be transferred to at least some other *Peperomia* species (Figure 4D).

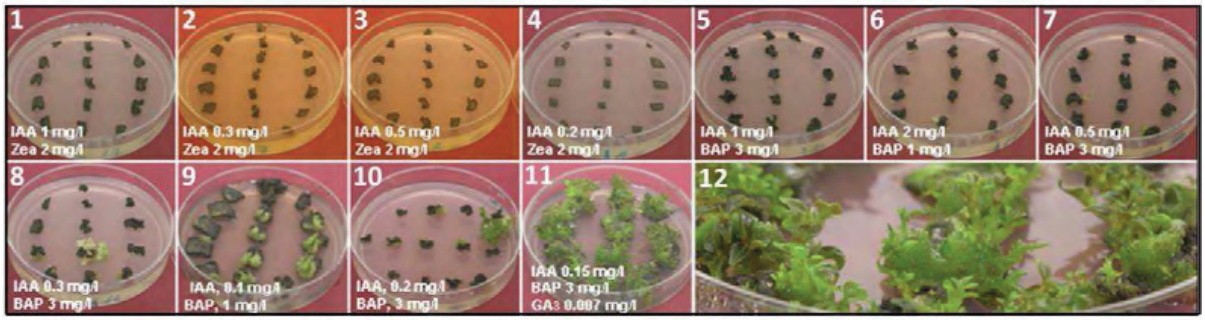

Figure 2. In vitro regeneration response of *P. metallica* leaf tissues cultured on different media containing various hormone combinations (1-11). A close-up of the regenerated plantlets from leaf segments of *P. metallica* is shown in image 12.

Table 2. In vitro regeneration response of *P. metallica* leaf tissues cultured on MS medium supplemented with various hormone combinations. The data are averages of 3 replicas.

Medium	No. of explants per petri dish	Explants responded to organogenesis (%)	Average no. of regenerated plants per plate
1	14	0 [a]	0 [a]
2	16	0 [a]	0 [a]
3	15	0 [a]	0 [a]
4	15	0 [a]	0 [a]
5	15	4.49 [ab]	1 [a]
6	14	7.14 [b]	1.33 [a]
7	14	26.21 [c]	3.67 [a]
8	15	24.47 [c]	4.33 [a]
9	15	24.47 [c]	3.33 [a]
10	14	28.57 [c]	9.33 [b]
11	14	100 [d]	210.33 [c]
12	14	100 [d]	206.67 [c]

Hormone composition of media 1-12 is indicated in Table 1. Data were analysed using SPSS, and means were compared using Duncan's test. Values indicated by different letters in the superscript show significant difference at $P < 0.05$ in comparison with the corresponding row values.

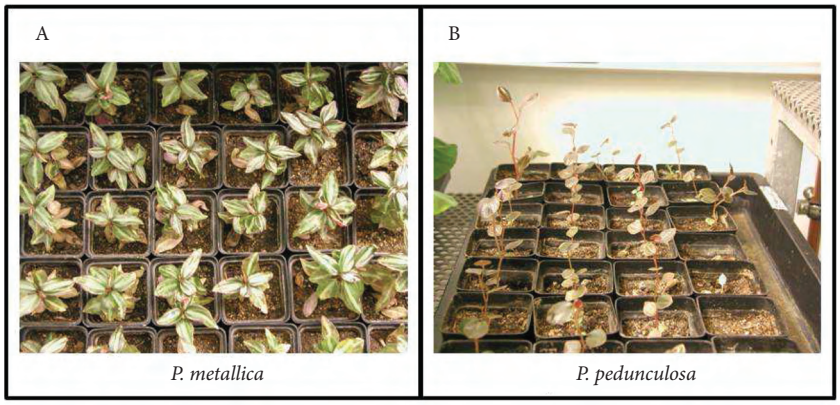

Figure 3. Regenerated plants of *Peperomia metallica* (A) and *P. peduncularis* (B) transferred to soil and acclimated in greenhouse conditions.

Discussion

Because of the unique feature of having giant chloroplasts in the leaf palisade parenchyma, *P. metallica* has become a useful model plant for studying various aspects of plastid biology, including plastid division, ultrastructure, and electrophysiology. However, the lack of efficient regeneration and transformation systems has limited these studies. Here, we reported a highly efficient and reproducible in vitro regeneration system for *P. metallica* and *P. peduncularis*, another species of genus *Peperomia*, which contain giant chloroplasts in their leaf palisade cells. Regeneration occurs through direct organogenesis from leaf discs at high frequency.

