MODIFICATION OF FLOWERING TIME IN *OSTEOSPERMUM ECKLONIS* L.
BY *CONSTANS* GENE

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**Abstract**

Altered onset of flowering in crop plants can be achieved by modifying the expression of flowering-time genes. In a few cases, these genes can change the flowering time of species unrelated to the plant from which they were isolated. We pursued this strategy in order to modify the flowering characteristics of the ornamental plant *Osteospermum ecklonis*. The flowering-time gene *CONSTANS* (*CO*) from *Arabidopsis thaliana* Landsberg erecta was the candidate in our work. This choice was based on literature demonstrating that *A. thaliana* transgenic plants containing extra copies of the *CO* gene flower earlier than wild type plants, suggesting that *CO* expression is sufficient to trigger flowering irrespective of day length. The *CO* cDNA was cloned in the expression vector pGREEN under the control of the constitutive promoter 35S. Genetic transformation of leaf tissue was performed using *Agrobacterium tumefaciens* strain AGL1. *CO* constitutive expression was detected in a checked transgenic clone. The flowering performance of potted transgenic plants was monitored in the greenhouse in comparison to control plants. 35SCO plants produced 30% more flowers than control, retained their ability to flower in June when control plants entered vegetative phase and were still able to produce new flower buds in August.

1. Introduction

Flowering depends on the co-ordinated expression of several classes of genes that may interact with environmental factors. The flowering-time gene *CONSTANS* (*CO*) has been cloned from *A. thaliana* Landsberg erecta (Putterill *et al.*, 1993). This gene encodes a putative transcription factor that is required to promote flowering under long day conditions in the original species (Putterill *et al.*, 1995). It has been also observed that transgenic plants of *A. thaliana* Landsberg erecta, containing extra copies of the gene (35S::CO), flower earlier than wild type plants even in short day conditions, suggesting that *CO* activity limits flowering time (Suarez-Lopez *et al.*, 2000). *O. ecklonis* (*Compositae*) is a popular pot plant native to South Africa. The plant, also known under the common name “African Daisy”, is a half-hardy perennial characterised by vigorous growth and plenty of flowers. The flowering time of cultivated varieties is late-winter to early-spring, and in summer the plants enter a vegetative phase. This is likely to be a consequence of either long day length condition or high temperature. Ornamental trait modification and virus tolerance have been achieved in *Osteospermum* by genetic engineering (Allavena *et al.*, 2000). In this work we have transformed *O. ecklonis* with the *CO* gene with the aim to modify the flowering time of the species.
2. Material and methods

The CO cDNA, kindly provided by Dr. George Coupland from John Innes Centre, Norwich U.K., was cloned in the pGREEN vector (a gift of Dr. Phil Mullineaux, John Innes Centre, Norwich, U.K.) under the control of the 35S promoter (cauliflower mosaic virus (CaMV) constitutive promoter) besides the NPTII gene. Genetic transformation of leaf tissue was performed using the engineered A. tumefaciens AGL1 strain, according to Giovannini et al. (1999). O. ecklonis leaf fragments of micropropagated plants were cocultivated with the bacterial suspension culture and after three days they were transferred to the regeneration medium (MS basal salts and vitamins, 30 g/l sucrose plus 2.8 µM IAA and 1.3 µM BA) added with kanamycin sulfate (100 mg/l) and cefotaxime (100 mg/l). Shoots regenerated from several independent transformation events were further propagated and rooted on basal medium and then acclimatized in the greenhouse to provide transgenic plant clones; plants regenerated from non co-cultivated leaves were used as control material. Ten clones were screened for the presence of the foreign CO gene by PCR analysis. DNA amplification was carried out on alkali treated leaf tissue using the procedure of Klimyuk et al. (1993). Based on the sequence of A. thaliana mRNA for CO protein (Gene bank accession number X94937) two synthetic primers were designed: 5' end primer 5’GTTCACTCTGCCAATCGCGT, 3' end primer (complementary strand shown) 5’AGCGGAACAACTCTATCTCCCC. PCR conditions were: thirty-five cycles of denaturation at 94°C, annealing at 56°C (1 min) and extension at 72°C (1 min), followed by a final extension of 7 min at 72°C. The PCR reactions were conducted in a total volume of 50 µl. Buffers and Taq DNA polymerase purchased from Life Technologies Italia S.r.l. (Italy) and the thermocycler PTC100 (MJ Research, Inc., USA) were used. The presence of the expected 509 bp DNA fragment was checked by electrophoretic agarose gel analysis (2% agarose, 1X TAE, 10 µg/ml ethidium bromide). CO gene expression was investigated in clone 301 by RT-PCR analysis. Total RNA was isolated from micropropagated plants of control and transgenic clone 301; 100 mg of leaf tissue were used for each sample according to the RNeasy Plant Mini Kit protocol (Quiagen, Germany). RNA concentrations were determined by absorbance at 260 nm, and purity was verified by the 260/280 nm absorption ratio. Contaminating DNA was eliminated in RNA samples using Sigma’s Amplification Grade Deoxyribonuclease I. Approximately 1 µg of total RNA was used in reverse-transcriptase reactions to generate cDNA. Two-step RT-PCR reactions of Sigma’s Enhanced Avian RT-PCR Kit (Sigma-Aldrich S.r.l., Italy) were performed; 5 µl of cDNA was finally amplified by AccuTaq LA DNA polymerase with the primers previously indicated and the following PCR conditions: thirty cycles of denaturation at 94°C, annealing at 56°C (1 min) and extension at 72°C (1 min), with a final extension of 5 min at 72°C. The amplified DNA fragments were visualised by electrophoretic agarose gel analysis (2% agarose, 1X TAE, 10 µg/ml ethidium bromide). The flowering performance of transgenic clone 301 (8 plants) was monitored in comparison with the parent variety (6 plants), in a well-lit greenhouse, from 19th February to the end of April 2001. The number of flower buds per plant, the number of flowers at anthesis and the total number of flowers per plant (buds, flowers at anthesis and wilted flowers) were counted weekly, during all the flowering time. At the beginning of May plants were potted in larger vases and data were collected until June, when O. ecklonis enters the vegetative phase (not permissive conditions for flowering). The monitored period covered the normal flowering time of O. ecklonis in our environment. Data were reported as means ± standard errors.

