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TRANSIENT EXPRESSION IN TOBACCO BRIGHT YELLOW 2 CELLS AND POLLEN GRAINS: A FAST, EFFICIENT AND RELIABLE SYSTEM FOR FUNCTIONAL PROMOTER ANALYSIS OF PLANT GENES

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Abstract – Gene expression is mediated by DNA sequences directly upstream from the coding sequences, recruited transcription factors and RNA polymerase in a spatially-defined manner. Understanding promoter strength and regulation would enhance our understanding of gene expression. The goal of this study was to develop a fast, efficient and reliable method for testing basal promoter activity and identifying core sequences within its pollen specific elements. In this paper we examined the functionality of buckwheat metallothionein promoter by a histochemical GUS assay in two transient expression systems: BY2 cells and pollen grains. Strong promoter activity was observed in both systems.

Abbreviations: GUS, beta-glucuronidase gene; MT, metallothionein; MU, 4-methyl umbelliferyl; BY2, Bright Yellow 2;

Key words: Promoter, transient expression, BY2 cell, pollen grain

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INTRODUCTION

Promoter analysis represents an important procedure for understanding the regulation of gene expression. Moreover, already characterized and well-examined plant promoters enable controlled expression of relevant genes and can be used for the production of transgenic plants carrying traits of interest.

The first step in characterization of a potential promoter region is determination of its nucleotide sequence and computer-assisted prediction of putative cis-regulatory elements that can facilitate and direct further study. Electrophoretic mobility shift and DNaseI protection assays provide some information about the interaction of these putative regulatory elements with nuclear protein extracts or purified protein transcription factors. However, only functional analysis in transgenic plant cells or tissues, carrying the promoter upstream of a reporter gene, can provide certain evidence of promoter activity. Deletion analysis of different fragments of the same promoter can define the minimal promoter or narrow the region considered to be involved in stress response or tissue-specific activity. Mutational analysis identifies oligonucleotides critical for specific promoter functions. Generation of stable transformed plants allows determination of tissue and developmental stage promoter specificity, as well as revealing stress response regions. However, this approach is time-consuming and accompanied by large differences in the obtained results due to varying transgene copy numbers, chromatin position effects and transgene locus organization (Bhattacharya et al., 1994). Transient expression analyses (performed in plant cell suspension cultures, protoplasts or in pollen

grains) have been developed in order to overcome these problems.

One of the model systems employed for this purpose is the tobacco BY-2 plant cell suspension, which is especially useful for cell localisation studies (Kawai - Yamada et al., 2001), understanding viral infection mechanisms (Bendahmane et al., 2002), characterisation of metabolic pathways (Zhang et al., 2005) as well as promoter analyses (Karine et al., 2001) etc. Stably transformed BY2 cells are usually used in laboratory practice, while promoter analysis with a transiently expressing reporter gene in transgenic BY2 cells is not commonly employed, although this system could provide fast screening of basal promoter activities and minimal promoter region determination (Ito et al., 2001).

Since they are haploid, single-celled and develop in a synchronous fashion, **pollen grains** were suggested as an ideal target for genetic manipulation. Particle bombardment of pollen grains has been used successfully to achieve transient expression and production of transgenic plants, e.g. tobacco (Touraev et al., 1995). There are no published results based on promoter analysis by transient expressed reporter genes in pollen cells. This system could be accurate for identifying promoter sequences potentially containing pollen specific cis- elements.