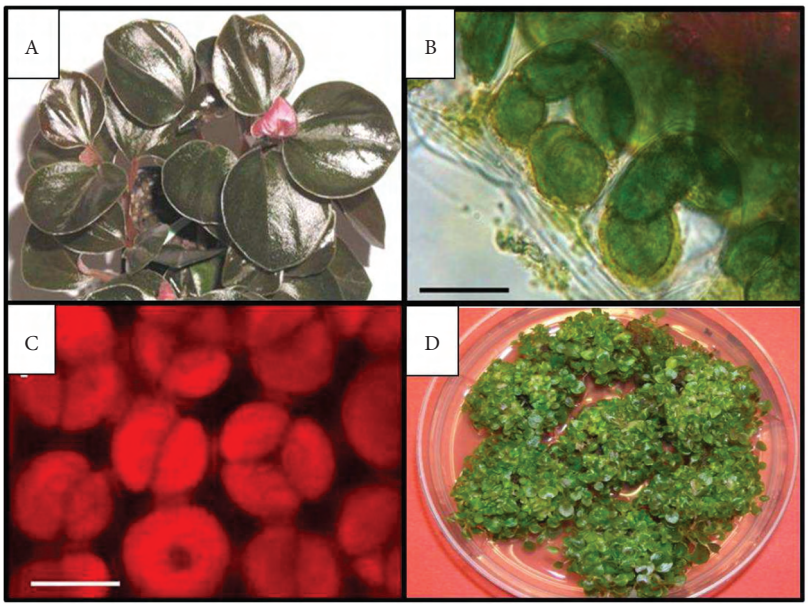

Figure 4. Plant morphology (A) and chloroplasts (B, C) in palisade mesophyll cells of *Peperomia peduncularis*. Part (D) shows the in vitro regeneration response of *P. peduncularis* leaf tissues cultured on the developed tissue culture medium. Image (B) was taken from thin leaf cross sections using high-resolution light microscopy. Image (C) shows visualisation of chloroplasts by confocal laser-scanning microscopy based on chlorophyll fluorescence in leaf palisade cells. Scale bars: 20 μm.

Induction of cell division and subsequent plant regeneration in in vitro cultures usually require the exogenous application of hormones such as auxin and cytokinin (Riou-Khamlichi et al., 1999). In plant tissue culture, exogenously applied auxins play an important role in the in vitro regeneration response. In sugarcane (*Poaceae*), for example, auxin concentration in tissue culture media has been shown to play an important role in determining the embryogenic and organogenic potential of cultured leaf tissues (Lakshmanan et al., 2006). While auxin seems to have a key role in the in vitro regeneration response, it does not act alone, but rather in combination with other regulators of plant growth and development. Cytokinins play an important role in the promotion and maintenance of plant cell division in cell and tissue cultures (Hartig & Beck, 2006). In addition, the application of other hormones, such as gibberellic acid, in growth media has been reported in several studies. However, different results have been observed. In tobacco, the addition of GA_3 has led to the repression of shoot formation. This repression could not be reversed by increasing the levels of auxin or cytokinin (Thorpe & Meier, 1973). In buckwheat, GA_3 has accelerated root initiation and increased root number, whereas bud shoot induction was inhibited (Srejovi & Neakovi , 1985). It has also been shown that addition of gibberellic acids to maize culture media results in either no effect or a slight inhibitory effect on callus induction (Sheridan, 1975). However, other data have shown that GAs can be beneficial to plant embryogenesis (Singh et al., 2002). In *Peperomia*, the effects of several hormones have been investigated on plant regeneration from leaf segments of 2 species, *P. scandens* and *P. eburnea* (Kukulczanka et al., 1977; Klimaszewska, 1979). The results showed that the 2 species respond differently to the plant growth regulators, and the best result for regeneration was observed when an auxin (NAA) was added to the medium in combination with a cytokinin (kinetin). Note that regeneration was not uniform in all regions of the leaf segments and that the main regeneration zone was the leaf blade basis and the petiole. In this study, in addition to an auxin and a cytokinin, application of GA_3 at low concentrations has led to a dramatic increase in shoot induction from

