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ORIGINAL ARTICLE

Agrobacterium-mediated transformation of the wild orchid *Cattleya maxima* Lindl

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Abstract

Protocorms are unique anatomical structures; they are akin to rhizoids and are formed by young orchid seedlings under physiological conditions. Explanted orchid tissues produce similar structures called protocorm-like bodies (PLBs) when exposed to appropriate *in vitro* growing conditions. Both the propagative nature of PLBs and the easiness by which they can be generated, make these structures an attractive alternative to seed-mediated production for growing large numbers of plants. To increase somatic embryogenesis and optimize the procedure, PLBs of *Cattleya maxima* were transformed using the *Agrobacterium tumefaciens* method. The T-DNA carried a Hygromycin-resistance gene, a visible marker (GFP5-GUSA) and a rice gene encoding the Somatic Embryogenesis Receptor Kinase, deemed to be important for somatic embryogenesis. Treated PLBs generated somatic embryos developing Hygromycin-resistant plantlets. The insertion of T-DNA was confirmed by PCR, and GFP expression was observed using a fluorescent stereomicroscope. Transformed *Cattleya maxima* PLBs were more efficient in forming somatic embryos (60 - 80 %) than untransformed controls (45 - 57 %), and this contrast was maximized in hormone-free, Murashige and Skoog (MS) medium (80 % of the transformed plants compared to 57 % of the untransformed ones). This finding supports the notion that SERK plays an important role in Orchid embryogenesis.

Keywords: Protocorm-Like-Bodies; Transformation; Orchids; pCAMBIA; *SERK*; *Agrobacterium tumefaciens*

Introduction

The Orchidaceae is the largest vascular plant family in Ecuador, with nearly 4 000 species, and is of economic importance for this country due to its significant share in the international ornamental plant market (Simpson 2006).

Cattleya is one of the most noted genera of the family, for its popularity and richness of colors (Krapiec 2003). One native representative species of the genus is *Cattleya maxima* (Dodson & Escobar 2005). Unfortunately, an intense specimen harvest, linked to habitat loss and deforestation threatens most native and endemic Ecuadorian orchid species (Dodson 2005). To counter this situation, *ex situ* clonal propagation methodologies for orchids are being developed (Cuoco & Cronan 2009). For instance, several techniques of clonal propagation have been developed for the genus *Cattleya* (reviewed by Arditti 2008; Krapiec 2003). Transgenesis is a powerful tool to aid the success of clonal propagation of orchids. Pioneering work in the early eighties (Fraley *et al*. 1983) paved the way for the production of transgenic plants engineered for several purposes (Deo *et al*. 2010). Currently, successful orchid transformation protocols rely on an efficient regeneration procedure based on Somatic Embryogenesis (SE) and on culture conditions favoring the formation of the specific structures called Protocorm-Like-Bodies (PLBs), as a prerequisite for optimal clonal propagation (Young *et al* 2000, Texeira *et al* 2006, Cueva *et al* 2014).

During SE, differential expression of several genes takes place (Chugh & Paramjit 2002). However, only few of the differentially expressed genes are considered embryogenesis-specific (Ikeda *et al*. 2003). The Somatic Embryogenesis Receptor-like Kinase (*SERK*) gene, is one of such genes expressed during SE as well as in zygotic embryogenesis. *SERK* expression is characteristic of embryo-forming masses in induced carrot suspension cultures (Schmidt *et al*. 1997). Homologues of carrot *SERK* have been described in different species and are encoded by small gene families of 1-6 members (described in Cueva *et al* 2012). In rice *Oryza sativa*, two SERK genes have been identified, *OsSERK1* and *OsSERK2* (Ito *et al*. 2005). Presumably, by modulating the expression of genes such as *SERK* during SE, the success of clonal propagation procedures could be improved. However, for orchids, and particularly for those of the genus *Cattleya*, reaching to this point first requires the development of the appropriate transgenesis toolkit.

