VOL. 6, NO. 11, NOVEMBER 2011 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science


www.arpnjournals.com

MULTIPLE COPIES OF THE ACTIVATOR INTERACT WITH A HETEROLOGOUS PROMOTER TO REGULATE GENE EXPRESSION

Shijuan Liu and Mei Yan
Qufu Normal University, Qufu, China
E-Mail: sjliusj@tom.com

ABSTRACT

To extend our knowledge of gene expression regulated by multiple copies of the same activator, a chimeric promoter was constructed, which contained five copies of the *ocs* activator (-294 to -116 relative to the transcription start site) added upstream of the same truncated *mas* promoter (-189 to +65 relative to the transcription start site). The chimeric promoter was linked to a β-glucuronidase reporter gene and introduced into tobacco plants. The results of the fluorometric assays exhibit that addition of five copies of the *ocs* activator to the *mas* promoter increases the level of GUS activity 3.5-fold in leaves but 3.3-fold in roots, respectively. This indicates that affixing multiple copies of the same element can produce higher gene expression. Further study has shown that multimerization of the *ocs* activator also influences wound inducibility of the chimeric promoter.

Keywords: gene expression, *ocs* activator, *mas* promoter, GUS activity, multiple copies, transgenic tobacco.

INTRODUCTION

The control of expression of many genes is frequently mediated at the level of transcription and involves the interaction between promoters and enhancers [1]. These *cis*-acting DNA sequences have been well characterized in yeast and animal systems and have been shown to be highly specific binding sites for trans-acting regulatory proteins [2]. Enhancer-like elements have also been identified in several gene promoters [3]. These gene elements act independently of their orientation and can activate the expression of heterologous promoters [4].

One of the best characterized *cis*-acting DNA sequences is the mannopine synthase (*mas*) promoter. The *mas* gene is found on the tumor-inducing plasmid of the plant pathogen *Agrobacterium tumefaciens* [5]. The *mas*l' and *mas*2' genes encode enzymes of a two-step pathway for the synthesis of the opine mannopine. These genes share a dual bidirectional promoter. Although born on a prokaryotic plasmid, the promoter of *mas* gene contains all the *cis*-acting signals required for function in plants. This promoter also contains various *cis*-acting elements necessary for the regulated transcription [6]. Detailed deletion analysis using different reporter genes has revealed that *mas* promoter harbors redundant functional domains necessary for the developmental, tissue-specific and inducible properties of the two genes it regulates [7]. For example, the region from -103 to +66 is responsible for wound inducibility [8]. Because it could function like a plant promoter, *mas* promoter has been widely employed as a model to study plant gene regulation.

Many eukaryotic genes contain upstream activating sequences or enhancer elements that are essential for gene transcription. These sequences are not promoters in themselves but activate transcription from linked promoters [9]. They are independent of their orientation relative to the promoters and can be either independent (enhancers) or dependent (upstream activating sequences) upon their position relative to these promoters. Enhancers can be tissue specific or inducible. They may also be modular, in that they may be composed of a limited number of basic sequence motifs that interact in a synergistic fashion. One of the best characterized *cis*-acting DNA sequences is the octopine synthase (*ocs*) enhancer element. The *ocs* gene is found on the tumor-inducing plasmid of *Agrobacterium tumefaciens* [10]. Since the *ocs* gene is well expressed in a variety of tissues in transgenic plants, the transcriptional signals of the *ocs* gene must function as endogenous plant sequences controlling constitutive expression [11]. The upstream region of the octopine synthase gene contains a transcriptional activator element that activates the *ocs* promoter independent of orientation. Ellis *et al*., [12] showed that sequences upstream of the *ocs* gene can activate a heterologous promoter. The chlormphenicol acetyltransferase (CAT) gene under control of the maize alcohol dehydrogenase promoter is poorly expressed in tobacco. When the *ocs* activator was cloned, in either orientation or at either end of the CAT gene, CAT activity was expressed at high levels in tobacco. The *ocs* activator therefore could stimulate the other promoter. A 176 bp DNA sequence between -292 and -116 relative to the start of transcription of the *ocs* gene functions as an enhancer in both orientations in transgenic tobacco or in plant protoplasts [13]. This DNA region contains a 16 bp palindromic *ocs*-element (-193 to -178), which is essential and sufficient for enhancing activity in transient expression assays [14].

