Analysis of T2 sunflower transgenic plants: high expression level and stability achieved by the *rbcS1* promoter regulation.

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ABSTRACT

- Sunflower genetic transformation, traditionally presented some limitations such as gene instability and low expression levels of transgenes. These same problems were also described in lettuce and chrysanthemum, species also belonging to the *Compositae* family, due to the use of the constitutive viral promoter *CaMV35S*. To analyze whether this promoter (used in our previous works and in all existing literature on sunflower transformation) was also the cause of these problems in sunflower, our group performed stable transformation assays with two constructs differing only in the promoter regulating *uidA* reporter gene (which encodes the betaglucuronidase enzyme): *CaMV35S* with the 5' leader sequence of the TMV (*CaMV35S*-TMV) and *rbcS1* (small subunit of ribulose-1,5-bisphosphate carboxylase). Results showed that the use of the *rbcS1* promoter increased levels of expression of betaglucuronidase when compared with the use of *CaMV35S*-TMV. We also observed a modification of the *nptII* gene expression (kanamycin resistance) with a shift in the transformation efficiency increasing from 2.23% to 7.06%. Moreover, response and aspect of the obtained plants (T0) was improved when they were transferred to the greenhouse (both by direct passage to earth or grafting), they were large-sized and with larger floral heads, resulting in an increase in the number and size of the obtained achenes. Our results are consistent with a study published in *Arabidopsis* where removal of the *CaMV35S* promoter resulted in the disappearance of the affected and altered expression pattern of transgenes which changed the phenotype of transgenic plants.
  The aim of this work was to analyze the gene stability and expression levels of sunflower T2 transgenic plants.
- Sunflower HA89 genotype was transformed with EHA105 *Agrobacterium* strain carrying the pKGWFS7,0 *Gateway* vector with the reporter gene *uidA* under the *rbcS1* promoter regulation and the *nptII* gene under the *nos* promoter regulation. Shoots rooted in kanamycin media where transferred to the greenhouse. T1 and T2 seeds were obtained and germinated, in sterilized sand, in the greenhouse. Transgene presence was analyzed by PCR or GUS histochemical staining.
- In the work presented here, we show the presence of the transgene in T2 transgenic plants by amplification of fragments corresponding to different regions of the construction. Also, GUS staining of leaves shows that a good expression level is maintained. These results reveal the genetic stability of this construction.
- Our results reveal that the change of the viral promoter *CaMV35S* for the chrysanthemum *rbcS1* promoter allows obtaining stable sunflower transgenic plants that are able to express the transgene in their second generation. In our previous analysis of sunflower transgenic plants with constructions containing *CaMV35S*, expression was almost undetectable and transgenes were lost in T2 (unpublished results). As shown in other species from the *Compositae* family, *rbcS1* promoter proved to be a more suitable promoter than *CaMV35S* for sunflower.
- The use of *rbcS1* promoter shows a promising future for sunflower genetic transformation, achieving transgene stability and improved expression levels, not only to obtain transgenic plants as a final product, but also, to evaluate candidate genes obtained *in silico* or from functional genomics studies, in order to assist plant breeding.

Key words: genetic transformation, *rbcS1* promoter, T2 stable expression

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important sources of edible oil and is becoming important for biofuel production. Sunflower oil is considered a good quality oil for its light taste and appearance but especially because it supplies more vitamin E than any other vegetable oil. Sunflower and peanut are the only major vegetable oil yielding crops that have no GM varieties authorized for commercial use. Sunflower biotechnological improvement is limited to molecular markers by the lack of availability of an efficient transformation protocol. One of the main reasons is because, until nowadays, sunflower is described as a recalcitrant species to *in vitro* propagation (Mayor et al., 2010). Besides, all published protocols suffer from low overall transformation efficiencies (Bidney et al., 1992; Knittel et al., 1994; Malone-Schoneberg et al., 1994; Burrus et al., 1996; Lewi et al., 2006; Radonic et al., 2006).