Transgenic orchid production has been reported only for a few orchid genera (Men *et al*. 2003). *Agrobacterium tumefaciens*-mediated and microprojectile particle bombardment (biolistic protocol) have been the most successfully used methods for orchid transgenesis (Deo *et al*. 2010, Texeira da Silva *et al*. 2011). The biolistic method is thus far the most employed approach for orchid transformation, as in the genera *Dendrobium* (Men *et al*. 2003, Kuehnle & Sugii 1992, Chia *et al*. 1994, Yu *et al*. 1999, Tee *et al*. 2003), *Phalaenopsis, Cymbidium* (Yang *et al*. 1999), *Cattleya*, *Brassia*, and *Doritaenopsis* (Knapp *et al*. 2000). The *Agrobacterium*-mediated method

has been used for *Dendrobium* (Wasana *et al*. 2015, Men *et al*. 2003, Yu *et al*. 2001), *Phalaenopsis* (Belarmino & Mii 2000, Chai *et al*. 2002, Mishiba *et al*. 2005, Sjahril & Mii 2006), *Cymbidium* (Chen & Chang 2002, Chin *et al*. 2007), *Oncidium* (Liau *et al*. 2003), *Vanda* (Pavallekoodi *et al*. 2014), *Erycina* (Lee *et al*. 2015), and *Cattleya* (Zhang *et al*. 2010). Compared to the biolistic method, *Agrobacterium*-mediated transformation offers the following advantages: delivery of a lower transgene copy number, lower level of transgene rearrangement, transfer of relatively large segments of DNA, and no special equipment requirements (Hei *et al*. 1994, Cheng *et al*. 1997). The *Agrobacterium* method proved to be efficient for most dicotyledonous species, but it appears to be less suited for monocots. The genus *Cattleya* was found to be recalcitrant to *Agrobacterium*-mediated transformation. Only one successful application of the method has been reported for a commercial *Cattleya* variety (CM2450) (Zhang *et al*. 2010).

Selection of transformants relies on the use of the two types of markers: reporter genes and selectable markers. The most commonly used reporter genes for orchid transformation are: β-glucuronidase (*GUS*), the anthocyanin pigmentation gene, the firefly luciferase gene, and the Green Fluorescent Protein (*GFP*) gene. As for selectable markers, several antibiotic, or herbicide resistance genes have been utilized, including neomicyn phosphotransferase (*nptII*), hygromycin phosphotransferase (*hpt*), and phosphinotricinacetyltransferase (*bar*) (Suwanaketchanatit *et al*. 2007). However, orchid cells are normally poorly sensitive to most selective agents. For instance, a high concentration of gentamicin and kanamycin (600 mg l^{-1}) was used to eliminate untransformed cells and plants of various *Dendrobium* species (Chia *et al*. 1994). Instead, hygromycin (Hyg) was reported to be a reliable selective agent for the transformation of orchids (Men *et al*. 2003, Belarmino & Mii 2000, Chai *et al*. 2002, Liau *et al*. 2003, You *et al*. 2003, Liao *et al*. 2004). In this study, an *Agrobacterium*-mediated protocol for the transformation of *C. maxima* was established as a mean to promote the overexpression of the *Os-SERK* gene on SE in this orchid species.

Materials and Methods

Plant Material

Protocorms of *C. maxima* were obtained from seeds germinated *in vitro* on half strength Murashige & Skoog (MS) medium containing 20 g/L of sucrose and grown at 57 μmol m^{-2} s^{-1} photon flux density (fluorescent lamps of 40 W), at 21 ± 2 °C with a 12 h photoperiod.