In this study, multiple copies of the *ocs* enhancer fragment were introduced into *mas* promoter to enhance promoter activity and to determine how multiple copies of the *ocs* activator affect the expression upon linked *mas* promoter.

MATERIALS AND METHODS

Materials

Restriction endonucleases and T_4 DNA ligase were purchased from Biolabs and used according to the manufacture's specifications. Reagents for GUS assays and antibiotics were purchased from sigma.

Bacterial strains and media

Escherichia coli strains were grown in LB medium at 37°C. *Agro bacterium tumefaciens* LBA4404 containing various plasmids were grown in YEP medium at 28 °C. Antibiotic concentrations, when used, were for *E. coli*: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; for *A. tumefaciens*: kanamycin, 50 μg/ml; rifamplcin, 10 μg/ml. These experiments were conducted under P1 containment conditions as specified by the National institutes of Health Recombinant DNA Guidelines.

Plasmid constructions

In order to construct the chimeric promoter GUS gene fusion, we used a genomic 254 bp fragment that contains 189 bp 5' upstream and 65 bp 3' downstream of the *mas* transcription start site. This fragment was amplified by PCR using the primer pair pr-1and pr-2 (Table-1) and pKan2 [15] DNA as a template. Pr-1 and pr-2 had additional nucleotides at the 5' ends to provide the *Hind* III and *Xba* I restriction sites, respectively. The CaMV 35S promoter of pBI121 was replaced with the *Hind* III/*Xba*I-cut PCR products to generate pMAS. Vector pMAS contained the GUS (*uidA*) coding region driven by the *mas* promoter and the *NOS* terminator. Further upstream was the neomycine phosphotransferase II gene providing resistance against kanamycin fused with a nopaline synthase promoter and terminator.

The fragment of *ocs* enhancer region, -294 to -116 relative to the transcription initiation site, was generated by PCR using the primers pr-3 and pr-4 (Table-1) and pEN1 DNA as a template. Both primers had additional nucleotides at the 5' ends to provide the *Hind* III restriction sites. The *ocs* activator was produced and the *Hind* III/ *Hind* III restriction endonuclease fragments were subsequently cloned as five copies into the *Hind* III site of the pMAS upstream of the *mas* promoter to create constructions pOMS5. The sequence of each of the PCR-generated promoter fragments was verified by the dideoxy chain-termination method [16] to exclude errors introduced into the sequence during PCR, and verification of all constructs was carried out by restriction enzyme digests, PCR, and sequencing. The resulting plasmids contained five copies of the *ocs* transcriptional activating element linked to the *mas* promoter. The constructs were subsequently transformed into *E. coli* DH5α cells [17]. Finally, plasmids were introduced from *E. coli* into *A. tumefaciens* LBA4404 [18] by transformation. *A. tumefaciens* transconjugants were selected on YEP medium supplemented with 10 μg/ml rifampicin and 50 μg/ml kanamycin.

Table-1. Oligonucleotides used in PCR to create constructs.

Primer	Sequence (from 5' to 3')
Pr-1S	AAGCTTCCAACTTTTTCTTGAT
Pr-2A	TCTAGACATCGATTTGGTGTAT
Pr-3S	AAGCTTCGGTGCGATGCCCCCATC
Pr-4A	AAGCTTGGATCCCTGAAAGCGACG

S and A indicate sense and antisense primers, respectively.

Plant materials and transformation

Leaf disks from 6-week-old in vitro sterile shoot tip cultures of *Nicotiana tabacum var. Wisconsin* 38 were infected with 2-days old cultures of *A. tumefaciens* transconjugants harboring the different recombinant plasmids, and transgenic plants were generated by the leaf disk transformation regeneration method [19].

GUS activity assay

To assay GUS activity, tobacco tissues were harvested from the leaves and roots of transgenic plants. The tissues (100-200 mg) were ground in extraction buffer consisting of 50 mM pH7 sodium phosphate, 10 mM EDTA, 0.1 M Sarkosyl, 0.1 M Triton X-100, and 10 mM β-mercaptoethanol. The extracts were centrifuged for 5 min (4 °C) and then the supernatant was used for assays of GUS. According to the fluorescent method described by Jefferson [20], GUS activity was assayed by using 10 μl extract (about 20 to 30 μg protein) and MUG (4-methylumbelliferyl-β-D-glucuronide) as a substrate. Protein concentrations were determined by the method of Braford [21].

