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Analyses of the Genomic Sequence and Promoter Activity of a Gene for a Protein Similar to Tat Binding Protein Isolated from *Brassica rapa*.

Hiroyasu KITASHIBA, Rieko OTA, and Kinya TORIYAMA *

Laboratory of Plant Breeding and Genetics, Faculty of Agriculture, Tohoku University, Sendai 981-8555, Japan
Corresponding author E-mail address: torikin@bios.tohoku.ac.jp

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Abstract

We have previously isolated a cDNA clone, which is similar in sequence to the Tat binding protein (TBP) of human immunodeficiency virus 1, from the anthers of *Brassica rapa* and designated it *BrTBP*. In the present study we isolated a genomic clone of *BrTBP* from *B. rapa* and found several sequence motifs conserved in various pollen-expressed genes. The 1.7-kb promoter region of *BrTBP* was fused to the GUS gene and introduced into tobacco and *Arabidopsis thaliana*. GUS expression was observed exclusively in mature pollen after anthesis in transgenic tobacco, while it was observed in tricellular pollen starting two days before anthesis in *A. thaliana*. Such expression patterns at the late stage of pollen development have not been reported in other pollen-specific promoters. These results indicate that the *BrTBP* promoter region used in this study has a unique activation pattern in tobacco and *A. thaliana*.

1. Introduction

Anther-specific genes and their promoters have been extensively studied in terms of analysis of the cis-elements necessary for tissue-specific gene expression and genetic engineering of male-sterile plants. For example, some tapetum-specific promoters have been well characterized in tobacco (*TA29*; Koltunow *et al.*, 1990) and *A. thaliana* (*A9*; Paul *et al.*, 1992). These promoters have been reported to be active in the anther tapetum from the meiotic stage until the microspore stage. The promoters of genes such as *LAT52, LAT59*, and *Bp19* have been reportedly activated exclusively in pollen (Twell *et al.*, 1990; Twell *et al.*, 1991; Kim *et al.*, 1997). The transcripts have been detectable first in the young pollen grains either before or after microspore mitosis and have been found to accumulate thereafter, reaching their maximum concentration in the pollen grains just before anthesis. Promoters such as *Bgp1* have been reported to be active in both the tapetum and microspores (Xu *et al.*, 1993). It is known that promoter activities in transgenic plants sometimes differ among host plants although the same promoter sequence has been introduced.

BrTBP cDNA was isolated from the anthers at the microspore stage of *B. rapa* (Kitashiba and Toriyama, 1997). It is similar in sequence to Tat binding protein 1 (TBP1; Ohana *et al.*, 1993), although the function of BrTBP is not known. When RNA *in situ* hybridization in flower buds was carried out, the transcripts were detected specifically in the tapetum and the middle layer of the anthers as well as in the pollen grains. The transcripts were also detected in the floral apex including immature flower buds and in the anthers at the microspore and bicellular stages by Northern blot analysis (Kitashiba *et al.*, 1996; Kitashiba and Toriyama, 1997). It is therefore expected that the promoter region of the *BrTBP* gene contains some cis-elements which are necessary for expression in pollen and have a promoter activity in pollen.

In this paper, we isolated a genomic clone of *BrTBP* from *B. rapa* to investigate the features of the promoter region. Then the promoter activity was investigated in transgenic tobacco and *Arabidopsis thaliana*.

2. Materials and Methods

2.1 Construction and screening of a genomic library

Total DNA was isolated from young leaves of a *Brassica rapa* S^9 homozygous plant and its genomic library was constructed using EMBL-3 SP6/T7 (Clontech), as described in Suzuki *et al.* (1995). The library was screened by DIG-labeled *BrTBP* cDNA

(Kitashiba and Toriyama, 1997). A part of the phage DNA corresponding to *BrTBP* cDNA was subcloned into the pBluescript SK(-) vector. Sequences were determined by the dye-terminator method using PRISM™ 377 (Perkin Elmer).