Plasmid Construction

The well-characterized monocot *SERK1* gene from rice (*OsSERK1*) (Kikuchi *et al*. 2003) was used in this study. The complete *OsSERK1* cDNA (Clone, PS-JO33117E16-National Institute of Agricultural Sciences NIAS-Japan) was amplified using primers bearing the S*pe*I and S*ph*I restriction sites (S*pe*I-OsSERK-fw: 5'ACT AGT ATG GCG GCG CAT CGG TGG 3' and S*ph*I-OsSERK-rv: 5'GCA TGC TCA CCT CGG CCC TGGA TAG 3') Subsequently, it was cloned in the pGEM®-T Easy vector (Promega). The DNA was then digested with S*pe*I and S*ph*I and subcloned in an pFF19 vector (Timmermans *et al*. 1990) that contains 35S promoter and terminator sequences of the cauliflower mosaic virus (CaMV) using X*ba*I and S*ph*I sites. The recombinant *pro35S*::*OsSERK1*::*ter35S* cassette was then digested with H*ind*III and *Eco*RI and subcloned into a pCAMBIA 1301 vector (Center for the Application of Molecular Biology to International Agriculture, Australia) harboring the hygromycin resistance marker gene (*hpt*) (Chin *et al*. 2007, Kei-ichiro *et al*. 2005). The recombinant cassette was introduced in two *A. tumefaciens* strains (GV3101/pMP90) by electroporation, with the following conditions: 2.5 kV, 25 μF, 400 Ω. The map of the recombinant T-DNA is shown in **Fig. 1**.

Assessment of *A. tumefaciens* strains

The transformation efficiencies of the *A. tumefaciens* strains GV3101/pMP90 (Koncz & Schell 1986) and EHA105 (Hood *et al*. 1993) were assessed. GV3101/pMP90 carries a gentamycin-resistance gene on the Ti plasmid and a rifampicin-resistance gene on the chromosome, and EHA105 carries a gene for resistance to rifampicin on its chromosome. Three-month old protocorms, in eight replicates (flasks), were transformed with each strain.

Inoculation and co-cultivation

A. tumefaciens was grown for 2 d at 28 °C in 5 mL of LB medium containing 100 mg/L of kanamycin and 100 mg/L of rifampicin. The culture was transferred to 50 mL of LB medium and cultured overnight at 28 °C. Four hours prior to transformation, acetosyringone at a final concentration of 200 μM was added. After centrifugation at 3 000 rpm, the pellet was resuspended in 20 mL of MS medium. About 20 mL of resuspended bacteria were added to each plate containing pre-cultured protocorms and incubated at room temperature for 3 h. The protocorms were blotted dry on sterile filter paper and co-cultivated at 25 °C in the dark for 4 d in MS medium containing 200 μM acetosyringone.

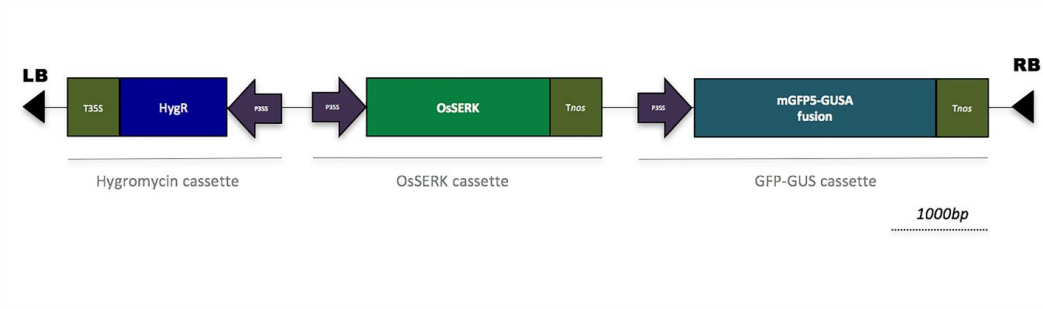

Figure 1. Schematic structure of the T-DNA region in the constructp CAMBIA1301-OsSERK. LB-Left border, RB-Right border, T35S: 35SCaMV terminator, P35S: 35SCaMV promoter. HygR (hygromycin phosphotransferase gene), OsSERK- *Orysa sativa* Somatic Embryogenesis Receptor like Kinase Gene; mGFP5-GusA fusion. All the coding regions are under the control of CaMV35S promoter and CaMV35S terminator. The orientation of each single cassette is indicated in the figure.

Selection of Transgenic PLBs and plant regeneration

To eradicate *A. tumefaciens* after co-cultivation, the protocorms were washed with MS medium containing 200 mg/L of timentin, transferred to MS medium with 200 mg/L of timentin and 50 mg/L of cefatoxime and subcultured weekly for five additional weeks. A preliminary test using *C. maxima* PLBs (primary) showed that the minimal selective concentration of hygromycin was 20 mg/L over a period of two months (data not shown). In this study, the selection was extended to five months, with monthly transplant to fresh medium.