leaf tissue through organogenesis. Interestingly, regeneration occurs in all leaf segments, from bottom to tip, uniformly. Genotype dependency and applicability of the method for plant regeneration from other *Peperomia* species remain to be investigated. Our results show that GAs can play an important role in the process of plant regeneration; however, the requirement for GA addition might be variable among different species. These differences could be due to the complex cross-talk between different plant hormones, differences in the endogenous hormone concentrations, and/or a variety of developmental and metabolic signals (Gazzarrini & McCourt, 2003). Our results obtained with *Peperomia* regeneration confirm the hypothesis that complicated molecular relationships between many factors, including plant hormones, are involved in determining the in vitro regeneration response in plants.

In conclusion, we reported a protocol for *Peperomia* regeneration that can provide a reliable tissue culture system for genetic transformation experiments, including the future development of a plastid transformation system for this model plant. Due to the presence of giant chloroplasts in the leaf mesophyll cells and the ease with which *Peperomia* cells can be regenerated into normal plants using our optimised protocol, the targeting of foreign genes to the chloroplast genome in *Peperomia* plants might be easier than in species with normal-size chloroplasts.

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References

Bartels F (1965). Die Plastiden von *Peperomia metallica* (Plastidenzählungen). *Z Bot* 52: 572-599.

Bulychev AA, Andrianov VK, Kurella GA & Litvin FF (1972). Micro-electrode measurements of the transmembrane potential of chloroplasts and its photoinduced changes. *Nature* 236: 175-177.

Gazzarrini S & McCourt P (2003). Cross-talk in plant hormone signalling: what *Arabidopsis* mutants are telling us. *Ann Bot* 91: 605-612.

Hartig K & Beck E (2006). Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle. *Plant Biol* 8: 389-396.

Klimaszewska K (1979). The regenerative potential of two species of *Peperomia* in tissue culture and biological activity of endogenous growth regulators. *Acta Hort* 91: 281-286.

Kukulczanka K, Klimaszewska K & Pluta H (1977). Regeneration of entire plants of *Peperomia scandens* Ruiz. from different parts of leaves in vitro. *Acta Hort* 78: 365-371.

Lakshmanan P, Geijskes RJ, Wang L, Elliott A, Grof CP, Berding N & Smith GR (2006). Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. *Plant Cell Rep* 25: 1007-1015.

Lincy A & Sasikumar B (2010). Enhanced adventitious shoot regeneration from aerial stem explants of ginger using TDZ and its histological studies. *Turk J Bot* 34: 21-29.

Murashige T & Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473-497.

Neumann D (1973). Zur Ultrastruktur der Riesenplastiden aus dem Palisadenparenchym von *Peperomia metallica* Lind et Rodig. *Protoplasma* 77: 467-471.

Riou-Khamlichi C, Huntley R, Jacqmard A & Murray JA (1999). Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science* 283: 1541-1544.

Sahai A, Shahzad A & Anis M (2010). High frequency plant production via shoot organogenesis and somatic embryogenesis from callus in *Tylophora indica*, an endangered plant species. *Turk J Bot* 34: 11-20.

Schürhoff P (1908). Ozellen und Lichtkondensatoren bei einigen Peperomien. *Beih Bot Zbl* (Abt I) 23: 14-26.

Sheridan WF (1975). Tissue culture of maize. I. Callus induction and growth. *Physiol Plant* 33: 151-156.

Singh DP, Jermakow AM & Swain SM (2002). Gibberellins are required for seed development and pollen tube growth in *Arabidopsis*. *Plant Cell* 14: 3133-3147.

Srejović V & Nešković M (1985). Effect of gibberellic acid on organogenesis in buckwheat tissue culture. *Biol Plant* 27: 432-437.

Thorpe T & Meier D (1973). Effects of gibberellic acid and abscisic acid on shoot formation in tobacco callus cultures. *Physiol Plant* 29: 121-124.

Wanke S, Samain MS, Vanderschaeve L, Mathieu G, Goetghebeur P & Neinhuis C (2006). Phylogeny of the genus *Peperomia* (Piperaceae) inferred from the trnK/matK region (cpDNA). *Plant Biol* 8: 93-102.