pVR37, a new binary promoter-probe vector with xylanase reporter

Constitutive or spatial and/or temporal expression of transgene is essential for various applications in plant biotechnology. Several promoters conferring such controlled expression of transgene have been characterized[1,2]. Since most of them are protected by patents, novel promoters are to be cloned and functionally validated. Promoters from different origins with less sequence homology would also reduce the risk of homology-dependent gene silencing[3].

Approaches to find novel promoters include the use of computational methods[4], promoter trapping[5–7] and expression pattern of genes under a variety of conditions[8–10]. Though the former two methods directly identify the promoter sequences, the latter method involves identifying the differentially expressed transcripts, and recovering the upstream sequences by techniques such as inverse-PCR, TAIL-PCR and chromosome walking. Once identified, its activity, nature and pattern of expression can be studied using a promoter probe-vector. A promoterless reporter gene downstream of one or more restriction sites is the key feature of a promoter-probe vector. The DNA fragment to be tested for promoter activity can be ligated into these restriction sites, and expression of the reporter gene can then be analysed in various tissues over time/conditions[11–13]. A variety of reporters such as resistance to antibiotics (kanamycin and hygromycin), green fluorescent protein (GFP), β-glucuronidase genes (GUS) and luciferase (LUC) have been used and compared previously[14,15]. Recently, *XynA*, a codon-optimized gene encoding xylanase enzyme (endo-1,4-β-glucanase) catalysing the cleavage of xylan has been employed as the reporter in plant transformation[16]. Xylanase acts on AZCL-linked xylan substrate to release the soluble dye- (AZCL-) labelled fragments, resulting in a blue colour which can be quantified by measuring the absorbance at 590 nm. Stability of xylanase allows many samples to be processed without affecting the accuracy of the assay. Vickers *et al.*[16] showed that the xylanase assay is simple and cost-effective. In this study, an attempt was made to construct and functionally validate a binary promoter-probe vector, pVR37, with *XynA* as the reporter gene for validating novel plant promoters.

Sequence of *XynA* gene and its 5′ untranslated region (UTR) was obtained from pD4XynR (kindly donated by Gang-Ping Xue, CSIRO, Brisbane, Australia). The coding region and the 5′ UTR were synthesized and cloned into *Kpn*I and *Sac*I sites of pGA4 by GENEART, Regenburg, Germany. The fragment of 778 bp containing 5′ UTR + *XynA* was released by *Bam*HI + *Eco*RI digestion, eluted from agarose gel, purified using QIAGEN Gel Extraction kit (QIAGEN, #28006) and ligated with pCAMBIA1305.1 (accession no. AF354045, obtained from CAMBIA, Canberra) cut with the same enzymes (*Bam*HI + *Eco*RI). Ligated products were transferred to *Escherichia coli* DH5α. The recombinant clones carrying promoter-probe vector (pVR37) were identified by blue/white colony assay. pVR37 released the expected 778 bp fragment upon restriction with *Bam*HI + *Eco*RI. PCR of pVR37 with RB29_M13_F/R primers yielded a product of 778 bp indicating that pVR37 carried a promoterless 5′ UTR + *XynA* reporter gene[16]. The multiple cloning site (MCS) in pVR37 included the target sequence for *Hin*dIII, *Pst*I, *Xba*I and *Bam*HI for inserting the DNA sequence to be tested for promoter activity. The sequence and annotation features of pVR37 (Figure 1 *a*) have been deposited at GenBank of NCBI (EU744547).