PCR screening of transformants

DNA was extracted from 30 mg of leaves, using the Wizard® SV Genomic DNA Purification System (Promega) and following the manufacturer's instructions. Genomic DNA (8 ng) was directly used for PCR screening with primers annealing on the *OsSERK1* gene (5'-TGCTCGTCTAGCCAATGATG-3'), and. (5'-ATGAGGGCCAAGCTCTACC-3'). PCR amplification was carried out in an Applied Biosystem Thermocycler as follows: 94 °C (5 min), 32 amplification cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min) and a final extension cycle of 5 min at 72 °C using GoTAQ polymerase (Promega, Spain).

GFP Analysis

The pCAMBIA-*OsSERK1* construct contains a mGFP5-gusA fusion under the control of 35SCaMV promoter and terminators for visual selection of transformants. Detection of GFP epifluorescence was performed using a fluorescent stereomicroscope (Olympus DFPL 0,5X-4, Japan) equipped with a U- RFL-T Filter (Olympus). Images were taken with a Camedia camera (C5060 Olympus, Japan) connected to the stereomicroscope and adjusted using ImageJ software (Rasband, 2016)

Somatic embryogenesis in transformed lines carrying *OsSERK1*

A total of 80 leaf explants of transformed and control *C. maxima* plants were cultured either on MS hormone-free medium or MS medium containing TDZ (0.3 mg/L) or BA (0.3 mg/L). The percentage of developing somatic embryos was evaluated after 30 d. The statistical significance of the results was assessed via F-test.

Results and Discussion

Selection of transformed explants

The hygromycin resistance marker gene was chosen because it was previously used for *A. tumefaciens*-mediated transformation of *Oncidium* species (You *et al*. 2003, Liao *et al*. 2004), and for *Phalaenopsis* hybrids along with a β-glucuronidase gene sequence (Belarmino & Mii 2000, Chai *et al*. 2002, Mishiba *et al*. 2005, Chan *et al*. 2005). Based on these observations, in the present study we have used a pCAMBIA 1301 vector harboring gene sequences for hygromycin resistance, GUS (uidA), and GFP in addition to an *OsSERK1* gene copy to evaluate its possible role in the stimulation of somatic embryo formation.

During the 5-month selection step, plants derived from PLBs, characterized by the presence of one or two leaves (**Fig. 2a**, white arrow) died after two months, while juveniles PLB without leaves formed calli producing new PLBs (**Fig. 2b**, black arrow) that further developed into plantlets (**Fig. 2c**). It is worth noting that the developmental stage of the explant is crucial for orchid transformation success. For instance, it was reported that for *Oncidium* the age of the plantlets used for transformation influenced hygromycin tolerance (Liau *et al*. 2003). Additionally, in a review (Texeira da Silva 2013) mentioned that coniferyl alcohol, which is known as a *vir* gene inducer, is present in PLB at a level higher than in any other tissues, thus suggesting that PLBs are ideal

for *Agrobacterium*-mediated transformation. In this work, this observation is confirmed also for *C. maxima*.

PCR and *OsSERK1* gene analysis of putative *C. maxima* transformants

After a month of growth on the MS selection medium, 89.5 % of the of the PLBs had survived. To verify whether secondary PLBs were transformed, a PCR-based test was used to verify the presence of the *OsSERK1* gene. An expected fragment (612 bp) was observed in 4 out of the 30 putative transformants (**Fig. 3**). This low transformation efficiency (approximately 10 %), confirmed the need for a more stringent selection step to eliminate false positives. To this end, the selection time was prolonged to five months.