2.2 Construction of promoter-GUS fusion

A 1.7 kb DNA fragment from position -1836 to -68 bp (the first ATG was marked as +1) was amplified by PCR using two primers: the forward side sequences are 5'-*CAAGCTT*CCGAACCCAATCAATATCCG-3' containing the *Hin* dIII site, and the backward side sequences are 5'-AAG*CCTAGG*GGAACTGAAGAGGC-3' containing the *Bam* HI site. PCR was carried out in a 50-μl reaction volume containing 50 ng of the plasmids including the subcloned *BrTBP* genomic DNA, 50 pmol of each primer, 20 pmol of each dNTP and 2.5 units of pfu DNA polymerase in 1 x PCR buffer (STRATAGENE) using a DNA thermal cycler (Perkin Elmer). Amplification was carried out at 94 ℃ for 30 sec, 57 ℃ for 1 min and 72 ℃ for 2 min (35 times). Amplified DNA was digested with *Hin* dIII and *Bam* HI, and this DNA fragment was subcloned into pBI221 in which CaMV35S promoter was removed by digestion with *Hin* dIII and *Bam* HI enzymes. This construct in which the *BrTBP* promoter was located upstream of the GUS gene was named pBTPGUS1. Since the *Eco* RI site was present at -1701 of *BrTBP* genomic DNA, the pBTPGUS1 plasmids were digested with *Eco* RI and the resulting *Eco* RI fragment containing *BrTBP* promoter, GUS gene and Nos terminator was ligated at the same site in the SLJ 7292 binary vector (Jones *et al.*, 1992). This construct was designated pBTPGUS2 (**Fig. 1**).

2.3. Transformation of tobacco and A. thaliana

pBTPGUS2 was transferred to *Agrobacterium tumefaciens* strain A136 carrying the helper plasmid pCIB542 (Toriyama *et al.*, 1991). Leaf disks of *Nicotiana tabacum* L. cv. Petit Havana SR1 were transformed as described by Horsch *et al.* (1988). The same construct was transferred to *A. tumefaciens* strain C58 that carried the helper plasmid pGV2260 (Deblaere *et al.*, 1985) for the transformation of *A. thaliana* ecotype Columbia by the *Agrobacterium* vacuum infiltration method (Bechtold *et al.*, 1993). Transgenic plants were selected based on kanamycin resistance.

2.4 DNA gel blot analysis

Genomic DNA was isolated from young leaves of non-transformed and transgenic plants as described by Murray and Thompson (1980). Genomic DNAs (1.0 μg) were digested with *Eco* RI. Then they were electrophoresed in 1.0% (w/v) agarose gel and blotted onto a nylon membrane (Schleicher & Schuell). The probe, an *Eco* RI-*Pst* I fragment of the promoter region (-300 to -1701; **Fig. 1**), was labeled with digoxigenin (Boehringer Mannheim Biochemica). Hybridization, washing and detection were performed according to the instructions of the manufacturer of the DIG DNA Labeling and Detection Kit (Boehringer Mannheim Biochemica).

2.5 GUS assay

Leaves, stems, infloral meristems, sepals, petals, anthers and pistils of tobacco were sliced using a microslicer (DOSAKA EM) and incubated at 37 ℃ with 1mM 5-bromo-4-chloro-3-indryl-β-D-glucuronide in GUS assay buffer (De Block *et al.*, 1992) supplemented with 2mM DTT. Histochemical GUS assay of *A. thaliana* was carried out as described by Toriyama *et al.* (1991) using the GUS assay buffer of De Block *et al.* (1992).

3. Results

3.1 Identification of the genomic sequence of BrTBP

We have previously reported that the *BrTBP* exists as a single copy gene in the genome of *B. rapa* (Kitashiba and Toriyama, 1997). We screened a genomic library of *B. rapa* and subcloned the genomic fragment corresponding to the *BrTBP* sequence. Finally, sequences of 4780 bp were determined to contain the sequences corresponding to *BrTBP* cDNA. Compared with *BrTBP* cDNA, the gemomic DNA was found to consist of ten exons and nine introns (**Fig. 1A**). The fragment contained the sequence of 1854 bp of the 5' upstream region from the ATG initiation codon (**Fig. 1B**).

