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Tissue-specific expression in transgenic rice and *Arabidopsis thaliana* plants of *GUS* gene driven by the 5′ regulatory sequences of an anther specific rice gene *YY2*

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ABSTRACT

The identification and characterization of promoters, *cis*-elements and transcription factors are critical for studying gene expression during growth and development in any organism. Use of specific promoters is an absolute requirement for the expression of foreign genes in plants in a developmentally, spatially and/or temporally regulated manner. The YY2 cDNA has been shown to be expressed specifically in the tapetum cells of rice anther. In this study, a 968 bp upstream regulatory sequence (GenBank accession no. FJ957881) of the gene was isolated using genome walking method. The isolated fragment was shown to drive anther-specific expression of GUS reporter gene in rice as well as in the dicot plant *Arabidopsis thaliana*. Deletion analysis revealed that a ~400 bp 5′ regulatory region is sufficient to confer anther-specific expression of GUS gene.

1. Introduction

Anther development in plants is the result of a well-orchestrated network of gene-expression [1]. Tapetum is the layer of tissue that is highly active; provides cell wall components and nourishment for pollen and the enzymes required for release of microspores from the tetrads [2,3]. Many anther-specific genes and their promoters have been studied extensively and have led to the identification of *cis*-elements necessary for tissue-specific gene expression. Many tapetum and pollen-specific promoters such as *TA29* has been well characterized in tobacco [1], *A9* in *Arabidopsis thaliana* [4], and *Osg6B* in rice [5], *LAT52* [6], *LAT59* [7] in tomato, *Bp1* [8], and *Bra r 1* [9] in Brassica. Some of these are expressed exclusively in the tapetum or pollen while others are expressed in both like the *Bgp1* from *Brassica napus* [10].

Among the possible applications for an anther specific promoter is its usefulness in engineering male sterility in plants. This could be achieved by cloning a toxic gene under anther specific promoter that could disrupt the anther development like *Bacillus* RNase (Barnase) [11]. They also demonstrated that by using another gene barstar, which is an inhibitor of Barnase, restoration of fertility can be achieved in F1 generation [12]. This tool could be used in hybrid seed industry for production of restorable male sterile parent lines.

This study describes the isolation of a rice anther specific promoter by using a genome walking method based on Siebert et al. [13]. The required adapters and primers were synthesized *de novo* and were used in the method which we will refer to as random amplification of genomic end (RAGE). Many recent studies have also demonstrated the use of this method in other plants like tomato [14] Brassica [15] and Pepper [16]. We used sequence information of the rice gene YY2 [17] for designing primers to walk upstream of the coding sequence to clone the promoter. We describe studies on the expression of this promoter using a GUS reporter in the anthers of transgenic rice. In an attempt to test the promoter function in dicot plants, we also transformed *A. thaliana* plants. Further characterization of the promoter was carried out by 5′ deletion analysis of the promoter in *A. thaliana*.

Today complete genomes of many plant species including rice are available and therefore isolation of promoters is easy and straightforward. This work was initiated before the rice genome sequence was published as an attempt to isolate and clone an anther specific promoter based on the available sequence data of the gene in the database during that period. However, there are many plants whose genetic resources are unexploited and could be utilized by employing similar technique as described here.

Abbreviations: GUS, β-glucuronidase; GSP, gene specific primer; ASP, adpater specific primer; RAGE, random amplification of genomic ends.

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2. Materials and methods

2.1. Plant material

Rice Pusa Basmati 1 was obtained from Indian Agricultural Research Institute, Delhi. The plants were grown in soil collected from rice fields and maintained in pots in the green house.

Arabidopsis seeds were from Lehle seeds, USA and obtained as a gift from Rockefeller Foundation, USA. The seeds were sown either directly or were germinated on damp filter paper and transferred to a mixture of Sand:exfoliated vermiculite (Tamilnadu Minerals, Chennai) in 1:1 ratio or in Soilrite Mix (Keltech Energies, Bangalore) which is a mixture of Irish Peat moss, Exfoliated vermiculite and Horticulture grade expanded Perlite in equal proportions. The plants were watered with nutrient solution once a week as per Clough and Bent [18] till the seeds matured on the plants. Plants were maintained at 22–27 °C in a growth chamber under 16 h/8 h light/dark periods. Primary bolts of healthy plants were clipped to encourage growth of multiple secondary bolts. The plants which were healthy with flowers were chosen for transformation.

Rice calli were grown and maintained on callus induction medium (MSR1) composed of MS basal medium, 2,4-D 3 mg/L, proline 500 mg/L, casein hydrolysate 500 mg/L and Phytagel 2.25 g/L, pH 5.8 (MSR1) [19]. AA–AS medium [20] was used during transformation and composed of MS Salts, B5 Vitamins, Amino acids, 50 μM acetosyringone. Rice regeneration media (MSR2)was composed of MS Salts, B5 Vitamins, BAP 3 mg/L, NAA 0.5 mg/mL, 4% sorbitol, and phytagel 2.25 g/L and rooting was carried out on 40% MS Salts, B5 Vitamins, 3 g/L phytagel (MSR3).

Arabidopsis seeds were germinated on MSA1 medium which contained MS salts and 0.6% Agar.