Promoter-probe vector (pVR37) was functionally validated by cloning CaMV 35S promoter in the multiple cloning site upstream of 5′ UTR + *XynA*. CaMV 35S in pWBVec8 (kindly donated by Ming-Bo Wang, CSIRO PI, and Canberra) was isolated using specific primers (RB19_35S_F: 5′ ACGAAGCTTCGTCAACATGGTGGAGCA 3′ and RB19_35S_R: 5′ GACGGATCCGTCCTCTCCAAATGAAAT 3′) designed to contain sites for *Hin*dIII and *Bam*HI. A PCR product of 423 bp was amplified from pWBVec8

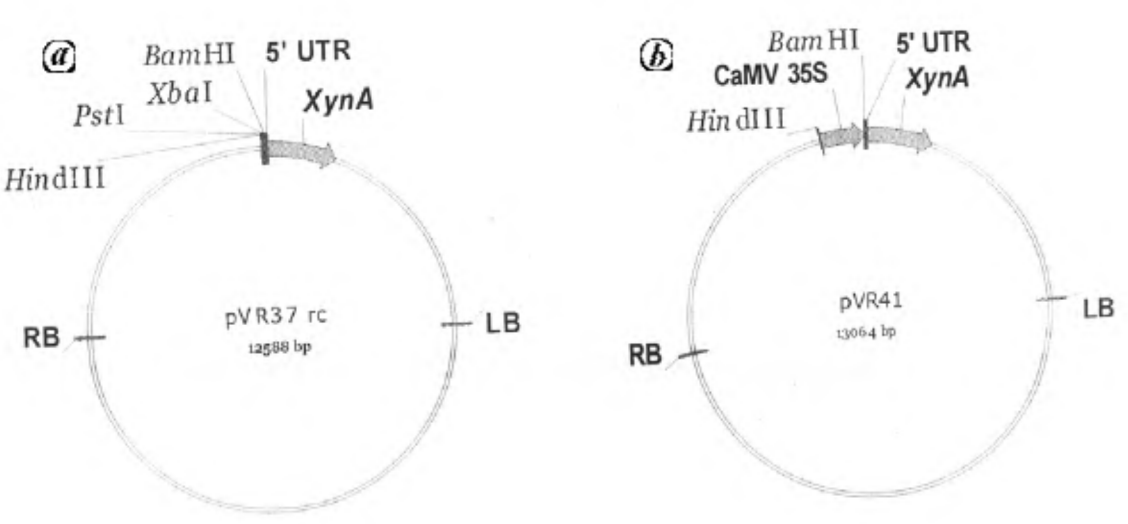

Figure 1. Vector map of pVR37 (***a***) and pVR41 (***b***).

Table 1. Absorbance units for AZCL-labelled fragments over time

Sample	Time (h)						
	1	6	12	18	24	30	36
pVR41	0.139	0.428	1.172	1.554	1.810	2.193	2.422
pVR37	0.014	0.014	0.017	0.017	0.021	0.020	0.024
pCAMBIA1305.1	0.011	0.016	0.015	0.017	0.017	0.020	0.024
Blank	0.013	0.018	0.015	0.016	0.016	0.020	0.021

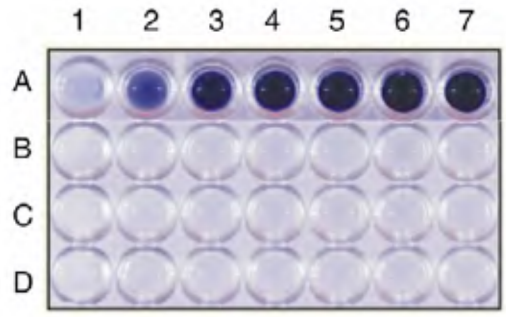

Figure 2. Colourimetric enzymatic activity of xylanase over time. A, pVR41; B, pVR37; C, pCAMBIA1305.1; D, Blank. 1–7, Samples measured at 1, 6, 12, 18, 24, 30 and 36 h after incubation respectively.