The 1854 bp of the 5'-flanking region of *BrTBP* genomic DNA was revealed to contain sequences similar to cis-elements identified in pollen-specific genes of tomato (**Fig. 1B**). A sequence motif, TGTGGTT, which is identical to the pollen box (PB) core motif (Twell *et al.*, 1991) was present at -811 and -1201. Three repeats of sequence motif TTTGGTT, in which six of seven nucleotides matched those of the PB core motifs, were present at -1663, -666, and -439 (**Fig. 1B**). Sequence motifs TGAGGTT, TATGGTT, TGTGATT, and TCTGGTT, in each of which six of seven nucleotides matched those of the PB core motifs, were present at -1566, -566, -513 and -197, respectively (**Fig. 1B**). A sequence motif GGAATGGTGA, in which eight of ten nucleotides matched those of the *LAT56/59* box (GAAATTGTGA: Twell *et al.*, 1991), and sequence motifs GAAATGGTTG,

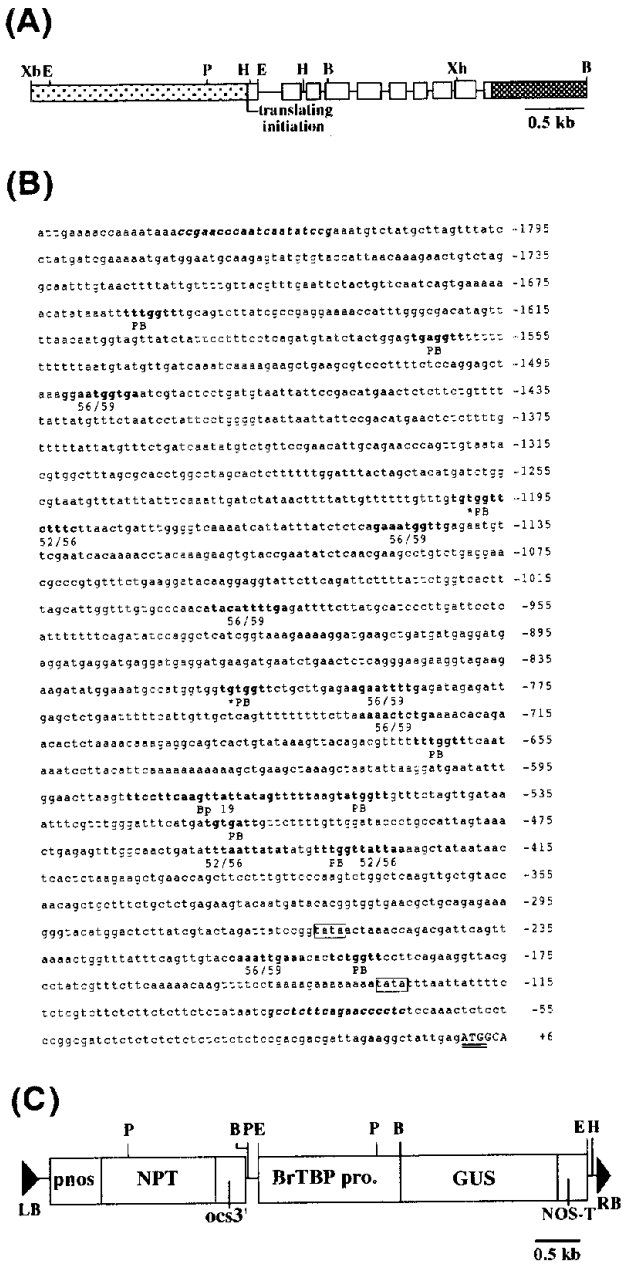

Fig. 1 Structure of genomic DNA, nucleotide sequence of the 5'-flanking region of *BrTBP*, and transformation vector pBTPGUS2.

(A)Genomic DNA structure. White boxes and horizontal lines represent the exons and the introns, respectively. The light-shaded box indicates the genomic regions containing a putative promoter sequence and the dark-shaded box indicates the 3' region of genomic DNA. B, *Bam* HI; E, *Eco* RI; H, *Hin* dIII; Xb, *Xba* I; Xh, *Xho* I; P, *Pst* I. The nucleotide sequence of the genomic DNA appears in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number AB044593.