2.2. Primers used for PCR amplifications

GSP1—5′-AGGTGCGGCCGCCTTGTTGCAGGATCGTCGCAGATG-3′; GSP2—5′-ACGTGCGGCCGCTGCCATTGCCACTCCTGGCATCGC-3′; ADAPrimer1—5′-GGATCCTAATACGACTCACTATAGGGC-3′; ADA-Primer2—5′-AATAGGGCTCGAGCGGC-3′ (all custom synthesized by Metabion, Germany); RASP-F—5′-GGGATCCCGCGGCCGCCCGGG-CAGGTATT-3′; RASP-R2—5′-CATGCCATGGCTTCGCCACCCGGTATCC-TTT-3′; RASP-D1—5′-GCGTCGACGCTCGTCAACAAGCCAGCTCG-3′; RASP-D2—5′-GCGTCGACCAGAGATCACACCGAATGGATG-3′; RASP-D3—5′-GCGTCGACGTCTTCCCTCATGCCGTCGTC-3′; RASP-R2—5′-C-ATGCCATGGCTTCGCCACCCGGTATCCTTT-3′; Hph-F—5′-GACGATT-GCGTCGCATCGACC-3′; Hph-R—5′-AGCGTCTCCGACCTGCTGCA-3′; GUS I-F—5′-GCGGATCCATGGTAGATCTGAGGGTAA-3′; GUS I-R—5′-CGAGCTCGAGCTGGTCACCTGTAATTCAC-3′ (all custom synthesized by (Sigma Genosys, Bangalore, India).

2.3. PCR amplification of a rice anther specific promoter

The rice anther specific promoter (RASP) was isolated using RAGE. This method is an extension of the genome walking method described by Siebert et al. [13]. Rice genomic DNA was isolated and digested with different blunt cutting enzymes. The digested DNA was purified by extraction with a mixture of phenol: chloroform (1:1 ratio) and precipitated with 95% ethanol. After washing with 70% ethanol, the DNA was air dried and dissolved in sterile deionised water. Adapter strands were synthesized commercially (Metabion GmbH, Germany) and were as follows. Long strand—5′ CTAATACGACTCACATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ and short strand—5′ ACCTGCCC $-NH_2$-3′. The strands were dissolved individually and annealed to obtain the adapter solution and the adapter was ligated to the digested genomic DNA. The ligation reaction contained digested DNA (5 μg), Adapter 5 μM, 1× ligase buffer (MBI Fermentas) and ligase 10 units (MBI Fermentas). The reaction was carried out at 22 °C overnight. Gene specific primers GSP1 and GSP2 were designed based on the cDNA sequence of YY2 [17].

The ligation mix was diluted 1:10 with sterile deionised water. The RAGE-PCR was carried out using gene specific primer (GSP1) and Adaptor Specific primer (ADAPrimer1) as the primers. 1 μl of the diluted mix was used as template in a 50 μl reaction containing the following components: 0.4 μM of the primers of GSP1 and ADAPrimer1, 200 μM dNTPs (Amersham Bioscience, Piscataway, NJ, USA), 1× XT-20 buffer (Bangalore Genei, India) and 2 units of XT-20 PCR polymerase (Bangalore Genei, India). The reactions were carried out under the following conditions. Initial denaturation 94 °C for 4 min, 35 cycles of 94 °C for 1 min, followed by a single step annealing and extension of 68 °C for 3 min, final extension of 68 °C for seven min. The PCR products were analyzed on 1% agarose gel with 0.5× TBE buffer.

The PCR product was diluted 1:50 and 1 μl was used as template for a 25 μl reaction using nested primers GSP2 and ADAPrimer2. The reaction contained 0.2 μM of the primers, 200 μM dNTPs (Amersham Bioscience, Piscataway, NJ, USA), 1× *Taq* DNA polymerase buffer (Bangalore Genei, India) and 1 unit of *Taq* DNA polymerase (Bangalore Genei, India). The reactions were carried out under the following conditions: Initial denaturation 94 °C for 4 min, 25 cycles of denaturation 94 °C for 1 min, annealing 65 °C for 30 s and extension 68 °C for 1 min 30 s, final extension of 68 °C for 5 min. The PCR products were analyzed on 1% agarose gel with 0.5× TBE buffer.

2.4. Amplification of the YY2 promoter from the RAGE clone and cloning of the promoter

Primers RASP-F and RASP-R2 were designed to amplify the region upstream of the translation start site of the YY2 gene. PCR conditions for 20 μl reaction were as follows: Template DNA 10 ng, dNTP mix 200 μM, Primer RASP-F, 0.2 μM, Primer RASP-R2 0.2 μM, 10× *Taq* polymerase assay buffer 2 μl, 1 unit of *Taq* polymerase enzyme (XT-20 PCR system, Bangalore Genei, India), sterile deionised H_2O to 20 μl. Restriction sites *Nco*I and *Bam*HI were engineered into the primers so that they could be cloned into pCAMBIA vectors by replacing the CaMV35S promoter of the GUS reporter gene. The cycling conditions for PCR were: Initial denaturation at 95 °C for 5 min; followed by 30 cycles of denaturation at 95 °C for 30 s; annealing and extension at 68 °C for 2 min; and a final extension step at 68 °C for 7 min. The blunt PCR product was cloned into pBlueScript SK(−) (Stratagene) and sequenced (National DNA Sequencing Facility, University of Delhi, India). Sequence analysis for presence of *cis*-elements were carried out using the online PLACE tool [21].

2.5. PCR amplification of deletion fragments and cloning

The deletion fragments were generated by PCR using three different forward primers and a common reverse primer (RASP-R2). RASP-D1 was used for the longest of deletions (483 bp), RASP-D2 for the second deletion (323 bp) and RASP-D3 for the third 184 bp deletion in combination with common reverse primer used was RASP-R2. The amplified products were cloned into pBlueScript SK(−), sequenced and the sequence was analysed as described for the 968 bp fragment except that the forward primers had *Sal*I restriction sites instead of *BamH*I.

2.6. Construction of binary vector used for transformation

All the vectors used for transformation experiments were constructed by cloning the promoters into pCAMBIA 1305.1