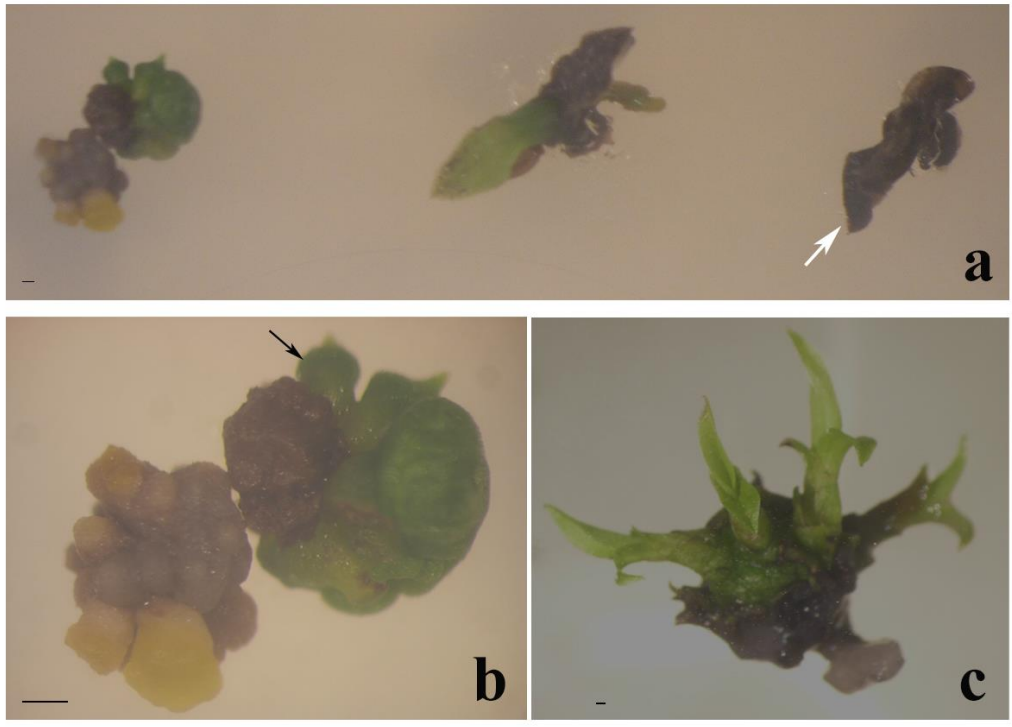

Figure 2. Selection of putative transformants of *Cattleya maxima*. **a**) Protocorms of different ages growing on the selection medium (20 mg L^{-1} hygromycin, MS). The white arrow indicates a dead protocorms. **b**) Surviving protocorms on the same selective medium (black arrow). **c**) Shoots developed six months after co-cultivation. (Bar = 0.5 cm).

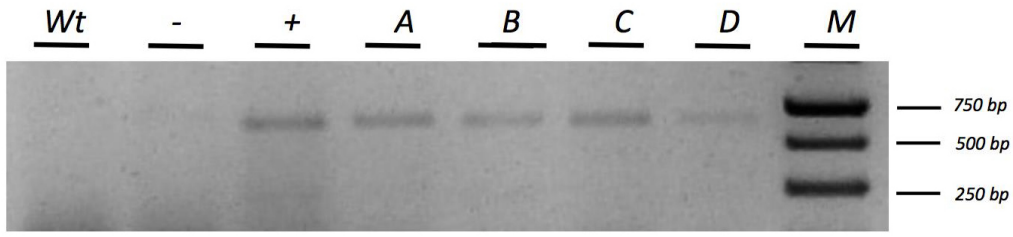

Figure 3. Control of the presence of OsSERK gene in transgenic lines. A band of the expected size (612bp) was observed in selected plants. As expected, the 612bp fragment failed to be amplified in both blank and wild-type, untransformed plant. "Wt" =Wild type plant "A, B, C, D" = selected plants "–" = blank, negative control "+" = pCAMBIA-OsSERK plasmid, positive control.

The ability to transfer a given T-DNA to a host cell depends on its chromosomal background (Yasmine & Debener 2010), the efficiency of the two *A. tumefaciens* strains was assessed by evaluating the percentage of protocorms surviving to hygromycin selection. GV3101/pMP90 failed to give any transformant while EHA105 strain, typically used for monocot species, yielded (7.4 ± 1.8) % viable PLBs after protracted following hygromycin selection.