Wound induction

For wound induction studies, leaves were dissected in half through the midrib, one piece (non-wound-induced) was immediately ground in extraction buffer and assayed, the other was injured on the leaves by puncturing numerous small holes employing a stainless steel brush. Wounded plants were immersed in distilled water for 24 h before samples were ground and processed for fluorometric assays.

RESULTS AND DISCUSSIONS

Construction of chimeric constructs

We constructed a chimeric *gusA* gene derived from *ocs* and *mas* elements. To be convenient for study of understanding how specific enhancer-promoter interactions regulate gene expression, the upstream regulatory region of *mas* promoter was deleted and the truncated *mas* promoter (-189 to +65) was used in the construct. The chimeric promoter contained five copies of the *ocs* activator (-294 to -116) placed upstream of the -189 *mas* promoter deletion. The chimeric promoter was fused to the *gusA* reporter gene. Figure-1 presents the

structure of the chimeric promoter and genes we were constructed.

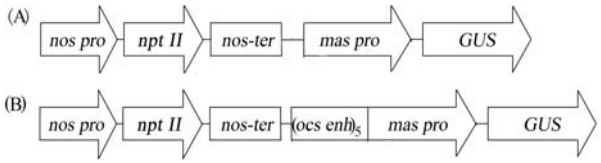

Figure-1. Structure of the fusion genes.

Expression level of the chimeric promoters in transgenic tobacco plants

Previous study has shown that *mas* promoter confers gene expressing in almost tissues in transgenic tobacco plants, so we wanted to determine how multiple copies of the *ocs* activator influence the expression upon linked truncate *mas* promoter. Then constructions (Figure-1) were introduced into tobacco through *Agro bacterium*-mediated transformation. For each construct leaves and roots were harvested separately from 16 or more *in vitro* grown plantlets. GUS activity in the leaves and roots of transgenic tobacco plants for each construct was quantified.

As anticipated, there were variations in the amount of GUS activity among transgenic lines for each construct (Figure-2 and Figure-3). However, expression level variability between different transgenic plants does not correlate with the copy number of stably integrated transgenes [22]. The observed variability has often been referred to as 'position effect', based on the as yet unproved assumption that expression levels of the introduced genes are directly influenced by host DNA sequence or chromosomal structure/composition at or near to the site of integration. Comparison of the ranges provided a good indication of the strength of each promoter.

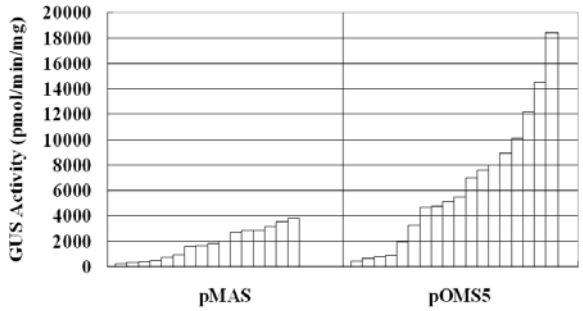

Figure-2. GUS activity of the two constructions in leave extracts of transgenic tobacco. Each bar represents the activity of an individual transformant. The different constructions are as indicated below the graph.

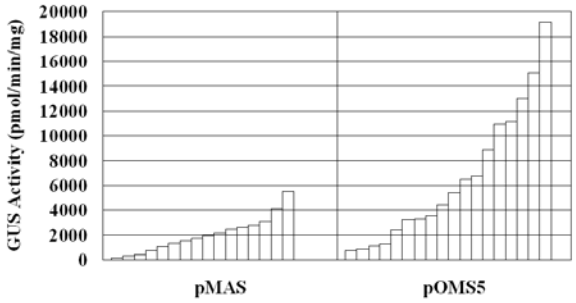

Figure-3. GUS activity of the two constructions in root extracts of transgenic tobacco. Each bar represents the activity of an individual transformant. The different constructions are as indicated below the graph.

Our results confirm that *ocs* activator enhances the expression of a heterologous promoter, and the relative strength of each construct was estimated (Table-2 and Figure-4). As shown in Table-2 and Figure-4, roots from in vitro grown plants displayed higher GUS activities in all constructs than leaves. In all tissues examined, the *mas* -189 deletion directed the lower level of GUS activity, which was 1806 and 1996 pmol MU min^{-1} mg^{-1} protein in leaves and in roots, respectively. Addition of five copies of the *ocs* activator to the truncated *mas* promoter resulted in a significant alteration of GUS activity in leaves (average 6371 pmol MU min^{-1} mg^{-1} protein). Moreover, there was a substantially elevation of GUS activity in roots (average 6542 pmol MU min^{-1} mg^{-1} protein). The level of expression using pOMS5 harboring five copies of the *ocs* activator was 3.5- and 3.3- fold higher than that directed by the *mas* promoter lacking the extra *ocs* activator in leaves and in roots, respectively. This indicates that affixing multiple copies of the same element does produce higher gene expression.

Table-2. Average GUS activity in different organ of transgenic plants containing various constructions.

Constructs		pMAS	pOMS5
Leaf	N	16	18
	X	1806	6371
Root	N	16	18
	X	1996	6542

GUS activity is assayed using total protein prepared from leaf and root tissue respectively. Activity is given in pmol MU min^{-1} mg^{-1} protein. n, the number of individual transgenic plants assayed for each construction. x, the average GUS activity for each construction.

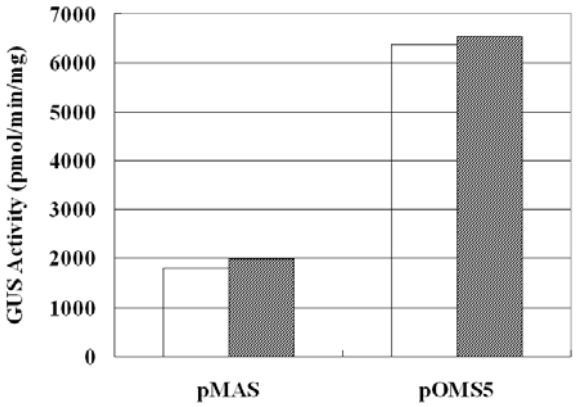

Figure-4. Average GUS activity of various constructions in transgenic plants. Average GUS activity in the leaves (white bar) or in the roots (hatched bar) of transgenic tobacco plants harboring different promoter-*uidA* fusions. The constructions are as indicated below the graph.

Our work has shown that the activity of the *mas* promoter is lowly regulated in plant, but the chimeric promoter shows high levels of activity in various types of plant tissues. In addition, the activity of the chimeric promoter is induced by wounding. Thus, this promoter may serve as a model to study gene regulation in plants. From this and other analysis, it is clear that the combinatorial properties of several *cis*-regulatory elements are required to define the overall regulatory program of the *mas* promoter. Such combinations of promoter 'subdomains' have been described for numerous genes and in plants have perhaps best been documented with the CaMV 35S promoter.

Previous study shows *ocs* activator of the upstream promoter region of the *ocs* gene has the properties of a eukaryotic enhancer element. It is essential for high-level expression for the *ocs* promoter and enhances the expression of a heterologous promoter (Adh-1). The *mas* promoter (construct pMAS) directed a similar level of GUS activity in roots and in leaves, which suggests that deletion of the DNA sequence from -318 to -189 can also enhance the activity of the promoter. Besides the results above mentioned, considering that the *mas* promoter directed a minimal background level of GUS activity in roots and leaves examined, and that addition of a heterologous *ocs* activator to the *mas* promoter did substantially increased the level of GUS activity relative to the *mas* promoter in leaves, we came to the conclusion that the *ocs* activator region is responsible for the enhancement of the GUS activity in the leaves. Our results also suggest that the action of the chimeric promoter in increasing expression of the hybrid gene override each other, namely, the enhancer element and the truncated promoter are dependent on each other and act cooperatively. However, the expression pattern of the *mas* promoter alters when it is fused with *ocs* activator.

Gradient expression of GUS activity directed by two constructs

Previous study showed that the expression of the *mas* promoter was under developmental control. In other words, the *mas* promoter activity is dependent on the age of the tissue, being higher in older parts of the plant. To extend and refined our present knowledge of the chimeric promoter constructed by us, the influence of age on the expression of the two *gusA* constructs was studied. GUS activity for each construct was examined in leaves of different developmental stages from independent transgenic plants. A gradient of activity resulted in higher expression of two promoters in the lower leaves than in the upper leaves of the plant (Figure-5), which meant that GUS activity increased with ageing of the tissues. Since pMAS lacking *ocs* activator still could direct a gradient pattern of expression, there must have been a *cis*-acting DNA elements in *mas* promoter sufficient to direct this developmental expression.

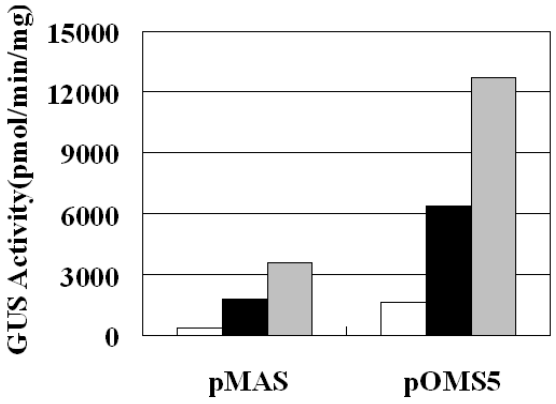

Figure-5. Basipetal GUS expression gradient driven by different promoters. Relative GUS activity driven by different promoters were assayed in young leaves (white bar), older leaves (black bar), and old leaves (hatched bar).

Wound induction assays

A number of plant genes are inducible by mechanical wounding of plant tissue. These genes include *mas*, *nos*, and one encoding a tobacco proteinase inhibitor I. To gain our knowledge about how various specific protein-DNA interactions are integrated into the overall pattern of gene regulation, the protein extracts were analyzed by enzyme assays from wounded and unwounded leaves of transgenic plants harboring two promoters. It could be observed that for two constructs leaves showed stimulation of GUS expression by mechanical damage (Figure-6), but the extent of wound inducibility differed. Transgenic plants harboring construction pMAS demonstrated a 1.5-fold induction of GUS activity. Addition of five copies of the *ocs* activator led to a 2.5- fold additional increase of GUS.

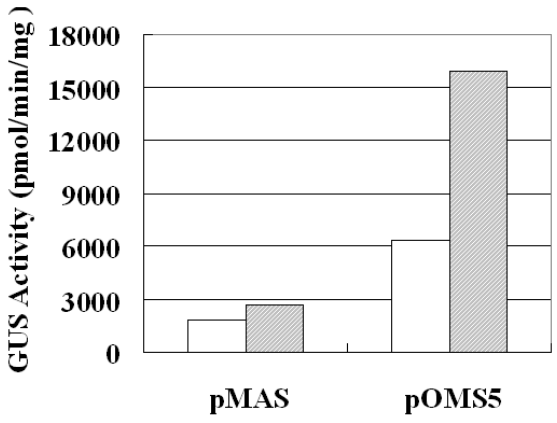

Figure-6. Wound inducibility of different chimeric promoters. GUS activity is driven by different promoters in unwounded leaves (white bar) and wounded leaves (hatched bar). The constructions are as indicated below the graph.

The results of our experiments exhibit that the *mas* promoter fragments were inducible by wound, and that the *cis*-acting DNA elements existed in the truncated *mas* promoter are still responsible for wound induction. Our results also indicate that adding multiple copies of the *ocs* activator alters this wound inducibility in some sort. These results demonstrated that the *ocs* element play a role in the expression of the chimeric promoter in the stable expression assay. We believe that this activating region must be quite close to *ocs* element since others reported that *ocs* element were quite active and directed wound inducibiltiy. The wound inducibility of gene expression implies the existence of a signal transduction pathway in response to the environmental stimulus. At the terminus of this signal transduction pathway, a wound-specific factor(s) would bind to wound-responsive promoter elements and activate gene expression.

CONCLUSIONS

We compared two promoters in transgenic plants using the *gusA* gene marker. Because of the variability exhibited by each individual transformant, a number of plants were analyzed in order to reach a significant conclusion. Based on the results of this study the following conclusions are drawn:

Multimerization of the *ocs* upstream region (the *ocs* enhancer) did have a major effect on expression, because a tendency toward higher levels was observed.

The *mas* promoter expression increased with plant age; the chimeric promoter expression also tended to increase with plant age. Elements localized in the +65 to -189 region of *mas* must therefore be responsible for increasing basipetal expression of *gusA*.

The *mas* promoter has the wound inducibility shown by the +65 to -189 5' *mas* region since both *mas* and chimeric promoters exhibited consistent wound inducibility.

REFERENCES

[1] Blackwood E.M. and Kadonaga J.T. 1998. Going the distance: a current view of enhancer action. Science. 281: 61-63.

[2] Ptashne M. 1986. Gene regulation by proteins acting nearby and at a distance. Nature. 322: 697-701.

[3] Schaffner G., Schirm S., Muller-baden B., Weber F. and Schaffner W. 1988. Redundancy of information in enhancers as a principle of mammalian transcription control. J. Mol. Biol. 201: 81-90.

[4] Ptashne M. 1988. How eukaryotic transcriptional activators work. Nature. 335: 683-689.

[5] DiRita V.J. and Gelvin B.S. 1987. Deletion analysis of the mannopine synthese gene promoter in sunflower crown gall tumors and Agro bacterium tumefaciens. Mol. Gen. Genet. 207: 233-241.

[6] Langridge W.H., Fitzgerald K.J., Koncz C., Schell J. and Szalay, A.A. 1989. Dual promoter of Agro bacterium tumefaciens mannopine synthase genes is regulated by plant growth hormones. Proc. Natl Acad. Sci. USA. 86: 3219-3223.

[7] Leung J., Fukuda G., Wing D., Schell J. and Masterson R. 1991. Functional analysis of cis-elements, auxin response and early developmental profiles of the mannopine synthase bidirectional promoter. Mol. Gen. Genet. 230: 463-474.

[8] Ni M., Cui D. and Gelvin S.B. 1996. Sequence-specific interactions of wound-inducible nuclear factors with mannopine synthase 2' promoter wound-responsive elements. Plant Mol. Biol. 30: 77-96.

[9] Dillon N. and Sabbattini P. 2000. Functional gene expression domains: defining functional unit of eukaryotic gene regulation. BioEssays. 22: 657-665.

[10] Bruce W.B., Bandyopadhyay R. and Gurley W. 1988. An enhancer-like element presents in the promoter of a T-DNA gene from the Ti plasmid of Agro bacterium tumefaciens. Proc. Natl Acad. Sci. USA. 85: 4310-4314.

[11] Fox C.P., Vasil V., Vasil K. and Gurley W.B. 1992. Multiple ocs-like elements required for efficient transcription of the mannopine synthase gene of T-DNA in maize protoplasts. Plant Mol. Biol. 20: 219-233.

[12] Ellis J.G., Llewellyn D.J., Dennis E.S. and Peacock W.J. 1987. Maize Adh-1 promoter sequences control anaerobic regulation: Addition of upstream promoter

elements from constitutive genes is necessary for expression in tobacco. EMBO J. 6: 11-16.

[13] Koncz C., De Greve H., Andre D., Deboeck F., Van Montagu M. and Schell J. 1983.The opine synthase genes carried by Ti plasmids contain all signals necessary for expression in plants. EMBO J. 2: 1597-1603.

[14] Leisner S.M. and Gelvin S.B. 1988. Structure of the octopine synthase upstream activator sequence. Proc. Natl Acad. Sci. USA. 85: 2553-2557.

[15] Franche C., Bogusz D., Schopke C., Fauquet C. and Beachy R.N. 1991. Transient gene expression in cassava using high-velocity micro projectiles. Plant Mol. Biol. 17: 493-498.

[16] Sanger F. Nicklen S. and Coulson A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl Acad. Sci. USA. 74: 5463-5467.

[17] Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 557-580.

[18] Hoekema A., Hirsch P.R., Hooykaas P.J.J. and Schilperoort R.A. 1983. A binary vector based on separation of vir and T-region of the Agro bacterium tumefaciens Ti plasmids. Nature. 303: 179-181.

[19] Horsch R.B., Fry J.E., Hoffmann M.L., Eichholtz D., Rogers S.G. and Fraley R.T. 1986. A simple and general method for transferring genes into plants. Science. 227: 1229-1231.

[20] Jefferson R.A. and Wilson K.J. 1991. The GUS gene fusion system. In: Plant Molecular Biology Manual. Gelvin S. and Schilperoort R., (Eds.), B14. Dordrecht: Kluwer Academic Publishers. pp. 1-33.

[21] Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

[22] Orphanides G. and Reinberg D. 2002. A unified theory of gene expression cell. 108: 439-451.