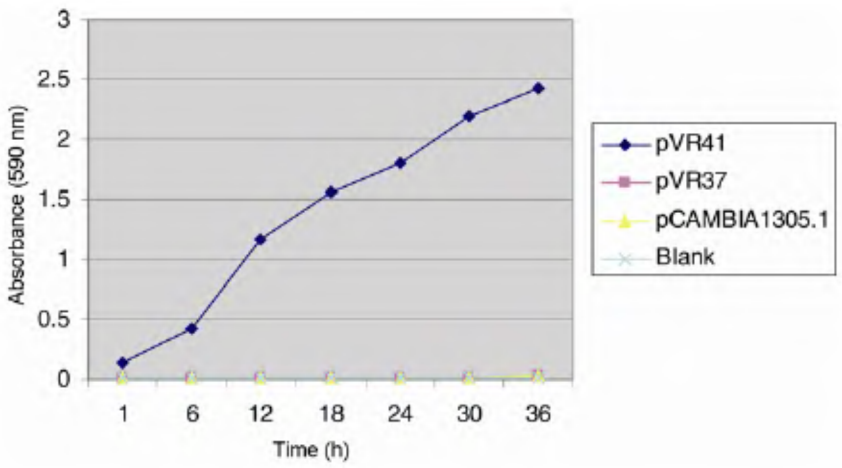

Figure 3. Overall enzymatic activity of xylanase over time.

with specific primers (RB19_35S_F/R) carrying *Hin*dIII and *Bam*HI sites, and cloned into the same sites of pVR37 to get pVR41 (Figure 1 *b*) carrying the complete expression cassette of CaMV 35S + 5′ UTR + *XynA*. pVR41 when subjected to PCR using CaMV 35S-specific primers or cut with *Hin*dIII + *Bam*HI, yielded 423 bp amplicon/fragment. PCR using RB29_M13_F/R primers produced an amplicon of 1201 bp, further confirming the presence of complete expression cassette (CaMV 35S + 5′ UTR + *XynA*).

pCAMBIA1305.1, pVR37 and pVR41 were transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating. Recombinant *A. tumefaciens* carrying pVR37 and pVR41 upon PCR with RB29_M13_F/R primers produced amplicons of 778 and 1201 bp respectively, whereas pCAMBIA1305.1 yielded a 794 bp expected size product upon *npt*II-specific PCR. *Agrobacterium* independently carrying these vectors was used for tobacco leaf disc co-cultivation following the standard protocol[17], with minor modifications. Co-cultivated leaf discs produced callus on the selection medium with hygromycin.

Protein was extracted from co-cultivated leaf disc-derived callus. Thirty-day-old calli (100 mg) were ground in 250 µl of extraction buffer (1 mM Na_2EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 10 mM β-mercaptoethanol, 50 mM $NaHPO_4$, pH 7.0). Extracts were centrifuged three times at 10,000 rpm, 4°C for 10 min to clarify the supernatant and then diluted to 1 mg/ml protein. Xylanase assay for callus extracts was performed in a solution containing 80 µl of the protein extract and 960 µl of xylanse assay buffer (pH 6.0, supplemented with 1% w/v AZCL-xylan and 4 mg/ml chloramphenicol). Aliquots (130 µl) of the above solution were incubated at 40°C with shaking at 200 rpm. Samples taken at regular intervals were placed on ice for 5 min and centrifuged at 4°C for 2 min. Absorbance by the supernatant was read at 590 nm at room temperature using ND-1000 Spectrophotometer (V, 3.5), NanoDrop Technologies, Inc., USA.

Expression of *XynA* was observed in 30-day-old calli derived from tobacco leaf discs co-cultivated with pVR41. But no activity was found in calli co-cultivated with either pVR37 or pCAMBIA1305.1. CaMV 35S promoter-driven expression of xylanase enzyme could catalyse the cleavage of AZCL-xylan, releasing soluble dye- (AZCL-) labelled fragments in the reaction solution (Table 1 and Figure 2). Overall enzyme activity as measured by accumulation of the dye linearly increased with time (Figure 3). However, xylanase enzyme was stable for 24 h at room temperature (~25°C). Reporter assay being simple and cost-effective, the *XynA* gene-based promoter-probe vector (pVR37) could be useful for high throughput screening of the promoter regions for spatio- and temporal expression analysis.