MATERIALS AND METHODS

Promoter constructs with the GUS reporter gene

Complete and truncated versions of the 5' regulatory region of the buckwheat *FeMT3* gene spanning from the ATG codon to -569 bp, - 416 bp and -163 bp upstream of the transcriptional start site, respectively, were amplified by PCR using the genomic clone *gFeMT4.1* (GeneBank accession no. AY361956) as the template and the corresponding primers. For all constructs primer R (5'-ctgatcaggttaatgagcttgagttacaaagag -3') was used as the reverse primer, while F1 (5'-cccaagctt*gcaggt*atcgatttgctaacg-3'), F2 (5'-cccaagcttattaaaaaaaattgggcacctgcg-3') and F3 (5'-cccaagcttacgttaaccttttgtttttgt-3') were used as forward primers for the -569, - 416 and -163 fragments, respectively. The *Bcl I* and *Hind III* sites needed for further cloning are underlined. Nucleotides in italics within primer F1 are from the

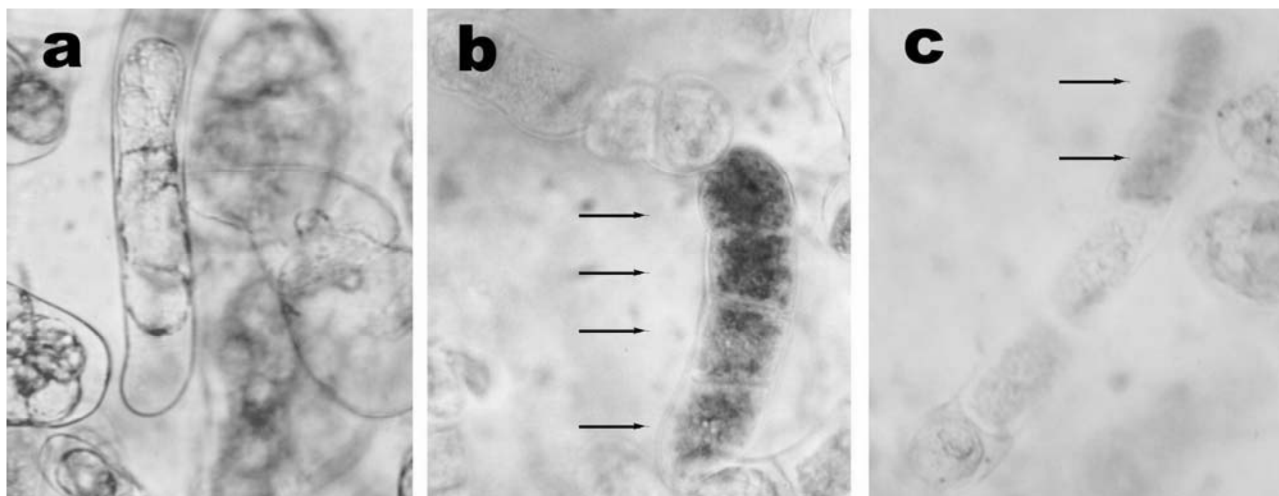

Figure 1. GUS staining of BY2 cells:
a) negative control- untransformed BY2 cells
b) positive control- BY2 cells harboring *Dc3* promoter::*gus* gene construct
c) BY2 cells harboring *FeMT3* promoter::*gus* gene construct