(B)Nucleotide sequence of the 5'-flanking region. The translation start codon ATG is marked as + 1 and indicated by a double underline. The putative TATA-boxes are in white boxes and cis-acting elements involved in pollen-specific gene expression (PB; 52/56; 56/59; Bp19) are indicated by bold letters. The motifs marked by asterisks show 100% identity with the known cis-acting elements. The sequences in itatics represent the primer sequences used in the amplification of the promoter region. Numbers indicate the positions of nucleotides.

(C)Transformation vector, pBTPGUS2 containing the *BrTBP* promoter and GUS fusion. NPT, gene for neomycin phosphotransferase; pnos, promoter sequence of the nopaline synthase gene; ocs 3', 3' signal of the octopine synthase gene; NOS-T, terminator sequence of the nopaline synthase gene; LB, Left border of T-DNA; RB, Right border of T-DNA; E, *Eco* RI; H, *Hin* dIII; B, *Bam* HI; P, *Pst* I.

TACATTTTGA, AGAATTTTGA, AGAATTTTGA, AAAACTCGA and CAAAATTGAAA, in each of which seven of ten nucleotides matched those of the *LAT56/59* box (Twell *et al.*, 1991) were present at -1491, -1151, -992, -794, -733 and -210, respectively (**Fig. 1B**). Sequence motifs TGTGGTTCTTTC, TTTAATTATATA, TTTGGTTATTAA in which nine of twelve nucleotides matched those of the *LAT52/56* box (TGTGGTTATATA: Twell *et al.*, 1991) were present at -1201, -453 and -439, respectively (**Fig. 1B**). In addition, a sequence motif TTCCTTCAAGTTATTATAGTTTTT, in which nineteen of twenty-four nucleotides matched those of the *Bp 19* region I (ATCCTTAAGTTACTTCTTATTTTT: Albani *et al.*, 1991), was present at -583 (**Fig. 1B**). We also found two putative TATA-boxes at -259 and -91 (**Fig. 1B**).

3.2 BrTBP promoter activity in transgenic tobacco

The genomic fragment from -69 to -1700 was amplified by PCR and then fused to the GUS gene. The resulting construct, pBTPGUS2 (**Fig. 1C**), was introduced into tobacco. We obtained four transgenic plants (#6, #12, #29 and #37) which were identified by Southern blot analysis (data not shown).

GUS activity was detected by histochemical GUS assay. The blue staining, GUS activity, was detected in the pollen grains after anthesis in plants #12 and #29. The representative GUS activity in the anther and pollen of plant #12 is shown in **Fig. 2** GUS activity was not detected in pollen mother cells, uninucleate microspores, bicellular pollen and mature pollen before anthesis (**Fig. 2A, C, E** and **G**). GUS activity was evident a day to three days after anthesis (**Fig. 2I** and **K**). GUS activity was also not

observed in the other floral organs (sepals, petals and pistils), leaves, stems or infloral meristems (data not shown). GUS activity was not observed in any tissues of the other two transgenic plants (#6 and #37) or wild-type plants (**Fig. 2B, D, F, H, J** and **L**).

Segregation of GUS positive and negative pollen grains was observed, as shown in **Fig. 2** The ratio of GUS positive and negative pollen grains was 197:188 in transgenic plant #12 and 200:188 in transgenic plant #29, indicating the segregation pattern of 1:1 (significant at the 5% level). The transgene was considered to be integrated in one locus in both transgenic plants.

3.3 BrTBP activity in transgenic Arabidopsis

The same construct , pBTPGUS2 (**Fig. 1C**) was also introduced into *A. thaliana*. We obtained 11 transgenic plants which were identified by Southern blot analysis (data not shown). GUS activity was observed only in pollen grains. No GUS activity was detected in sepals, petals, pistils, leaves, stems, infloral meristems or roots (data not shown). **Fig. 3** (**A, C, E, G** and **I**) shows the representative GUS activity in bicellular pollen and tricellular pollen of transgenic plant #1-6. Any GUS activity was not observed in bicellular pollen until three days before anthesis (**Fig. 3A** and **C**). A weak signal was first detected in tricellular pollen grains two days before anthesis (**Fig. 3E**). Blue staining was evident a day before anthesis (**Fig. 3G**) and a strong signal was obtained in pollen grains on the day of anthesis (**Fig. 3I**). Nine other plants showed the same expression pattern and one plant did not show any GUS activity (data not shown). Wild-type plants did not show any GUS activity (**Fig. 3B, D, F, H** and **J**).

