Construction and functional validation of a new promoter-probe vector pRR21

The promoter, a DNA sequence normally located upstream of the transcribed region, plays an important role in controlling transgene activity. Choice of the promoter to confer constitutive, spatial and/or temporal transgene expression is one of the key determinants in plant biotechnology applications. In recent years, a wide range of different promoters from plant, viral and bacterial origin have been characterized and used extensively in regulating transgene expression in plants[1,2]. However, most of them are protected by patents. Therefore, novel plant sequences that can function as promoters for transgene expression are to be identified and tested. A wider range of effective promoters would also make it possible to introduce multiple transgenes into plant cells while still avoiding the risk of homology-dependent gene silencing.

Novel promoters are generally isolated based on promoter trapping or gene expression (cDNA) data under a variety of conditions. In the cDNA method, based on the expression pattern of a gene, its genomic region is identified, and the promoter is obtained by chromosome walk, TAIL–PCR or inverse PCR, where the genome sequence is not available. Otherwise the promoter sequence can be retrieved from the genome sequence data (if available). The cDNA method has been commonly used to isolate tissue and organ-specific regulatory elements[3–5]. Since the DNA sequence of several higher eukaryotes is now available, the computational method of promoter-finding has become popular. This provides the opportunity to identify and analyse the parts of a genome believed to be the promoter[6].

The most common approach to study the activity of novel plant promoters is to employ a promoter probe-vector, which carries a promoterless reporter gene placed downstream of one or more restriction sites. 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 under various conditions[7–9]. In this study, an attempt has been made to construct and functionally validate a binary promoter-probe vector with *SgfpS65T* as reporter gene for use in plants.

An improved version of the green fluorescence protein (GFP) encoding gene, *SgfpS65T* was PCR-amplified from pKU352NA (kindly donated by Narayana Upadhyaya, CSIRO PI, Canberra) using specific primers 5′ CATGGATCC-ATGGTGAGCAAGGGCGAG 3′ (RB20_GFP_F) and 5′ AAGGGTACCCTTGTAC-AGCTCGTCCATGC 3′ (RB20_GFP_R), designed to contain *Bam*HI and *Kpn*I sites. PCR product of ~750 bp was restricted with *Bam*HI + *Kpn*I and cloned into pUbi1casER⁻ vector (kindly donated by Narayana Upadhyaya) to get pRR19. *SgfpS65T* + *Nos*-ter cassette from pRR19 was released with *Bam*HI and *Eco*RI and cloned into binary vector, pCAMBIA1305.1 (obtained from CAMBIA, Canberra), cut with the same enzymes to get the promoter-probe vector, pRR21 (Figure 1 *a*).

pRR21 upon restriction with *Bam*HI + *Kpn*I and *Bam*HI + *Eco*RI released 717 bp (*SgfpS65T*) and 988 bp (*SgfpS65T* + *Nos*-ter) fragments respectively. Also *SgfpS65T*-specific PCR yielded an amplicon of 717 bp. Sequencing of pRR21 with M13_F and M13_R primers showed continuous open reading frame for *SgfpS65T*. pRR21 carried a multiple cloning site (MCS) with target sequences for *Bam*HI, *Xba*I, *Sal*I, *Pst*I and *Hin*dIII to clone any DNA fragments whose promoter activity needs to be checked. Because pRR21 is constructed in the pCAMBIA1305.1 binary vector, it carries all other features of the latter, including hygromycin selection. Complete sequence and feature information of pRR21 has been deposited at the GenBank, NCBI, with an accession number EU760495. This promoter-probe vector will be made available upon request.

pRR21 was functionally validated by cloning the CaMV 35S promoter in its MCS. CaMV 35S in pWBVec8 (kindly

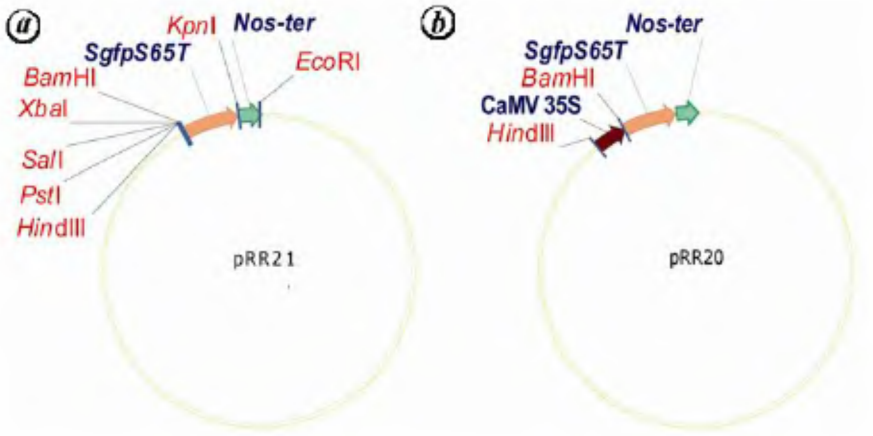

Figure 1. Vector map of (*a*) pRR21 and (*b*) pRR20.