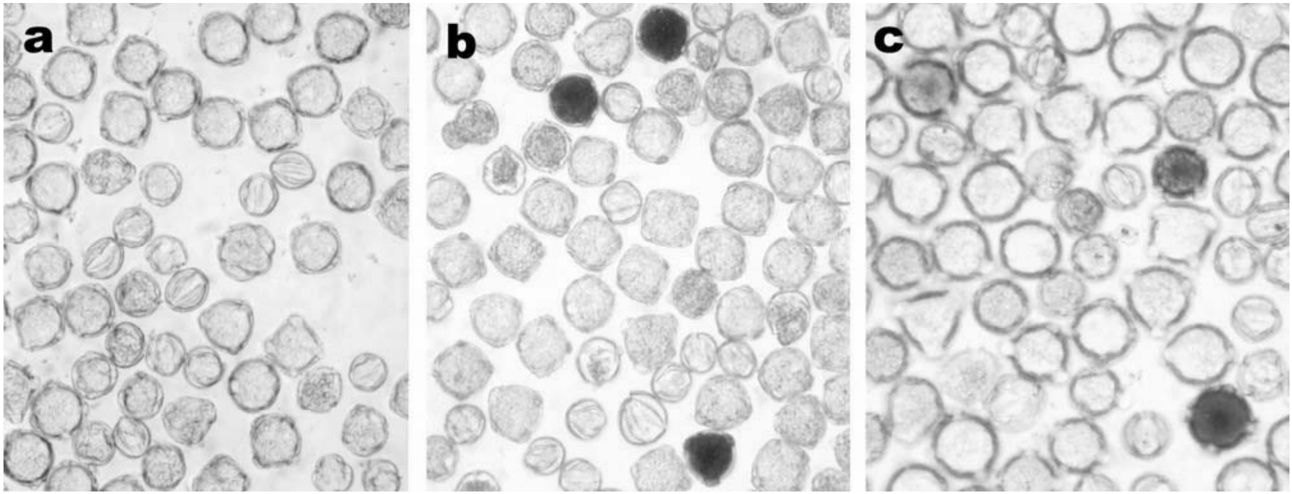

Figure 2. GUS staining of pollen grains:
a) negative control- untransformed pollen grains
b) positive control- pollen grains harboring *Dc3* promoter::*gus* gene construct
c) pollen grains harboring *FeMT3* promoter::*gus* gene construct

Marathon (Clontech) adapter sequence. Amplified promoter fragments were cloned upstream from the *GUS* reporter gene within the pRD410 binary vector, replacing the *Bam HI-Hind III* fragment containing the *CaMV35S* promoter. The obtained vectors were used for biolistic bombardment of pollen and BY2 cells. Vector pBI101 containing 1.5kb of the Dc3 (group III *lea*-class gene from *D. carota*) promoter upstream from the GUS reporter gene was used as the positive control. Negative controls were untransformed BY2 and pollen grain cells.

Particle bombardment parameters

The helium-driven PDS-1000/He particle delivery system (Bio-Rad, USA) was used for the biolistic transformation experiments. The following parameters were applied: 9 cm target distance, 1000- psi helium rupture pressures and a vacuum pressure of 27 in. Hg. Briefly, 3 µl (1µg/µl) of plasmid was precipitated onto gold particles with an average diameter of 1.1 µm (ChemPur Feinchemikalien und Forschungs-bedraf GmbH, Karlsruhe, Germany) under constant vortexing with 60 µl gold particles (30 mg/ml), 60 µl 2.5M $CaCl_2$, and 30 µl 1M spermidine. The mixture was placed on ice for 25 min, centrifuged and the pellet washed twice with 100% ethanol before being re-suspended in 22 µl of 100% ethanol. 11 µl of the coated–particle suspension was loaded onto a macrocarrier disk and left to dry completely before bombardment.

Transformation of tobacco microspores

Tobacco mid-bicellular pollen grains from 18 to 19 mm flower buds were isolated essentially as described (Touraev et al., 1995.). For bombardment, 60 µl of microspore suspension was dropped in the middle of a wet 3 cm Petri dish. Immediately after bombardment, the microspores were maintained in 1 ml of Tupy's medium (Tupy et al., 1991) at 25 °C in the dark. After 24 h, the cultures were diluted with 1 ml of Tupy's medium, centrifuged and washed once with 1 ml of 100 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4). Finally, microspores were again centrifuged at 100 x g and GUS localized histochemically (Jefferson et al., 1987). Stained pollen grains were viewed through a Leitz-DIAPLAN microscope equipped with a camera system.

Maintenance and transient transformation of tobacco BY2 cells

The tobacco BY2 cell line was maintained as described by (Nagata et al., 1992). For bombardment, 400 μl of 3-day-old BY2 suspension was dropped in the middle of a wet filter paper in the Petri dish. Immediately after bombardment, the BY2-cells were rinsed out and maintained in 5ml of LS medium modified to contain 1 mg/L of thiamine instead of 0.4 mg/L (Linsmaier and Skoog, 1965) at 25 °C in the dark. After 24 h, transformed cells were stained at 26°C for 16 h using X-Gluc solution (100 mM NaH_2PO_4/Na_2HPO_4, pH 7.2 mM X-Gluc, 40% methanol). Stained BY2 cells were pictured through a Leitz-DIAPLAN microscope equipped with a camera system.