In addition, sunflower genetic transformation, traditionally presented some limitations such as gene instability and low transgene expression levels, as it was determined that in T1 plants betaglucuronidase levels were low, restrained to the trichomes of the main nerve in the leaf abaxial face (Radonic, 2010) and that all T2 plants lack the transgene (unpublished results). These same problems were also described in lettuce and chrysanthemum, species also belonging to the *Compositae* family, due to the use of the constitutive viral promoter *CaMV35S* (Davey et al., 2007; Visser et al., 2007). In lettuce, the main difficulty was the low stability of transgenes, McCabe et al. (1999) analyzed the heritability of ammonium glufosinate resistance regulated by *CaMV35S* up to the third generation and only 2.5 % of the T0 plants transferred this resistance. Meanwhile in chrysanthemum low expression levels were described when transgenes were under the regulation of this promoter (Annadana et al., 2002; Outchkourov et al., 2003).

To analyze whether this promoter (used in our previous works and in all existing literature on sunflower transformation) was also the cause of these problems in sunflower, our group performed stable transformation assays with two constructs differing only in the promoter regulating the reporter gene *uidA* (which encodes the betaglucuronidase enzyme): *CaMV35S* with the 5' leader sequence of the TMV (*CaMV35S*-TMV) and *rbcS1* (small subunit of ribulose-1,5-bisphosphate carboxylase). Results showed that the use of the *rbcS1* promoter increased levels of expression of betaglucuronidase when compared with the use of *CaMV35S*-TMV promoter in T1 plants (Radonic, 2010).

It was also observed a modification of the *nptII* gene expression (kanamycin resistance) with a shift in the transformation efficiency increasing from 2.23 % with the *CaMV35*-TMV promoter containing vector to 7.06 % with the *rbcS1* promoter containing vector. Moreover, response and aspect of the obtained plants (T0) was improved when they were transferred to the greenhouse (both by direct passage to earth or grafting), they were large-sized and with larger floral heads, resulting in an increase in the number and size of the obtained achenes, while T0 plants with the *CaMV35S*-TMV promoter were mostly grafted (as its roots were smaller) and presented multiple smaller floral heads with empty or fewer smaller achenes. Our results were consistent with a study published in *Arabidopsis* (Yoo et al., 2005) where the *CaMV35S* promoter altered the expression pattern of transgenes expressed in the same construction and affected the phenotype of transgenic plants, all which disappeared with the removal of this promoter.

The aim of this work was to analyze the gene stability and expression levels of sunflower T2 transgenic plants.

MATERIALS AND METHODS

Helianthus annuus L. inbred line HA89 seeds were provided by the Sunflower Breeding Program located at EEA INTA Balcarce, Provincia de Buenos Aires, Argentina.

Agrobacterium tumefaciens EHA105 strain was used carrying the pKGWFS7,0 *Gateway* vector (Karimi et al., 2002) with the *uidA* reporter gene under *rbcS1* promoter regulation and the *nptII* gene under *nos* promoter regulation.

Protocol was carried out as described in Radonic et al. (2006) with modifications in the selection scheme (Radonic, 2010). Shoots rooted in kanamycin media where transferred to the greenhouse. T1 and T2 seeds (achenes) were obtained and germinated, in sterilized sand. First, they were maintained at 4°C for a week and later they were transferred to 22-24°C with 16 h light/8 h dark. Once cotyledons were fully expanded, they were transferred to the greenhouse and transferred to 8 liters pots with a mixture composed by 40% soil, 30% peat, 20% manure and 10% perlite.

Genomic DNA extraction was performed (Saghai-Maroof et al., 1984). Transgene presence was analyzed by PCR amplification of a fragment of *uidA* gene or GUS histochemical staining (Jefferson et al., 1987).

PCR reaction mixture in a final volume of 50 µL contained: buffer (1x, Invitrogen, USA), Platinum Taq DNA polymerase (1 U, Invitrogen, USA), $MgCl_2$ 2.5 mM, dNTPs 0,2 mM and 0,1 µM of each primer (5' CCCTTACGCTGAAGAGATGC 3' and 5' GTTCATGCCAGTCCAGCGTT 3'). PCR conditions were: 2 min at 94°C, 5 touchdown cycles of 30 s at 94°C, 1 min at 60-56°C, 1 min at 72ºC, followed of 30 cycles of 30 s at 94ºC, 1 min at 55ºC, 1 min at 72ºC and a final step of 10 min at 72ºC. Expected product was of 781 bp.

RESULTS

Nine different plants from the second generation of two different T0 plants were analyzed by PCR amplification or GUS staining.

DNA from five T2 plants was analyzed by PCR amplification of a fragment of *uidA* reporter gene, as it is shown in figure 1, all plants contained the transgene.

