The expression of CsLFY and bar genes at transcription and translation levels in transgenic tobacco plants

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Abstract

Transgenic tobacco plants containing CsLFY and bar under 35S CaMV promoter control in T1 generation were analyzed. Also, the presence and expression of these transgenes at molecular level were studied. The presence of the transgenes in the genome was shown by PCR and expression of these genes at the level of transcription and translation processes was investigated. Expression of these differential transgenes at RNA level was shown in all lines. Electrophoretic patterns revealed the similarity of protein spectra of transgenic lines and the tobacco control. Proteins with molecular mass 44 kDa and 23 kDa were detected in different plant organs (mainly in leaves, leafstalk, stalk and less in roots) which theoretically correspond to expression products of CsLFY and bar genes, showing their constitutive activity.

Keywords: gene expression, transgenes, tobacco, genetically modified plants.

In the past decade, a wide variety of genes that disrupt the vegetative phase transition or alter the meristem identity in the herbaceous annual plant species have been identified and characterized (BLÁZQUEZ M. A. et al., 1997).

LFY codes for a plant specific transcription factor that is considered to be a master regulator of floral meristem development. Both LFY and AP 1 genes have been identified in members of different plant families, including citruses. The genomic organization of the CsLFY gene is similar to that observed for other LFY homologues. The 1197 bp open reading frame of the CsLFY gene predicted a 44 kDa LFY protein. In citrus CsLFY transcripts were readily detectable in fully open flowers and not detected in most vegetative tissues as seed, root and leaf (PILLITTERI L.J. et al., 2004).

Previous results show that higher level of CsLFY gene expression lead to a shorted vegetative phase with earlier flower development (LUPAȘCU V., 2007). The higher levels of CsLFY mRNA expression in tobacco is correlated with elevated levels of AP1 transcripts, whereas the levels of TERMINAL flower mRNA were almost undetectable in these plants. These results suggest that the Citrus LFY gene alone may regulate the time to flowering by up-regulating the expression of AP1 and down-regulating the TFL gene in a heterologous plant species (PORT A. et al., 2005).

The bar gene was originally cloned from Streptomyces hygroscopicus, an organism which produces the tripeptide bialaphos as a secondary metabolite. Bialaphos contains phosphinothricin, an analogue of glutamate which is an inhibitor of glutamine synthetase (DE BLOCK M. et al., 1987; THOMPSON C.J. et al., 1987). The assay of bar gene expression in
transgenic canola has detected its activity in roots, leaves, buds and seeds, equivalent to 200-1000 ng *phosphinothricin acetyltransferase* (PAT) protein/mg of total plant protein, since the gene is linked to a constitutive promoter. Activity was highest in the bud and leaves (1000 ng/mg total protein). The PAT protein is expressed in the roots, leaves, buds and seeds. It was not detected in the stem. Maximum expression was 0.001% of the total plant protein (http://apec.bioteo.or.th/pdf/Textbook.pdf). In T-25 corn plants, the enzyme activity was detected in kernels, leaves, roots and the highest PAT activity was found in stems (Draft Risk Analysis Report, 2001).

The controlling possibility of plant vegetation phases and flowering, allowing their better adaptation to different cultivation regions and periods is one of the aspects related to obtaining GMPs. The fusion of *CsLFY* gene, which is involved in alternation of growth phases and the *bar* gene coding the PAT enzyme under constitutive promoter 35S CaMV represent a practical interest for the creation of new genetically modified plants (GMPs) varieties.

Accordingly, the goal of this research was the identification and analysis of the *CsLFY* and the *bar* transgenes expression at transcription and translation levels in transgenic tobacco plants used as genetic model systems.

**Materials and Methods**

**Plant material and growth conditions.** Tobacco plants (*Nicotiana tabacum* L. var. Xanthi) were transformed with *bar* marker-gene (0.5 kb), coding the *phosphinothricin acetyltransferase* enzyme and the *CsLFY* gene (2.2 kb) isolated from "Washington" navel orange (*Citrus sinensis* L. Osbeck) coding for the *CsLFY* protein. The open reading frame and terminator region of the *CsLFY* and *bar* genes, regulated by the *CaMV 35S* promoter was stably integrated into the genome of tobacco plants using an *Agrobacterium*-mediated transformation system (Fig. 1.). The transgenic plants used in our experiments were kindly provided by Plant Transformation Research Center, UCR.