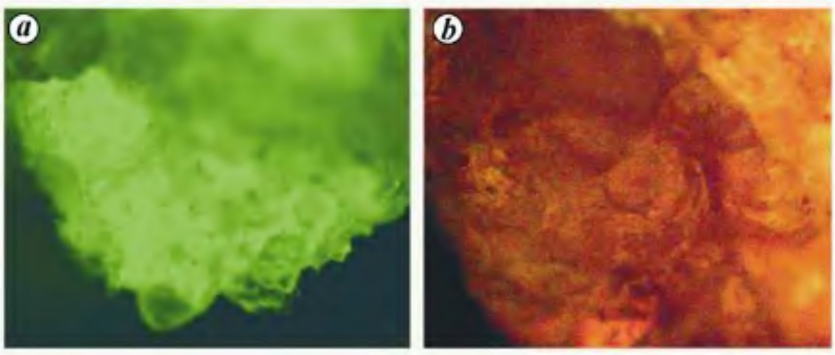

Figure 2. SgfpS65T expression in tobacco leaf discs co-cultivated with (*a*) pRR20 and (*b*) pRR21.

donated by Ming-Bo Wang, CSIRO PI, Canberra) was isolated using two specific primers (RB19_35S_F: 5′ ACGAAGCTT-CGTCAACATGGTGGAGCA 3′ and RB19_35S_R: 5′ GACGGATCCGTC-CTCTCCAAATGAAAT 3′) designed to contain sites for *Hin*dIII and *Bam*HI. PCR product of 429 bp and pRR21 were cut with *Hin*dIII + *Bam*HI and ligated, and the product was transferred to *Escherichia coli* DH5α to get pRR20 (Figure 1 *b*).

Recombinant promoter-probe vector (pRR20) containing CaMV 35S when restricted with *Hin*dIII + *Bam*HI gave a fragment of 429 bp, indicating the presence of complete expression cassette (CaMV 35S promoter + *SgfpS65T* + *Nos*-ter). pRR21 and pRR20 were transferred to *Agrobacterium tumefaciens* for tobacco leaf disc transformation by following the standard protocol[10]. Co-cultivated leaf discs, calli and plants regenerated on hygromycin selection medium were observed for SgfpS65T expression under stereo-microscope (Olympus, SZX-16) at the National Centre for Biological Sciences. Images of SgfpS65T-expressing tissues were captured. Of the 30 leaf discs co-cultivated with pRR20, 19 showed SgfpS65T expression (Figure 2 *a*). However, leaf discs co-cultivated with pRR21 failed to show green fluorescence (Figure 2 *b*). In addition, calli formed from all SgfpS65T-positive leaf discs also showed fluorescence. Putative transgenic plants (for pRR20) which survived hygromycin selection were confirmed by *SgfpS65T*-specific PCR. Eleven plants produced an amplicon of expected size upon *SgfpS65T*-specific PCR (data not shown) and all 11 transgenic plants showed SgfpS65T expression.

Though GUS reporter was used for validating the pathogen-inducible synthetic promoters[11], the GFP has advantages such as (i) it is a non-destructive method, (ii) permits both qualitative and quantitative assays[12,13] and (iii) does not need any substrate or cofactors. In the present study, an improved version (*SgfpS65T*) resulting from chromophore mutation, S65T in the wild *gfp* was used, which is known to result in 35-fold increased fluorescence[14] compared to wild-type when excited at 488 nm. The *SgfpS65T* gene has been successfully used as a visual marker for *iAc* in pKU352NA for use in rice functional genomics[15]. Because pRR21 is a binary vector with pCAMBIA1305.1 background, it is expected to work in many plant systems for probing novel promoters.

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Calcium deprivation markedly enhances guggulsterone accumulation in cell cultures of *Commiphora wightii*

Guggulsterone (Gs; two isomers -E and -Z, GsE and GsZ) is an effective anti-hyperlipidemic agent obtained from the gum resin of guggul tree, *Commiphora wightii* (Arnott.) Bhandari (syn. *C. mukul*)[1]. The recent reports explain the mechanism of its lipid lowering properties[2] and its potential role in cancer therapy, making it an attractive molecule for production through biotechnological methods using this species.

Production of metabolites in cell cultures and development of scale-up technology require standardization of various growth parameters for optimal yield[3]. To overcome the low secondary metabolites production in cell cultures, some strategies have been proposed to increase the productivity. Manipulation of culture medium constituents and culture conditions is the first step to increase the production of secondary metabolites[4,5]. There is sufficient evidence that Ca^{2+} serves as a secondary messenger in many growth and metabolic activities such as cell division and differentiation[6], cell polarity, cell elongation, photomorphogenesis, and biotic and abiotic stress responses[7]. Ca^{2+} influx is necessary for alkaloid accumulation in suspension cultures of *Catharanthus roseus* in response to the addition of cytokinin and cytokinin-enhanced alkaloid accumulation relies on the calcium–calmodulin-dependent process[8]. Ca^{2+} increases the accumulation of different sets of secondary metabolites, such as benzophenanthridine alkaloids in cell suspension of *Sanguinaria canadensis*[9], while Ca^{2+} deprivation enhances the alkaloid release in the medium in *C. roseus* cell cultures[10]. Earlier, we reported Gs accumulation in cell cultures grown in the growth medium[11] and production medium[5]. In our efforts to improve the production system, this report describes the marked influence of Ca^{2+} deprivation on the production of Gs in cell cultures of *C. wightii*.