The presence of GFP5-GUSA fusion protein in pCAMBIA-*OsSERK1* offers the possibility to identify transformants by observing epifluorescence. After a growth period of five months on Hyg-selective medium, putative transformants were randomly screened. At inspection, all putative transformants presented normal phenotypes, and no signs of dwarfism were observed. Fluorescence was detected in 70 % of both calli (**Fig. 4**) and regenerated plants (**Fig. 5**). No background fluorescence was observed in untransformed calli and plants (data not shown).

Table 1. Somatic embryogenesis responses of transformed and wild type *C. maxima* plants. *Percentage of leaves producing somatic embryos.

Medium	Somatic embryogenesis (%)*		
	Transformed plants	Wild type	F of Fisher significance
MS + 0.3 mg L^{-1} TDZ	60 ± 28	51 ± 14	0.757
MS + 0.3 mg L^{-1} BA	60 ± 25	45 ± 6	0.331
MS	80 ± 3	57 ± 3	0.002

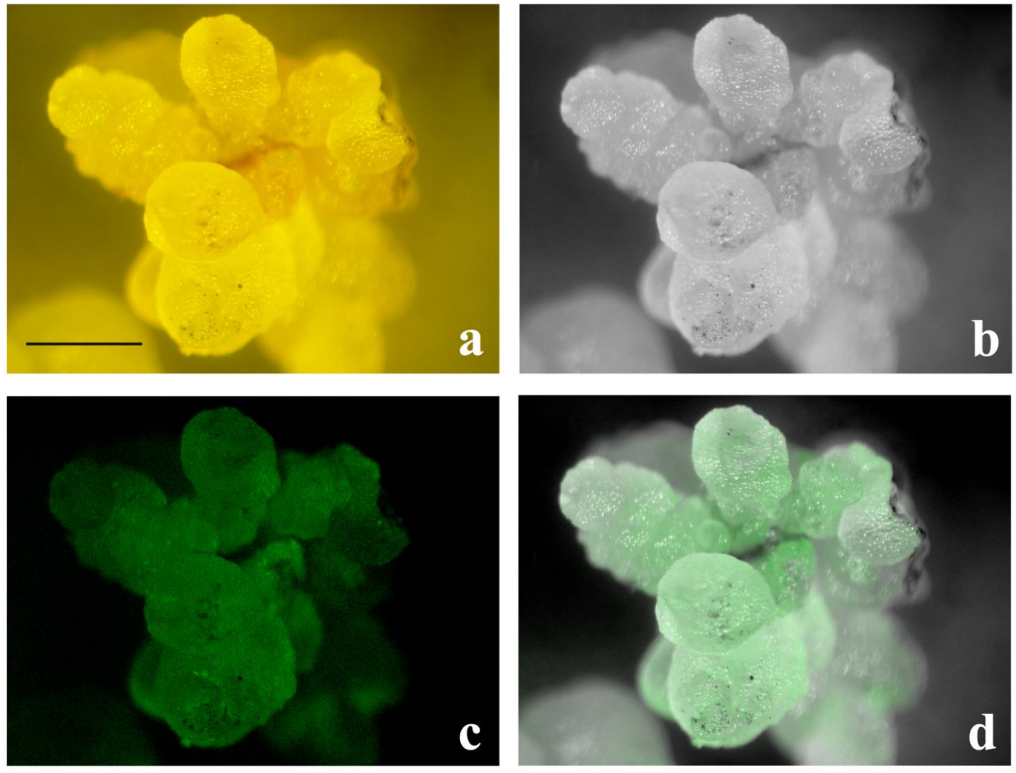

Figure 4. GFP-epifluoresence in a callus generated from a protocorm of *C. maxima* co-cultivated with *A. tumefaciens*. Panels **a)** and **b)** show a regenerated transformant examined using a fluorescence stereomicroscope. **a)** is in true color while, **b)** is the black-and-white version, **c)** GFP epifluorescence, **d)** overlay of **b)** and **c)**. (Bar = 0.5 cm)