Experiments were conducted under greenhouse conditions. Tobacco primary transformant seeds were sown directly into pots (2 to 3 seedlings per pot). Seeds were grown at 23°C under 16 h light/8 h dark regimen until the elongation phase of development (20 day old plants).

![Figure 1. The structure of T-DNA included into tobacco genome](image)

**DNA isolation and PCR analysis.** The total genomic DNA was isolated from 1 g of young leaves using the standard methods described by MURRAY M.G. and THOMPSON W.F., (1980). DNA was resuspended in 100 μl of water. A *bar* gene fragment of 242 bp was amplified using 5'-GAAGTCCAGCTGCCAGAAAC / 5'-AGTCGACCGTGTACGTCTCC primers and the *35SCaMV promoter* fragment of 175 bp with 5'-TGACAGATAGCGGGCAATG / 5'-CAACCACGTCTTTCAAAGCA primers.
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respectively. Each reaction employed: 5 µl of DNA (0.1 µg/ml), 1 µl sense primer (50 pmol/µl), 1 µl antisense primer (50 pmol/µl), 10 µl 10X buffer, 6 µl MgCl₂ (25 mM), 2 µl PCR dNTP (10 mM), 0.5 µl Taq DNA polymerase (5 u/µl) and 74.5 µl dH₂O. PCR amplification was performed in a Gradient Master Thermal Cycler (Corbett, Australia, 2007) with 40 cycles of denaturation at 95°C for 30 s, primer annealing at 54°C for 30 s and extension by Taq polymerase at 72°C for 50 s. The electrophoresis of the PCR products was run on a 2% agarose gel in the presence of ethidium bromide (0.5 mg/ml) and 1x TAE buffer.

Expression of transgenes was evaluated through RNA and protein analysis.

RNA isolation and Gel-Blot Hybridization. All RNA was extracted from leaves of untransformed and transformed plants. The leaves were frozen in liquid nitrogen ground to a fine powder and stored at – 80°C. RNA was extracted using TRIZOL reagent (Gibco BRL). The 15 µg of RNA was fractionated by electrophoresis in 1.4% (w/v) agarose gel with formaldehyde, blotted onto nylon membranes using cDNA fragments for bar, CsLFY and ARNr 18S genes labeled with ³²P-dCTP as in previously described procedures (OROZCO-CARDENAS M.L., RYAN C.A., 2003; OROZCO-CARDENAS M. et al., 2001).

Protein extraction and SDS-PAGE. Preparation of protein extracts from leaves, leafstalk, stalk and roots was carried out at 4°C. Plant material was homogenized using a mortar and pestle with 3 volumes (w/v) of extraction buffer (pH 6.8) containing: Tris HCl 0.06 M, 5% glycerin, 1 mM EDTA, 1% 2-mercaptoethanol. Protein samples (200 µg) were separated by SDS-PAGE of 12.5% concentration of acrylamide resolving gel (LAEMLI U.K., 1970).

Results and discussions

Twelve transgenic lines were obtained from T₀ transformants and analyzed for stable integration and expression of CsLFY and bar transgenes. A partial coding region of CsLFY and bar genes was amplified by basic PCR methods using specific primers. The identification of transgenes was achieved by specific primers for identification of bar gene and specific primers for identification of 35S-P (Fig. 3).

Six lines (# # 2, 6, 7A, 13, 25 and 30) showed the expected 242 bp amplicon and 175 bp. Thus, line # 19A has proved absence of both sequences, bar gene and 35S-P but other lines have shown one or both sequences.

