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Full Length Research Paper

Isolation, characterization and expression analysis of *BrMyb* from *Erwinia carotovora* subsp. *Carotovora* diseased Chinese cabbage

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Plant MYB Transcription factors play important roles in defense responses. We described here a novel gene *BrMyb* encoding MYB transcription factor homologue was isolated from *Erwinia carotovora* subsp. *Carotovora (Ecc*) pathogens diseased Chinese cabbage (Brassica rapa subsp. *pekinensis)* and named as *BrMyb*. The nucleotide sequence of the cloned *BrMyb* revealed a single open reading frame of 1047 bp coding for 348 amino acids with a theoretical protein size of 48 kDa. The deduced amino acid sequence showed significant homology with Brassica oleracea brassica MYB protein and Arabidopsis thaliana minor MYB protein by 89.2 and 81.2% amino acid sequence identity, respectively. The protein showed significant alignments with a SANT/Myb DNA binding domain in members of MYB family proteins from dicotyledon and monocotyledon. The expression of *BrMyb* could be detected in developmental leafstalks and enhanced by Ecc infection, injury, methyl jasmonic acid and salicylic acid treatments, respectively. These results suggested that *BrMyb* might be an old plant gene, and plays roles not only in plant development but also in the response to pathogen infection and other stresses.

Key words: Transcription factor, *Erwinia carotovora* subsp. *carotovora*, *Brassica rapa* subsp. *pekinensis,* methyl jasmonic acid, salicylic acid.

INTRODUCTION

Plant continuous exposure to environment has evolved a number of mechanisms to cope with different biotic and abiotic stresses for survival. As parts of these mechanisms, transcription factors function by directly binding to the promoters of downstream target genes in a sequence specific manner to either activate or repress the transcription in adjusting many biological processes in response to these stresses (Scott, 2000). In Arabidopsis, more than 1500 transcription factors (Riechmann et al., 2000) were found on the basis of sequence analysis, and among which, a number of families of transcription factors, such as AP2/EREBP, bZIP/HD-ZIP, Myb and some zinc finger family proteins, have been implicated in plant stress responses for their expression is induced or repressed under different stress conditions (Qu and Zhu, 2006; Chen et al., 2006).

The common characterization of MYB transcription factors is the highly conserved DNA-binding domain, the so-called MYB domain (Ogata et al., 1994), containing 50 - 53 amino acids. MYB domain with the helix-turn-helix motif is always located at the N-terminal and could be found in nearly all eukaryotes. In animals, most MYB proteins contain three imperfect repeats of this domain,

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for example, the well characterized mammalian transcription factor c-MYB is composed of the three repeats, R1, R2, and R3 (Klempnauer et al., 1982). While in plants, most of MYB proteins have only two repeats (R2 and R3), and were mainly divided into three families, the R2R3, R1R2R3 and MYB-related families, on the basis of the number and position of the MYB repeats (Rosinski and Atchley, 1998). The MYB super family comprises the largest number of members in any plants gene family, for example, in arabidopsis, there are 195 MYB genes, including 126 R2R3-MYB, 5 R1R2R3-MYB and 64 MYB-related, and among which, only a few genes show constitutive and ubiquitous expression (Qu and Zhu, 2006; Chen et al., 2006).

Accumulated evidence indicated that MYB proteins are involved in the regulation of an array of metabolic and developmental processes. For example, FLP and MYB88 have been predicted function in jointly restrict divisions late in the Arabidopsis thaliana stomatal cell lineage (Lai et al., 2005), the expression of PttMYB21a (Karpinska et al., 2004) was associated with development of secondary vascular tissue in hybrid aspen. MYB transcription factors are also crucial in response to various stress, for example, over-expression of Myb4 could enhance the accumulation of ectopic lignin and significantly increase cold and freezing tolerance in Arabidopsis (Newman et al., 2004; Vannini et al., 2004); BcMYB1 was predicted to be involved in the regulation of gene expression in response to dehydration stress through an ABA-independent pathway in Boea crassifolia (Chen et al., 2005); AtMYB30, could be specific, rapid, and transient transcriptional activated in response to the avirulence pathogens, and over expression of AtMYB30 accelerates the appearance of the HR in response to avirulent bacterial pathogens (Daniel et al., 1999; Vailleau et al., 2002). Up till now, many MYB genes has been extensively investigated, while the precise roles of most of MYB family genes still remain unclear (Chen et al., 2006). Thus, it is necessary to investigate the functions of MYB transcription factors in development and various stresses, and even in different organisms.

Chinese cabbage (*Brassica rapa* subsp. *pekinensis)* originated from China and is one of the favorite vegetables and widely planted in China, Korea and Japan. Soft-rot is one of the most important diseases caused by *Erwinia carotovora* subsp. *Carotovora* (Ecc) pathogens, which can make great damages in field and storage stage and in certain years, over 50% reduction in the production in China (Liu, 1998). However, as is now known, little work was done in studying the mechanism of Chinese cabbage in resisting Ecc up till now. Recently, a suppressed subtracted hybridization (SSH) cDNA library was constructed from Ecc infected Chinese cabbage (unpublished) and many expressed sequence tags (ESTs) generated in our laboratopy. These ESTs will provide valuable help for elucidating the interaction between host and Ecc.

In the presented study, we report here the cloning of a MYB family transcription factor (GenBank accession: DQ903665), *BrMyb*, from soft-rot diseased Chinese cabbage by rapid amplification of 3` cDNA end (RACE) method, and show that its expression could be induced / enhanced by wound, pathogen infection and hormones.

MATERIALS AND METHODS

Plant material, bacteria culture and experimental treatments

Chinese cabbage cultivar “longbai NO.2” has better soft rot disease resistance and is widely cultivated in northern area of China. