

DEVELOPMENT OF A TRANSIENT PROMOTER ASSAY SYSTEM FOR OIL PALM

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ABSTRACT

Optimization of the physical parameters was carried out to produce transient β -glucuronidase (GUS) and green fluorescence protein (GFP) activities in oil palm tissues (leaf, mesocarp and root) for promoter analysis.

The optimum conditions for DNA delivery into mesocarp slices and root tissue were 1550 psi helium pressure, 9 cm distance from macrocarrier to the sample and using 1 μ m gold particles as microcarrier to penetrate the tissues. For leaf explants, a lower pressure of 1350 psi sufficed with the same size gold particles and distance. The specificity of the Type 3 metallothionein-like gene (MT3-B) promoter was determined using the optimized system developed. The MT3-B promoter was cloned into an expression cassette (pBI221) containing GUS as the reporter gene with the removal of the existing Cauliflower Mosaic Virus (CaMV) 35S promoter to form a pBI-4C vector. The original pBI221 plasmid containing the CaMV 35S promoter was used as a control. In our study, expression of the MT3-B promoter was only observed in the root tissue at a low level but not in the other tissues (mesocarp and leaf). In contrast, with the constitutive promoter (CaMV 35S), expression was observed in all the tissues tested. These results correlated with the expression profile obtained for the MT3-B gene using northern blot analysis.

Keywords: oil palm, particle bombardment, transient expression assay, MT3-B gene promoter.

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INTRODUCTION

The ability of the biolistic technique to deliver DNA into intact plant cells has provided an opportunity for genetically engineer crop plants (Klein *et al.*, 1987) and as a tool for analysing tissue-specific promoters via the transient gene expression system (Vasil *et al.*, 1989). The basic principle of the biolistic method is to use high velocity microprojectiles to penetrate the outer tissue layers in order to introduce genetic materials into living cells, which then survive to express the introduced gene. This process involves a consideration of the physical, chemical and ballistic interactions involving DNA, high velocity microprojectiles, target cells and tissue type. The microprojectiles are commonly DNA-coated gold or tungsten particles.

The transient expression frequency provides the most convenient measure of the success rate of DNA introduction into a tissue by particle bombardment.

This assay, by means of reporter genes, allows rapid analysis of the expression of a sequence cloned into a gene cassette prior to its transformation into plant cells (Hunold *et al.*, 1995; Ainley and Key, 1990). The most popular reporter systems for monitoring genetic activity in plant cells include firefly luciferase, GUS and GFP. There are also several reports on the differential expression patterns of promoter activity based on the transient expression assay of these reporter genes (Crone *et al.*, 2001; Takahashi *et al.*, 1992; Moriwaki *et al.*, 1999). Such an approach is a powerful and sensitive means of studying the activity of a gene promoter as a function of cell development, or tissue and organ expression (Takahashi *et al.*, 1992). For example, the promoters of individual plant heat shock genes have been fused to the GUS gene in order to characterize the developmental and tissue-specific expression patterns of the genes both with and without the heat stress in bombarded *Tradescantia* pollen (Crone *et al.*, 2001).

A cDNA clone encoding a metallothionein-like protein designated MT3-B was isolated from the oil

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palm. Northern analysis showed that the *MT3-B* gene is expressed in both the mesocarp [at 15 and 17 weeks after anthesis (WAA)] and root at low levels. The genomic clone containing the *MT3-B* promoter has been isolated and fully sequenced (Siti Nor Akmar *et al.*, 2002). The *MT3-B* promoter may be potentially useful for targeting the expression of introduced genes into the oil palm root or mesocarp.

The availability of temporal and tissue-specific gene promoters is essential to genetically engineer crop plants. The transient expression system has been developed using mesocarp and leaf tissues of the oil palm for analysing tissue-specific promoters using reporter genes (Zubaidah and Siti Nor Akmar, 2000; 2001). This paper therefore reports the optimized conditions for the introduction and expression of the GFP and GUS genes in bombarded oil palm tissues. The specificity of the oil palm *MT3-B* gene promoter was also determined using the transient assay system employing GUS as the reporter gene.

MATERIALS AND METHODS

Preparation of Target Material for Transformation

Preparation of plasmid DNA was carried out using the Qiagen™ mini prep kit according to the protocol of the manufacturer (Qiagen, Chatsworth, CA). Freshly excised leaf (frond 1), roots (from 3-month-old seedlings) and mesocarp (12 WAA) were sterilized in 20% Clorox (20 min), followed by rinsing three times with sterile distilled water. The explants were cut into 5 mm x 5 mm disks before placing onto Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2.5 mg litre⁻¹ benzylaminopurine (BAP) for leaf and mesocarp tissues while root tissues was cultured on MS medium supplemented with 0.5 mg litre⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) 24 hr before bombardment.

Particle Bombardment

DNA transfer was carried out using the Biolistic™ particle delivery system, PDS-1000/He (Bio-Rad Laboratories, Richmond, CA), following the manufacturer's recommendations with a few modifications as follows: 3 µg of 1 µm diameter gold particles were coated with 10 µg of plasmid DNA in the presence of 100 µl (2.5 M) calcium chloride and 40 µl (0.1 M) spermidine. After centrifugation (13 000 rpm) and washing the pellet with 70% ethanol, the DNA-coated gold particles were finally resuspended in 50 ml 100% ethanol. For each bombardment, a 7 µl aliquot was loaded on a macro-carrier and

allowed to air dry. The system was set up at 6 mm distance between the macro-carrier and stopping screen, and 11 mm distance from the macro-carrier to the rupture disc. The vacuum was maintained at 27 Hg. The samples were placed 6 cm or 9 cm beneath the stopping screen. Rapture disks ranging from 900 to 1800 psi were used and each experiment carried out in triplicate.

Transient Expression Studies

- Gus assay. GUS activity was measured fluorometrically using 4-methylumbelliferyl β-D glucuronide (MUG) and histochemical staining of GUS was performed as described by Jefferson *et al.* (1987).
- Microscopic detection of GFP. Bombarded mesocarp slices and leaf sections were observed under a Leica fluorescence microscope fitted with GFP filter set (excitation at 360-480 nm and emission spectrum at 480-500 nm). GFP gene expression was determined as bright-green fluorescence caused by GFP accumulation in the cells.

Cloning of the *MT3-B* Promoter into a Gene Reporter Plasmid

The *MT3-B* promoter was cloned into pBI221 plasmid carrying GUS as the reporter gene by replacing the cauliflower mosaic virus (*CaMV*) 35S promoter in the vector construct to yield plasmid pBI-4C. A few modifications were made to the ligation procedure given by the manufacturer (Roche):

- DNAs (insert and vector) incubated at 50°C for 5 min followed by immediate cooling before adding the ligation mixture;
- ligation was carried out at 4°C (instead of 16°C) for 24 hr; and
- molar ratio of vector: insert of about 1:4.

RESULTS AND DISCUSSION

Development and Optimization of the Transient Expression System in Oil Palm Tissues

Microprojectile bombardment, one of the most powerful transformation techniques, has been successfully applied to direct gene transfer in oil palm (Parveez and Christou, 1999). Based on our results, this system is also useful for transient expression studies. In this study, the physical parameters for transient transformation were optimized using the GFP and *uidA* reporter genes, coding for the GFP and GUS. Transient assay analysis was carried out to determine the successful delivery

of foreign genes into the oil palm tissues. In this study, GFP and GUS were used as the reporter genes. The expression of the GFP gene in the oil palm tissues bombarded with HBT1 α plasmid (Chiu *et al.*, 1996) carrying a constitutive promoter (*CaMV* 35S) was determined under a Leica fluorescence microscope. The number of green fluorescence spots indicated the expression level of the GFP gene. *Table 1* show that the highest expression was observed in mesocarp bombarded at 1550 psi rupture disc pressure, whereas in leaf the highest expression was obtained at 1350 psi. A previous study on transient expression of white spruce showed that higher pressure in microprojectile bombardment resulted in lower GUS expression in pollen, but a higher expression in embryogenic tissues (Li *et al.*, 1994). The other parameters tested in our study, including the amount of DNA delivered per shot and volume

age as well as the various technical parameters of particle bombardment are important factors to consider for DNA transfer into oil palm tissues.

Using the optimized biolistic system that has been developed, analysis of the bombarded mesocarp tissues was carried out using the GUS assay which allows determination of the strength and localization of the activity of the specific promoter tested. Plasmid DNA pBI221 (Clontech, USA) containing the GUS-coding region flanked by the *CaMV* 35S promoter and nopaline synthase (NOS) terminator was used to detect the expression of the GUS gene in the oil palm leaf and mesocarp. The oil palm tissues were bombarded with the plasmid and expression of the reporter gene in the bombarded tissues was determined using the GUS assay (fluorometric or histochemical). *Table 2* shows the expressions of the GUS gene in bombarded mesocarp

TABLE 1. EXPRESSION OF THE GREEN FLUORESCENCE PROTEIN (GFP) GENE ON BOMBARDED MESOCARP SLICES AND LEAF DISCS

Rupture disk pressure (psi)	Distance from microcarrier to target tissue (cm)	No. of green fluorescence spots/explant after two days' culture	
		Mesocarp (12 WAA)	Leaf (spear leaf)
Control (-DNA)	6	0	0
	9	0	0
900	6	4	6
	9	2	3
1 100	6	7	15
	9	5	10
1 350	6	15	17
	9	10	20
1 550	6	25	11
	9	20	10
1 800	6	8	3
	9	12	5

of gold suspension delivered per shot. Based on GUS fluorometric assay, we found that the results did not show any significant differences in the level of GUS expression. We have also used different age of fruits (data not shown) from eight to 15 WAA and found that explants from 12 WAA to be the best giving the highest expression of GFP. In this study, we found that it was very hard to handle matured fruits because of the high content of oil bodies. Previous reports on other plants showed that the choices of tissue line and age gave different transient expressions of the reporter gene (Charest *et al.*, 1993; Duchesne and Charest, 1991; Newton *et al.*, 1992). In this study, it also appeared that the explant type and

slices with (treated) and without (control) the plasmid using the histochemical assay. About 95% of the mesocarp slices bombarded with the pBI221 plasmid were GUS positive while in the control no GUS positive expression was observed.

The fluorometric assay was based on hydrolysis of the MUG substrate by GUS. The GUS expression was monitored by the addition of buffered MUG substrate to mesocarp extracts of the tissues bombarded with plasmid containing the constitutive promoter (*CaMV* 35S). The reaction of the GUS enzyme in the bombarded mesocarp was to produce methylumbelliferone (MU) which fluoresced under emission of 430 nm and excitation of 360 nm.

TABLE 2. β -GLUCURONIDASE (GUS) ASSAYS ON MESOCARP SLICES BOMBARDED WITH (treated) AND WITHOUT (control) THE pBI221 PLASMID

Tissue (mesocarp slices)	No. of mesocarp slices	No. positive
Control	90	0
Treated	90	85

TABLE 3. FLUOROMETRIC ASSAY OF MESOCARP TISSUES BOMBARDED WITH (treated) AND WITHOUT (control) THE pBI221 PLASMID

Tissue	GUS activity (pmol min ⁻¹ mg ⁻¹ wet tissue)			
	30 min	60 min	90 min	120 min
Control	3.5	3.2	3.5	3.5
Treated	34	45	56	50

Differences in the production of MU between the control (no plasmid) and treated tissues (with pBI221 plasmid) were clearly observed 30 min after addition of the substrate with the maximum activity of the enzyme at 90 min of 56.0 pmol min⁻¹mg⁻¹ wet tissue (Table 3). Thus, the optimum time to measure GUS activity in the oil palm tissues was 90 min after treatment. In the treated tissues, the GUS expression increased with time, while in the control tissues the expression level remained constant at approximately 3.5 pmol min⁻¹mg⁻¹ wet tissue. The GUS expression in the control tissue may be due to endogenous activity which can be considered the basal level of GUS activity. The GUS expression in the bombarded tissues was significantly higher than in the control tissues. Therefore, the increased expression must be due to the introduced gene. Histochemical assay supported these results.

Both the fluorometric (Table 3) and histochemical (Table 2) assays showed positive GUS expression on the bombarded tissues containing the GUS gene. The fluorometric assay was the more sensitive method

to quantify the GUS expression, requiring only a very small amount of the bombarded tissue. Further, it can also estimate the strength of the specific promoter which is important for promoter analysis. The histochemical assay, on the other hand, enabled us to determine the locality of expression, providing information on the specificity of the promoter.

Production of Transformation Vectors Containing the *MT3-B* Promoter and Reporter Gene

The *Hind* III and *Xba* I sites were introduced by PCR to flank the *MT3-B* promoter. The *CaMV* promoter in the pBI221 plasmid was removed by digestion with *Hind* III and *Xba* I and replaced with the amplified *MT3-B* promoter containing the introduced restriction sites. The resulting clone, designated pBI-4C, was digested with *Hind* III and *Xba* I to confirm the presence of the insert. Figure 1 shows the restriction analysis of the recombinant clone containing the expected insert size (lane 1). The pBI-4C clone was further verified by DNA sequencing data.

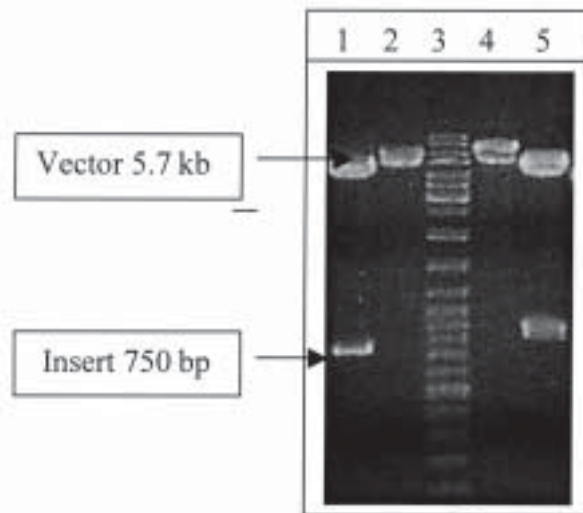
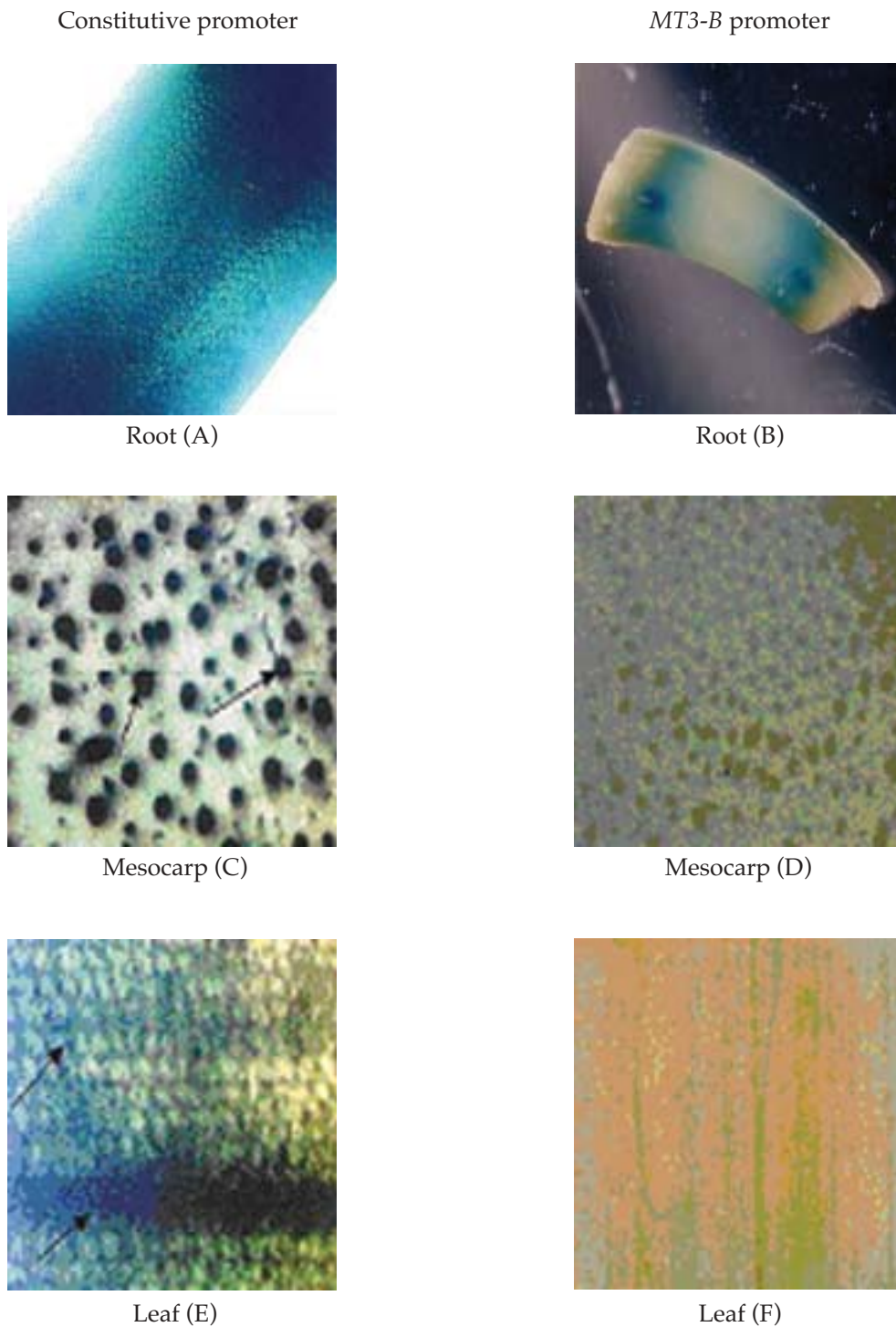