The expression of CsLFY and bar under 35S-P was tested at mRNA and protein levels. The expression of genes in leaves of transformed plants was confirmed by gene-blot analysis. When the accumulation of transcripts was analyzed marked differences were noted. Figure 4 shows mRNA-blot analysis of a wild-type tobacco plant used as control and five independent transformants (# # 6, 7A, 23, 25 and 30) that were selected to prove the
variability of gene expression. For the identification of the expression level the same quantity
of RNA (15 μg) was used. Transcripts of two different sizes were detected. The larger and
more abundant transcript (1.19 kb) was present in high levels in # # 6, 23, 25 and 30.

The line # 23 proved to here the highest level of bar gene expression. Lines # 25 and #
30 have middle level of bar gene transcript (0.5 kb). Low intensities of RNA band has been
observed for line # 7A and the lowest for line # 6 (Fig. 4, B).

It is possible that low expression of transgenes for some lines occurs as a result of co-
suppression phenomenon that could appear after integration of similar gene copies
(JORGENSEN R.A. et al., 1996).

Thus, although the both genes are included into the genome and controlled by the
same promoter 35S CaMV, and are theoretically inherited as a single locus, the level of their
expression at different transgenic tobacco lines did not correlate and different accumulation of
transcripts with exception of line # 23 were distinguished.

The 35S CaMV promoter exhibits a high level of transcription activity in a variety of
plant tissues and is one of the most widely studied and used promoter in transgenic plants.
The expression of transgenes in most GMPs is controlled by 35S CaMV promoter that
induces their constitutive expression (BOTTERMAN J., LEEMANS J., 1989; ARAGAO
F.J.L, BRASILEIRO C.M.B., 2002). However, the levels of expression of the two genes are
different. Under the control of the constitutive 35S CaMV promoter the transgene expression
is highest in young leaves.

The citrus plant CsLFY transcripts were not detected in most of the vegetative organs
(PILLITTERI L.J. et al., 2004). The regulation of CsLFY gene by 35S-P in the leaves of the
transgenic tobacco, however, is active (Fig. 4, A).

One of problems regarding transgenes expression is the instability of the proteins
coded by these transgenes, for instance, in soybean, the glutamine synthetase protein of
GmGlnβ1 gene was not detected (ORTEGA J. L. et al., 2001). Thus, to find the expression of
CsLFY and bar genes at translation level, protein analysis was performed at the elongation
phase of development. At this stage the protein synthesis is highest (HALFHILL M.D., et al.,
2003). Also, it is known that the intensity of the protein synthesis depends on the promoter
used (BLUMENTHAL A., et al., 1999; BRIAN K. HARPER and NEAL STEWART J. R.,
2000).

It is known that the CsLFY gene codes for proteins with a molecular mass of 44 kDa
(PILLITTERI L.J. et al., 2004) and the bar gene codes for the PAT protein with a molecular
mass of 23 kDa that has different levels of expression in different organs (BERIAULT J. N.,

To assess the effects of the constitutive expression of the transgenes in tobacco plants,
assays in different organs were performed. Total soluble proteins were extracted from leaves,
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leafstalk, stalk and roots. The wild-type protein extract and transgenic line # 23 showed similar profile. The basal levels of 44 kDa and 23 kDa protein syntheses found in transgenic line, however, were much higher than the control, showing their constitutive expression (Fig. 5). In contrast, the 44 kDa and 23 kDa protein biosynthesis positively correlated with mRNA accumulation in leaves.

**Figure 5.** SDS-PAGE of proteins isolated from leaves (1), leafstalks (2), stalks (3) and roots (4) of line # 23 and control tobacco. M – molecular markers.

**Conclusions**

The transgenic expression at the mRNA level of *CsLFY* and *bar* genes under the control of 35S-P was detected in all the analyzed tobacco lines, but with different levels. The levels of expression within the plant varied substantially too, being higher in leaves. The *CsLFY* gene had high levels in four lines (# # 6, 23, 25 and 30), and the *bar* gene, only in one case (line # 23). Consequently, at the protein level expression was observed in most plant organs, but mainly in leaves, leafstalks, stalks and less in roots, where proteins with molecular mass 44 kDa and 23 kDa were detected. These may correspond to expression products of *CsLFY* and *bar* genes, showing their constitutive expression.

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**References**


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