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Introduction of Astaxanthin Biosynthesis Gene *crtW* into Petunia and Calibrachoa Using *Agrobacterium*-mediated Transformation

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ABSTRACT

Agrobacterium-mediated gene transfer method for transferring *crtW* gene was investigated in Petunia and Calibrachoa by using *Agrobacterium tumefaciens* strain EHA105, harboring a plasmid containing the *crtW* gene from *Brevundimonas* sp. strain SD212 under 35S promoter with kanamycin resistant gene for selection. Co-cultivation was done using leaf disk method for 3 days in *Agrobacterium* solution with 20 ppm acetosyringone and selection was carried out on selective MS medium containing 200 ppm kanamycin. Inoculated leaf explants of Petunia and Calibrachoa produced putative transgenic callus tissue that demonstrated orange color and astaxanthin accumulation. The result of PCR analysis indicated the Petunia and Calibrachoa calluses contained the *crtW* gene and RT-PCR result showed *crtW* gene expression in the callus tissue.

1. Introduction

Genetic transformation is a technique to obtain specific desired characteristics in plant breeding by transferring a gene or genes for the desired trait from another species into a plant to make it express the expected phenotype. Genetic modification by *Agrobacterium* in *Petunia* has been reported, such as transferring antisense broccoli ACC synthase and ACC oxidase genes for delaying flower senescence (Huang *et al.*, 2007), pollination with pollen carrying the GUS gene obtained by vacuum-infiltrating pollen with *Agrobacterium tumefaciens* (Tjokrokusumo *et al.*, 2000), or pollen co-cultivation with *Agrobacterium tumefaciens* (Süssmuth *et al.*, 1991), and

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introduction of a cytokinin overexpression biosynthetic gene for delaying leaf senescence (Bai *et al.*, 2009). Genetic transformation has also been reported in *Calibrachoa excellens* by hairy root inoculation with *Agrobacterium rhizogenes* carrying *rol* genes (Gennarelli *et al.*, 2009).

Many research projects have been done that modified the carotenoid synthetic pathway in Solanaceace plants by using *Agrobacterium,* such as a project to achieve ketocarotenoid accumulation in leaves and flower of tobacco by transformation with *crtZ* and *crtW* genes from *Paracoccus* with CaMV 35S promoter, which showed ketocarotenoid accumulation in leaf and nectary (Ralley *et al.*, 2004), and transformation with *crtO* ketolase gene from *Synechocystis* with CaMV 35S promoter, which showed high ketocarotenoid accumulation in leaf, nectary and petal (Gerjets *et al.*, 2007; Zhu *et al.*, 2007). Researchers have also reported flower color alteration in *Petunia* by transferring a *DFR* gene from another plant species such as *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton (Mori *et al*., 2014) and *Calibrachoa hybrida* (Chu *et al*., 2014) to *Petunia hybrida*. The transgenic *P. hybrida* plants showed petal color alteration because they could express *DFR* gene and modified flavonoid content and composition. With another species, in *Lotus japonica* (Suzuki *et al*., 2007) using *crtW* gene from *Agrobacterium aurantiacum* with CaMV 35S promoter, the transgenic plants showed high accumulation of ketocarotenoid such as astaxanthin, adonixanthin, canthaxanthin and echinenone in petals and leaves, which indicated that the overexpression of *crtW* gene can change the petal's color by modification of carotenoid content and composition.

Petunia and *Calibrachoa* also belong to the family Solanaceae and are both commercial ornamental plants. But recently, there have been no reports about modification of the carotenoid biosynthetic pathway in *Petunia* and *Calibrachoa,* and there are no *Petunia* cultivars with dark yellow petals, only pale yellow ones. For this research, an experiment was conducted to modify the carotenoid biosynthesis pathway by using gene overexpression for changing the petal color in *Petunia* and *Calibrachoa*. This research aimed to obtain transgenic plants of *Petunia* and *Calibrachoa* that have high astaxanthin accumulation and novel petal color.