Segregation of GUS positive and negative pollen grains was not evident in these transgenic plants. Most of the pollen grains were stained blue. This would be due to the multiple integration of the introduced gene which was identified by Southern blot analysis (data not shown).

4. Discussion

BrTBP is similar in sequence to HIV Tat binding protein 1 (TBP1). TBP1 has been reported to function, via interaction with Tat protein, as a transcriptional factor of HIV-1 (Ohana *et al.*, 1993). Recently, some homologous genes have been reported in plants. *TBPOs-1* and *TBPOs-2* were isolated from rice (Suzuka *et al.*, 1994; 1998) and *LeMA-1* from tomato (Prombona *et al.*, 1995). The function of TBP1-like genes in those plants, however, has not yet been elucidated. There has been no report of genomic sequences of these genes. In the present study, we isolated the genomic clone of *BrTBP* and found the cis-elements characteristic of pollen-specific genes within 1.7 kb of the promoter region. We therefore postulated that the promoter region used in this study might be active in pollen.

We introduced promoter-GUS fusion into *A. thaliana* and tobacco, because efficiency of transformation is low in *B. rapa*. The promoter activity in transgenic tobacco and *A. thaliana* was restricted to mature pollen grains. The spatial and temporal activation pattern of the *BrTBP* promoter was different from that expected from the endogenous *BrTBP* expression in *B. rapa*, in which the transcripts of *BrTBP* were detected in pollen and in the anther wall from the microspore stage to the mature stage (Kitashiba and Toriyama, 1997). This discrepancy might be attributed to the difference of host plants into which the transgene had been introduced. A similar discrepancy in the promoter activity in different host plants has been reported in *Bgp 1* promoter activity in *A. thaliana* and tobacco (Xu *et al.*, 1993) and in the promoter of the *S*-locus glycoprotein gene of *B. oleracea* and *B. rapa* (Sato *et al.*, 1991; Toriyama *et al.*, 1991; Thorsness *et al.*, 1991). It is also possible that the 1.7 kb promoter region used in this study did not contain the cis-elements necessary for expression in immature microspores, the tapetum and the anther wall, as reported for the analysis of other pollen expressed genes such as *Bgp1* of *B. rapa* (Xu *et al.*, 1993). It is therefore necessary to characterize the promoter activity of defined promoter sequences in each host plant.

The promoter activities of pollen-specific genes have been reported in several genes, such as *Bp19, Npg1, NeIF-4A8* and *LAT52* (Kim *et al.*, 1997; Tebbutt *et al.*, 1994; Brander and Kuhlemeier, 1995; Twell *et al.*, 1990; Twell *et al.*, 1991). The promoter activity in tobacco has been reported to be detectable in pollen at microspore mitosis. In this study, the promoter activity of *BrTBP* in transgenic tobacco and *A. thaliana* was absent at the microspore stage until the last stage of anther development. It is quite unique that GUS activity was exclusively detected in the pollen grains after anthesis in transgenic tobacco and in the tricellular pollen in transgenic *A. thaliana*. Such activation patterns at the late stage of pollen development has not been reported previously.

We suggest that a promoter which is active only in mature pollen grains will be useful for producing male-sterile plants in which the pollen is normal during microspore development but aborts at the