RESULTS AND DISCUSSION

To test the usefulness and efficiency of the two transient expression systems, we performed transformation and analysis of two promoter types in both BY2 cells and immature pollen grains. One of the chosen promoters was the well-known pollen-specific Dc3 (Touraev et al., 1995), while the other was a buckwheat metallothionein type 3 (*FeMT3*) promoter (Brkljačić et al., 2005). Computer assisted analysis of the *FeMT3* promoter uncovered numerous putative cis-elements that could be involved in different stress responses (hormonal, heat shock, light or metal stimuli), as well as three pollen specific sites. In the following experiments, the FeMT3 promoter and its two 5' deletants proved to be active in the anthers, leaf and root of the stably transformed plants (Bratić et al., 2009). However, further investigations of pollen specific activity and mutational analysis of putative pollen specific elements could not be carried out in this time-consuming and prone-to-variation transgenic system. The goal of this study was to develop a fast, efficient and reliable method for testing both basal promoter activity and to identify the core sequences within pollen specific elements.

The FeMT3 promoter at full available length and its two deletion variants were functional in **BY2 cells** transformed by particle bombardment (Fig. 1). In accordance with previously published results (Bratić et al., 2009), there were no great differences in their activities. This three-day long method will enable fast and accurate promoter dissection and identification of the minimal functional promoter region.

The results of transformation and promoter activity in **pollen grains** are presented in Fig 2. Basically, this method allows plus/minus promoter activity analysis, as only large differences in promoter activity could be detected by this approach. Thus, signal differences between various FeMT3 constructs could not be observed. On the other hand, GUS signals in pollen grains carrying FeMT3 promoter constructs were less intensive than in those carrying the strong pollen specific Dc3 promoter construct (Touraev et al., 1995), which served as the positive control.

The confirmation of pollen-specific promoter activity in this fast and reliable promoter–testing system will make possible promoter dissection and mutation of specific putative regulatory elements with the aim of finding strict sequences responsible for pollen specificity in the FeMT3 or other promoters.

The two presented systems of transient transgenic expression enabled successful rapid screening and confirmation of the functionality of different promoter fragments. In addition, transiently expressing BY2 cells can be easily selected to give rise to stably transformed cell lines, which are used in stress-response promoter studies (Nagata et al., 2004). Both transgenic BY2 cells and pollen grains have great regeneration potential, allowing production of transgenic plants, which can be utilized to determine tissue-specific promoter activity patterns.

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ТРАНЗИЈЕНТНА ЕКСПРЕСИЈА У BRIGHT YELLOW 2 ЋЕЛИЈАМА ДУВАНА И ПОЛЕНОВИМ ЗРНИМА КАО БРЗ, ЕФИКАСАН И ПОУЗДАН СИСТЕМ ЗА ФУНКЦИОНАЛНУ АНАЛИЗУ ПРОМОТОРА БИЉНИХ ГЕНА

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Експресија гена је регулисана посредством промотора - ДНК секвенци узводно од кодирајућег региона. Промотори својом секвенцом одређују места везивања транскрипционих фактора, регулатора и РНК полимеразе. Структурна и функционална анализа промотора неопходна је за разумевање механизама генске експресије. Циљ овог рада је развијање брзог, ефикасног и поузданог система за тестирање базалне промоторске активности као и откривање секвенци полен-специфичних елемената промотора. У овом раду је тестирана функционалност промотора за металотионеин хељде, посредством хистохемијског *GUS*-есеја у два транзијентна система за експресију: *BY2* ћелијама и поленовим зрнима. У оба система је уочена изражена активност испитиваног промотора.