2. Methods

2.1 Plant material

Shoots of *Petunia* × *hybrida* Surfinia® 'Patio Yellow' (Suntory Flowers Ltd, Japan), *Calibrachoa* × *hybrida* Million Bells® 'Neon Yellow' (Suntory Flowers Ltd, Japan), and seeds of *Petunia hybrida* (Sakata Seed Corporation, Japan) were disinfected with 1% available chlorine sodium hypochlorite solution (Wako Pure Chemical, Japan) and transferred to MS medium (Murashige and Skoog, 1962) solidified with 2 g l^{-1} gellan gum (Wako Pure Chemical, Japan). Surface-sterilized plants were used for *Agrobacterium*-mediated transformation.

2.2 Plasmid construction

The *crtW* gene from marine bacteria *Brevundimonas* sp. strain SD212 was fused with transit peptide (tp) sequence of the pea (*Pisum sativum*) Rubisco small subunit and fused into the CaMV 35S promoter of binary vector pRI201-AN (TaKaRa, Japan). The kanamycin-resistance gene neomycin phosphotransferase (*nptII*) was also included in the vector. The vector was introduced into *A. tumefaciens* strain EHA105 by electroporation. (Figure 1)

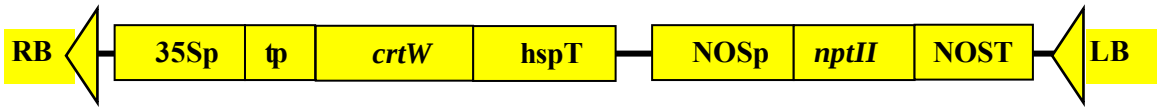

Figure 1: Schematic map of plasmid construct harboring *crtW* gene. *RB* right border, *LB* left border, *35Sp* cauliflower mosaic virus 35S promoter, *tp* transit peptide, *hspT* heat shock protein terminator, *nptII* neomycin phosphotransferase, *NOSp* and *NOST* promoter and terminator of the nopaline synthase gene.

2.3 *Agrobacterium*-mediated transformation

Leaf explants of surface-sterilized *Petunia* and *Calibrachoa* plants were co-cultivated with *Agrobacterium tumefaciens* containing binary vector *crtW* on co-cultivation MS medium containing TDZ 2 mg l^{-1}, NAA 0.1 mg l^{-1} and acetosyringone 20 mg l^{-1} in dark condition for 3 days. Then the explants were washed with autoclaved water 4-5 times, excess water was removed with autoclaved filter paper and explants were transferred to selection medium: MS medium contain TDZ 2 mg l^{-1}, NAA 0.1 mg l^{-1}, kanamycin 200 mg l^{-1} and meropen 10 mg l^{-1}. They were subcultured every week for *Agrobacterium* elimination.

2.4 Analysis of transgenic plants

2.4.1 Pigment analysis

Pigment analysis was performed by thin-layer chromatography (TLC) on callus of *Petunia* and *Calibrachoa*. First, 0.1 g of sample was collected and ground with 0.2 g silica gel. Then, it was transferred to a micro tube and 500 µl diethyl ether was added. The sample was mixed and only the extract solution was dropped on TLC silica gel aluminum sheet (MERCK, Germany), using petroleum ether: acetone (8:2) solution for mobile phase within a closed system.

2.4.2 Molecular analysis

2.4.2.1 Detection of crtW gene in explants by polymerase chain reaction

Total genomic DNA was isolated from calluses of transgenic *Petunia* and *Calibrachoa* according to Rogers and Bendich (1985). For PCR analysis, PCR was performed using Takara PCR Thermal Cycler Dice Touch (TaKaRa, Japan) under the following conditions: 94°C for 30 s, 55 °C for 40 s, 72 °C for 1 min; 30 cycles with total genomic DNA as template and the tp-*crtW*

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gene specific primer set, 5'-ATTCTAGAGAGCTTTGCAATTCATACA-3' and 5'-ATGAGCTCGGATCCTCAAGACTCTCCTCTCCAA-3', which generates a 950 bp DNA fragment. Amplified products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel (Wako Pure Chemical, Japan).

2.4.2.2 Expression analysis of crtW gene

Reverse transcription polymerase chain reaction (RT-PCR) was used for detection of *crtW* gene expression. Total RNA extraction from calluses of *Petunia* and *Calibrachoa* was carried out using RNeasy Plant Mini Kit (Qiagen, Japan). To detect expression of *crtW* gene, cDNA was amplified from total RNA by using RT-PCR, and *crtW* primer set was used for amplification with about 250 ng of RNA per one PCR reaction. Then, amplified cDNA samples were multiplied by PCR. Amplified products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel.