Somatic embryogenesis in transformed explants

A significantly higher percentage of embryos (80 % compared to 57 %) were formed from in *C. maxima* leaf explants, containing the *OsSERK1* cassette, obtained from plantlets regenerated from secondary PLBs (Fig. 2C) as compared to those derived from wild type plants when cultured on hormone-free MS medium (**Table 1**). Furthermore, the total number of embryos per leaf was significantly higher in leaves obtained from transformants grown on hormone-free MS medium compared to untransformed leaves: 2.3 ± 0.7 versus 1.5 ± 0.2 ($F = 0.022$). Interestingly, the presence of TDZ or BA resulted in a reduced percentage of embryo formation (Table 1). Since it is well known that a proper balance of auxin/cytokinin stimulates embryogenesis in orchids (Novak *et al.* 2014) it is tempting to speculate that the external addition of cytokinins might interfere with the endogenous physiological auxin/cytokinin ratio necessary for somatic embryogenesis.

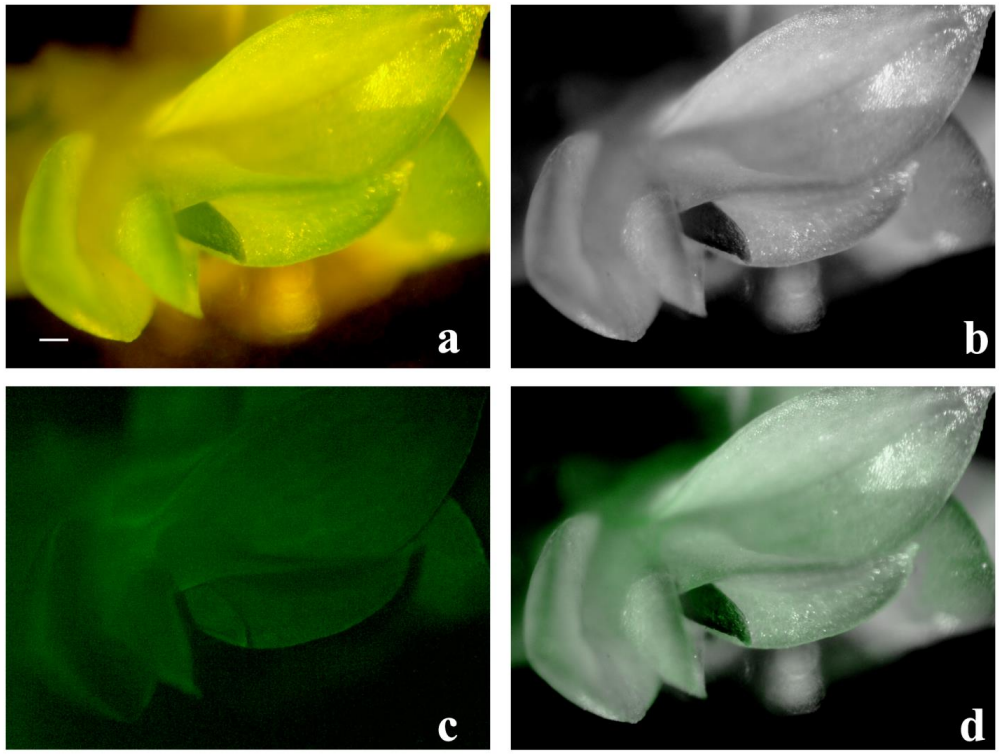

Figure 5. GFP epifluorescence in an eight-month regenerated plant of *C. maxima*. Panels **a)** and **b)** show a putative transformant examined using a fluorescence stereomicroscope; **a)** is in true color while **b)** is the black-and-white version; panels **c)** shows epifluorescence in the sample and panel **d)** shows the overlay of **b)** and **c)**. No background fluorescence was observed in wild type plants (data not shown). (Bar = 0.5 cm)

Conclusion

In the present work, we describe a method for the stable transformation of the wild orchid *C. maxima* mediated by *A. tumefaciens*. We have also developed a method for the regeneration of transformed plants *via* somatic embryogenesis through a dedifferention-regeneration cycle that includes the formation of secondary PLBs. The higher number of embryos obtained from leaves containing recombinant heterologous *OsSERK1* gene, as compared to untransformed plants, points to its involvement in orchid somatic embryogenesis.