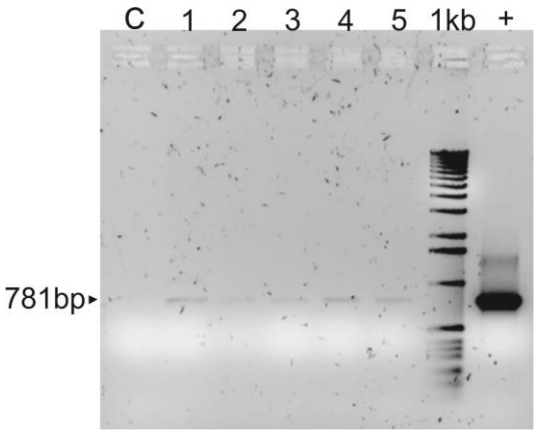

Fig. 1. PCR amplification of DNA from five T2 plants. C: reaction mixture negative control. 1-5: amplification of *uidA* gene. +: reaction positive control, pKGWFS7,0 Gateway vector. Expected fragment is indicated with an arrow.

Also, GUS staining of leaves from the four T2 plants analyzed showed that a good expression level is maintained (figure 2). These results confirm the stability obtained with the construction.

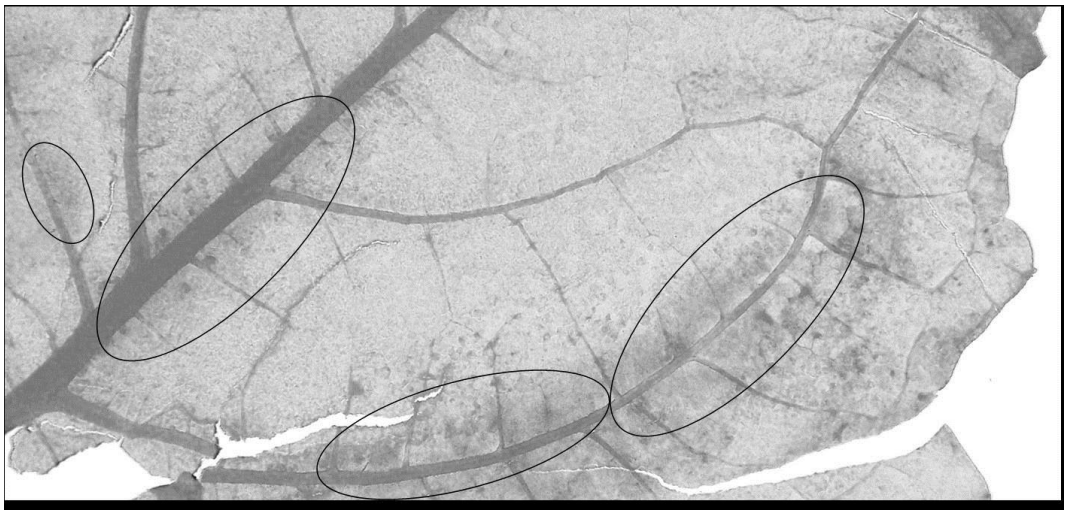

Fig. 2. GUS staining of a leaf from a T2 plant. Regions of positive staining are indicated.

DISCUSSION

In our previous analysis of sunflower transgenic plants with constructions containing *CaMV35S* promoter, expression was almost undetectable (Radonic, 2010) and transgenes were lost in T2 (unpublished results). In the case of *rbcS1* promoter T1, that gave origin to T2 plants analyzed here, presented higher expression levels (were staining could be seen throughout the entire leaf) than the obtained with *CaMV35S* promoter (Radonic, 2010). Our results reveal that the change of the viral promoter *CaMV35S* for the chrysanthemum *rbcS1* promoter allows obtaining stable sunflower transgenic plants that are able to express the transgene in their second generation. Thus, *rbcS1* promoter proved to be more suitable for sunflower transformation than *CaMV35S* promoter. This was also shown in other species from the *Compositae* family, chrysanthemum, were expression with *CaMV35S* promoter was almost undetectable, whereas *rbcS1* promoter was considered a strong promoter (Outchkourov et al., 2003).

The use of *rbcS1* promoter shows a promising future for sunflower genetic transformation, achieving transgene stability and improved expression levels, not only to obtain transgenic plants as a final product, but also, to evaluate candidate genes obtained *in silico* or from functional genomics studies, in order to assist plant breeding.

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