3. Results

The specific primers, designed for the CO gene, amplified the expected 509 bp DNA fragment in eight out of ten transgenic clones (Fig. 1). CO constitutive expression was detected in micropropagated plants of clone 301 by amplification of cDNA sample with the same set of primers (Fig. 2). Transgenic plants were successfully acclimatised
and cultivated in the greenhouse. The average number of flowers at anthesis per plant (Fig. 3B) was higher in the transgenic plants of clone 301 as a consequence of a more profuse flower bud production (Fig. 3A). The transgenic plants produced 30% more flowers per plant than control (Fig. 3C). Moreover, transgenic plants retained their ability to flower in June whereas control plants flowered sporadically and entered vegetative phase. In August only transgenic plants were still able to produce new flower buds (an average number of 7.9 buds per plant).

4. Discussion

The non-conventional breeding program based on genetic engineering was successful in *Osteospermum* to modify ornamental traits by using *rol* genes of *Agrobacterium rhizogenes* (Giovannini et al., 1999), and to obtain virus tolerant plants with the N protein gene of tomato spotted wilt virus (Vaira et al., 2000). Preliminary results from this work show that constitutive expression of a single *Arabidopsis* flowering time CO gene increases the production of flowers in the ornamental plant *O. ecklonis* during the normal flowering period (February-May) and extends the period of flowering during not permissive conditions (June to August). The early flowering phenotype reported over-expressing CO gene in *Arabidopsis* (Putterill et al., 1995) is not observed in 35SCO *Osteospermum* plants of clone 301, probably because in this species the transition from vegetative to reproductive phase is under the control of a different genetic pathway (e.g. vernalisation pathway). On the other hand the extension of flowering in the transgenic plants could be related to the continuous expression of the CO gene and its regulatory effect on other genes. It is our purpose to evaluate the behaviour of additional transgenic clones expressing CO gene and extend the evaluation period along the year. The final aim is the understanding of the CO gene potential for the production of *O. ecklonis* genotypes that flower even in not permissive conditions.

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References


Figure 1. PCR analysis of *O. ecklonis* plants derived from independent transformation events using *A. tumefaciens* Agl1 pGREEN NPTII 35SCO. 2% agarose gel showing the DNA fragments amplified from CONSTANS gene (509 bp): 1 to 10 putative transgenic plants (cl292, cl293, cl294, cl295, cl297, cl300, cl290, cl301, cl298, cl299); 11 pGREEN NPTII 35SCO plasmid; 12 control plant of the parental variety; 13 sample without DNA; M 1kb Ladder marker

Figure 2. Evaluation of *CO* expression. 2% agarose gel showing the PCR amplification product (509 bp) of specific primers for CONSTANS gene: 1 and 6 pUC18 Hae III marker; 2 control cDNA; 3 total RNA sample of cl301 with the amplified DNA contamination; 4 total RNA sample of cl301 after Dnase I treatment; 5 cDNA of cl301 with the amplification fragment of 509 bp
Figure 3. Flowering performance of *O. ecklonis* control and transgenic plants (clone 301) from the beginning of flowering (19 February: day 1) to the end of the normal flowering time (day 66). The number of flower buds per plant (A), the number of flowers at anthesis (B) and the total number of flowers per plant (buds, flowers at anthesis and wilted flowers) (C) were counted during all the flowering time. Following this, plants were potted in larger vases. In June, 105 days from the beginning of flowering, transgenic plants retained their ability to flower in non-permissive conditions developing new flower buds. Bars represent standard errors.