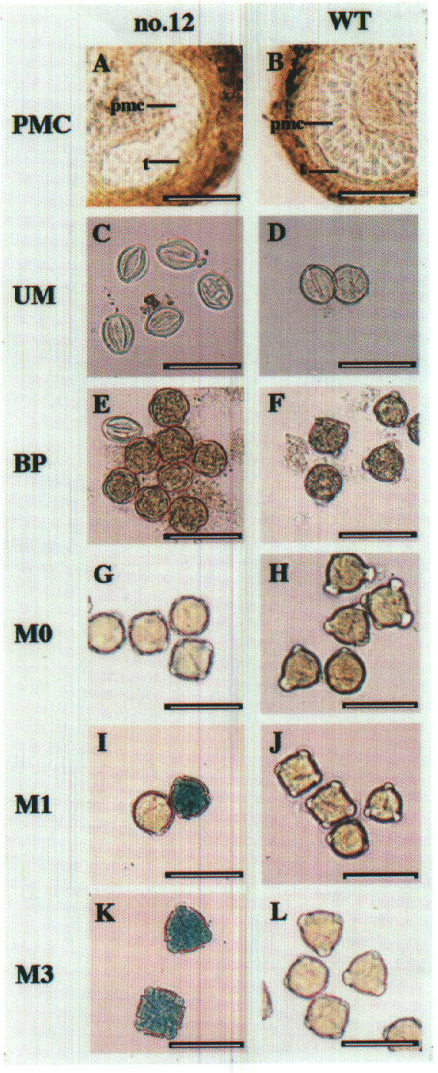

Fig. 2 Histochemical detection of GUS activity in anthers of transgenic tobacco (# 12), and nontransformant (NT). Blue staining indicates GUS activity. CUS activity was detecated only in mature pollen after anthesis. PMC, anther containing the pollen mother cells; UM, uninucleare microspore atage; BP, bicellular pollen stage; M0, mature pollen stage before antersis; M1, mature pollen a day after anthesis; M3, mature pollen three days after anthesis; pmc, pollen mother cells; t, tapetum cells. Ber=100 μm (in A and B) or 50 μm (in the other photographs).

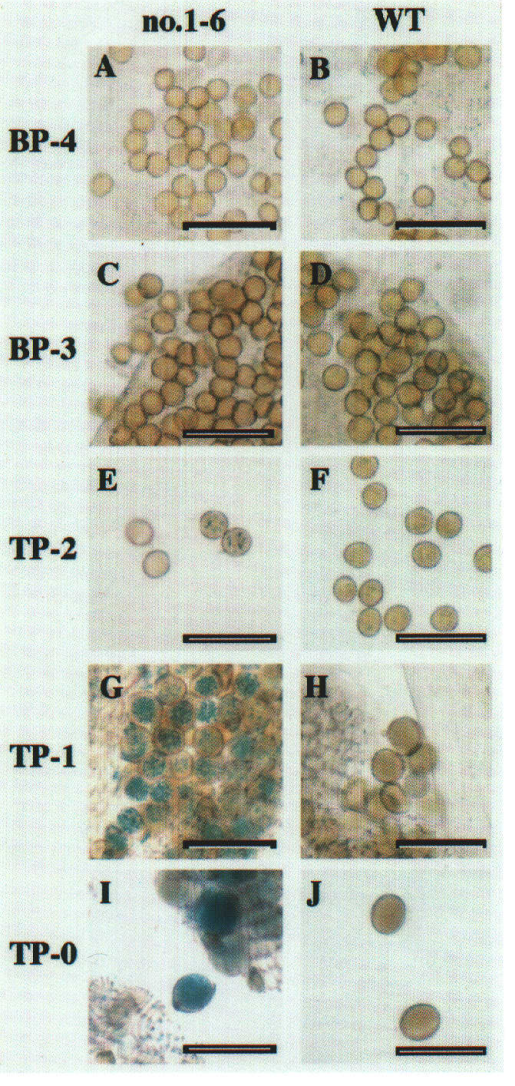

Fig. 3 Histochemical detection of GUS actuvity in pollen of transgenic *Arabidopsis* plant (# 1-6) and a wild-type plant (WT). Blue staining indicates GUS activity. BP, bicellular pollen; TP, tricellular pollen. Numbers indicate the age in days of flower buds before anthesis. GUS activity was detected only in tricellular pollen starting 2 days before anthesis (TP-2). Bar=50 μm.

last stage of maturation. Homozygous plants of such male-sterility will be produced by recovering plants through microspore culture. The promoter sequence reported here will be useful for genetic engineering of such a novel type of male-sterile plants.

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