3. Results and Discussion

3.1 Production of transgenic plants

In this study, *Petunia* and *Calibrachoa* were successfully transformed and produced calluses. This is consistent with a previous report of transformed gene expression in *P. hybrida* following leaf disk co-cultivation with *Agrobacterium,* which showed that the *Agrobacterium*-mediated transfer method is a simple and efficient (Horsch *et al*., 1986). Some of the transformed calluses in this study showed orange color, indicating the accumulation of ketocarotenoid compounds such as astaxanthin. This result was similar to a study on *Lilium* × *formolongi* that also used the *crtW* gene obtained from marine bacteria *Brevundimonas* sp. strain SD212 (Azadi *et al*., 2010).

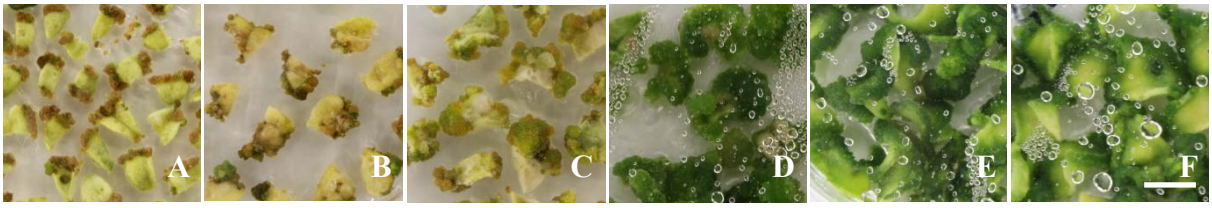

Figure 2: Some transgenic kanamycin-resistant calluses of (A) *Calibrachoa* (B) *Petunia* (Suntory Flowers Ltd) and (C) *Petunia* (Sakata Seed Corporation) that regenerated on MS medium containing kanamycin showed orange color compared to non-transgenic calluses of (D) *Calibrachoa* (E) *Petunia* (Suntory Flowers Ltd) and (F) *Petunia* (Sakata Seed Corporation) that only showed green color.(Note: *Bar* 1 cm.).

Petunia and *Calibrachoa* explants only showed callus formation. This is probably because the type and concentration of plant growth regulators might not have been optimum for shoot regeneration. But these calluses were successfully transformed with *crtW* gene, as was confirmed by PCR with specific primers. Transgenic *Petunia* and *Calibrachoa* calluses also exhibited

orange color compared with wild-type callus, demonstrating higher red carotenoid astaxanthin accumulation that was not present in wild-type calluses (Figure 2).

3.2 Pigment analysis

The TLC result showed transgenic *Petunia* and *Calibrachoa* calluses had astaxanthin and other keto-carotenoid compounds accumulation but showed low amounts of β-Carotene compared with wild-type because astaxanthin was synthesized from β-carotene (Mann *et al.*, 2000; Gerjets *et al.*, 2007; Suzuki *et al.*, 2007; Azadi *et al.*, 2014). Wild-type *Petunia* and *Calibrachoa* calluses had no accumulation of astaxanthin and other keto-carotenoid compounds (Figure 3). Similarly to our results, analysis of carotenoid pigments by TLC also revealed accumulation of astaxanthin and another keto-carotenoid compounds in transgenic *L. japonicas* when compared with wild-type plants (Suzuki *et al.*, 2007).