Figure 1. Restriction analysis of pBI221 and pBI-4C plasmids. Lane 1: plasmid pBI-4C digested with Hind III and Xba I, Lane 2: plasmid pBI-4C digested with Hind III, Lane 3: DNA size marker, Lane 4: plasmid pBI221 digested with Hind III, and Lane 5: plasmid pBI221 digested with Hind III and Xba I.

Analysis of the *MT3-B* Promoter Using the Optimized System

The activity of the *MT3-B* promoter was assayed by the GUS histochemical staining procedure after bombardment with the pBI221 and pBI-4C plasmids. Figure 2 shows the results of the histochemical assays on the leaf, mesocarp and root tissues bombarded with plasmid containing the *MT3-B* and *CaMV* 35S promoters using the optimized method that have been developed (Zubaidah and Siti Nor Akmar, 2001). Blue spots were observed in the root tissues indicating GUS expression but GUS expression was not detected in the leaf and mesocarp tissues. This provided a good indicator for the specificity of the *MT3-B* promoter because both constructs were based

on the pBI221 vector. The only difference was that in the constructed pBI-4C plasmid, the *CaMV* 35S promoter was replaced with the *MT3-B* promoter. These results confirmed the earlier result by Northern analysis (Siti Nor Akmar *et al.*, 2002) that expression of the *MT3-B* gene can only be observed in the root and mesocarp at low level late in ripening (15-20 WAA). Since 12 WAA mesocarp was used in this study, no expression of the *MT3-B* promoter was observed. Moreover, the type of vector used and the strength of the promoter have been shown to be important for the expression of specific promoters (Charest *et al.*, 1993; Ellis *et al.*, 1991). Further study on the *MT3-B* promoter activity using mesocarp from the later stages of fruit development is in progress.



Notes: A and B: localization of GUS activity in root bombarded with *CaMV 35S* and *MT3-B* promoters, C and D: localization of GUS activity in mesocarp bombarded with *CaMV 35S* and *MT3-B* promoters, E and F: localization of GUS activity in leaf bombarded with *CaMV 35S* and *MT3-B* promoters.

Figure 2. Distribution of β -glucuronidase (GUS) activity in oil palm tissues (root, leaf and mesocarp) (comparison between two promoters).

CONCLUSION

In this study, a transient expression assay system for promoter analysis in mesocarp slices and leaf discs with GUS and GFP as the reporter genes was established. This is a simple, versatile and fast method for analysing the strength and specificity of oil palm promoters. Using this method, results can be obtained within a week. The choices of explant type and culture conditions were important for high expression at all the tissue ages. The particles chosen must, however, be compatible with the explant materials.

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