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Role of BCG vaccination in tuberculosis control

Tuberculosis (TB) remains a major public health problem globally and its control is a daunting challenge in low-income country settings such as India. Our country contributes 20% of the global burden of new TB cases[1]. TB in children remains a major public health concern, especially as severe forms of the disease often occur, and there are many challenges to accurately diagnosing TB disease in children. BCG vaccination is an important component of the Universal Immunization Programme (UIP) in India and is administered at birth. However, the role of BCG vaccination in the prevention of the acquisition of TB infection amongst children and hence in TB control remains controversial. The protection provided by BCG vaccination against TB disease has been observed to vary between 0 and 80% across the many studies conducted on BCG in different countries[2]. The Tuberculosis Prevention Trial in South India reported that BCG did not offer any protection against adult forms of bacillary pulmonary TB disease[3]. A low protective effect of BCG vaccination (27%; 95% CI: –8 to 50%) against TB was observed recently amongst children vaccinated under the UIP[4]. Studies carried out amongst BCG vaccinated and non-vaccinated children have also shown that BCG vaccination does not affect the estimates of prevalence of infection and annual risk of TB infection (ARTI), thereby confirming that the children irrespective of BCG vaccination can be included into the overall calculation for prevalence of infection and ARTI estimates[5,6]. In view of this knowledge, we present the findings of a study which assessed the prevalence of infection and ARTI amongst BCG-scarred children as well as those with no BCG scar in the Central Indian State of Madhya Pradesh.

A community-based cross-sectional tuberculin survey was conducted amongst children from 11 selected districts of the state to estimate the prevalence of TB infection and ARTI. The children were tested using 1 tuberculin unit (TU) of purified protein derivative (PPD), RT 23, on the mid-volar aspect of the left forearm intra-dermally and the maximum diameter of the reaction sizes was read after 72 h. The number of infected children was obtained using the mirror-image technique by locating the mode at the right-hand side of the frequency distribution of reaction sizes of children. ARTI is defined as the probability of acquiring new tuberculous infection or reinfection over a period of one year, and was estimated using the formula ARTI = $1-(1-p)^{1/a}$, where p is the proportion of children infected and a the mean age of the children test-read.

Of the 4967 test-read children, 3150 (63.4%) had no BCG scar. The prevalence of infection and ARTI amongst vaccinated children was estimated to be 7.7% (95% CI: 6.4–9.0%) and 1.4% (1.2–1.7%) respectively (Table 1). The corresponding figures for non-vaccinated children were 6.8% (95% CI: 5.9–7.7%) and 1.3% (1.1–1.4%) respectively. Thus the prevalence of infection and ARTI was found to be similar in both groups of children, i.e. BCG-vaccinated and non-vaccinated, thereby suggesting that BCG appears to have had little, if any, impact on preventing the acquisition of TB infection by these children. Other studies conducted at various places in South India have reported similar findings amongst BCG-vaccinated and non-vaccinated children[5,6]. Our findings from amongst this population of Central India add further support to this observation.

Available information from different studies, however, indicates that in countries where BCG vaccination has been adopted, there was a decline in the incidence of the haematogenous form of TB in children (e.g. miliary and meningeal) and deaths attributable to these forms of TB[7,8]. Conversely, there was an upsurge of such cases in countries where BCG vaccination had been discontinued[9,10]. Recent studies have also demonstrated that BCG vaccination has a non-specific beneficial effect on infant survival and found that a BCG scar is a marker of better survival among children in areas with high child mortality[11,12].

Important contributing factors for the variable efficacy observed for the present BCG vaccine are said to include background immunity induced by non-tuberculous environmental mycobacteria, diversity of BCG strains, and over-

Table 1. Prevalence of infection and ARTI among BCG-vaccinated and non-vaccinated children

BCG scar	No. test/ read	No. infected			ARTI (%)	*P*-value
		Number	Percentage	95% CI		
No	3150	215	6.8	(5.9 –7.7)	1.3 (1.1–1.4)	NS
Yes	1617	124	7.7	(6.4–9.0)	1.4 (1.2–1.7)	
All*	4967	355	7.1	(6.4–7.9)	1.3 (1.2–1.5)	

*Children with doubtful scar and no information on scar included.