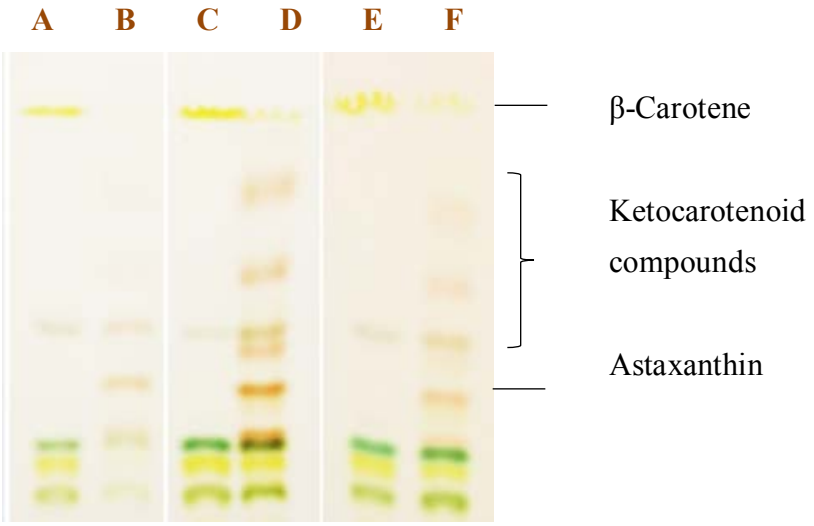

Figure 3: TLC result comparing between wild-type (right) and transgenic calluses (left) of (A-B) *Calibrachoa* (C-D) *Petunia* (Suntory Flowers Ltd) and (E-F) *Petunia* (Sakata Seed Corporation).

3.3 Molecular analysis

3.3.1 PCR analysis

Gel electrophoresis of PCR products amplified from transgenic calluses DNA that had survived on selection medium containing kanamycin indicated all of the transgenic calluses of *Petunia* and *Calibrachoa* showed the presence of bands of tp-*crtW* (950 bp), which confirmed that all of the transgenic calluses of *Petunia* and *Calibrachoa* were successfully transformed with *crtW* gene (Figure 4).

3.3.2 Expression analysis of *crtW* gene

For *crtW* gene expression analysis, *crtW* transcripts from transformed calluses of

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Calibrachoa and *Petunia* were detected that indicated these transformed calluses have *crtW* gene expression (Figure 5).

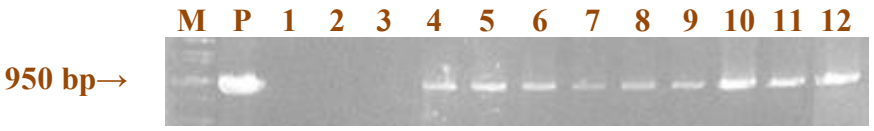

Figure 4: Gel electrophoresis of PCR products amplified from transgenic calluses. *Lane M* Molecular size marker, *Lane P* DNA plasmid containing LjUbip-*crtW*, *Lane 1* wild-type *Calibrachoa*, *Lane 2* wild-type *Petunia* (Suntory Flowers Ltd), *Lane 3* wild-type *Petunia* (Sakata Seed Corporation), *Lane 4-6* transgenic *Calibrachoa*, *Lane 7-9* transgenic *Petunia* (Suntory Flowers Ltd), *Lane 11-13* transgenic *Petunia* (Sakata Seed Corporation). (950 bp indicated by arrow).

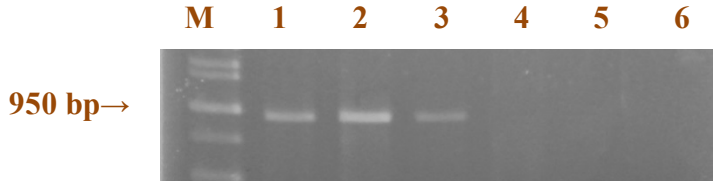

Figure 5: RT-PCR analysis of *crtW* expression in transformed calluses of *Petunia* and *Calibrachoa*. *Lane 1* transformed *Calibrachoa* calluses, *Lane 2* transgenic *Petunia* calluses (Suntory Flowers Ltd) *Lane 3* transformed *Petunia* (Sakata Seed Corporation), *Lane 4-6* wild-type of *Calibrachoa*, *Petunia* (Suntory Flowers Ltd), and *Petunia* (Sakata Seed Corporation) calluses respectively. (950 bp indicated by arrow).

4. Conclusion

Leaf explants of *Petunia* and *Calibrachoa* successfully developed transgenic calluses, which were revealed by molecular and pigment analyses. Although complete plants could not be regenerated from these calluses, still these findings pave the way for further research. If we work to find an appropriate culture method and optimum plant growth regulators for inducing shoot regeneration from callus, then it will lead to the production of transgenic *Petunia* and *Calibrachoa* plants that exhibit astaxanthin accumulation the same as in the calluses in this study.

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