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Conflict of interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject discussed in this manuscript.

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Transformación mediada por *Agrobacterium* de la orquídea silvestre *Cattleya maxima* Lindl

Resumen. Los protocormos son estructuras anatómicas únicas: son similares a los rizoides y se forman por vástagos jóvenes de orquídeas bajo condiciones fisiológicas. Los tejidos explantados de orquídeas producen estructuras llamadas Cuerpos Similares a Protocormos (PLBs) cuando están expuestos a condiciones apropiadas de crecimiento *in vitro*. Tanto la naturaleza propagativa de los PLBs como la facilidad con que se generan, hacen de estas estructuras una alternativa atractiva, frente a la mediada por semillas, para la producción de gran número de plantas en crecimiento. Para aumentar la embriogénesis somática y optimizar el procedimiento, se transformaron PLBs de *Cattleya maxima* usando el método de *Agrobacterium tumefaciens*. El T-DNA portaba un gen de resistencia a la Higromicina, un marcador visible (GFP5-GUSA) y un gen de arroz que codificaba para el receptor tipo quinasa de embriogénesis somática (SERK), considerado importante en la embriogénesis somática. Los PLBs tratados generaron embriones somáticos y desarrollaron plántulas resistentes a la Higromicina. La inserción del T-DNA se confirmó por PCR, y la expresión de GFP se observó usando un estereomicroscopio fluorescente. Los PLBs transformados de *Cattleya maxima* fueron más eficientes en desarrollar embriones somáticos (60-80 %) que los controles no transformados (45-57 %) y este contraste se maximizó en medio Murashige y Skoog (MS) libre de hormonas (80 % de las plantas transformadas en comparación con 57 % de las no transformadas). Estos hallazgos apoyan la noción de que SERK juega un papel importante en la embriogénesis de orquídeas.

Palabras clave: *Agrobacterium tumefaciens*; Cuerpos Similares a Protocormos; Transformación; Orquídeas; pCAMBIA; *SERK*.

Transformação da orquídea silvestre *Cattleya maxima* Lindl mediada por *Agrobacterium*

Resumo Os protocormos são estruturas anatômicas únicas: são similares aos rizoides e se formam por hastes jovens de orquídeas sob condições fisiológicas. Os tecidos explantados de orquídeas produzem estruturas chamadas Corpos Similares a Protocormos (PLBs) quando estão expostos a condições apropriadas de crescimento *in vitro*. Tanto a natureza propagativa dos PLBs como a facilidade com que se generam, fazem com que estas estruturas sejam uma alternativa atrativa, comparativamente a mediada por sementes, para a produção de grandes números de plantas em crescimento. Para aumentar a embriogênesis somática e otimizar o procedimento, se transformaram PLBs de *Cattleya maxima* utilizando o método de *Agrobacterium tumefaciens*. O T-DNA carregava um gen de resistencia a Higromicina, um marcador visível (GFP5-GUSA) e um gen de arroz que codificava para o receptor tipo quinasa de embriogênesis somática (SERK), considerado importante na embriogênesis somática. Os PLBs tratados geraram embriões somáticos e desenvolveram plântulas resistentes a Higromicina. A inserção do T-DNA se confirmou por PCR, e a expressão de GFP se observou utilizando um estereomicroscópio de fluorescência. Os PLBs transformados de *Cattleya maxima* foram mais eficientes em desenvolver embriões somáticos (60-80 %) que os controles não transformados (45-57 %) e este contraste se potencializou em meio Murashige y Skoog (MS) livre de hormônios (80 % das plantas transformadas em comparação com 57 % das não-transformadas). Estes resultados apoiam a noção de que SERK desempenha um papel importante na embriogênesis de orquídeas.

Palabras clave: *Agrobacterium tumefaciens*; Corpos Similares a Protocormos; Transformacao; Orquideas; pCAMBIA; *SERK*.

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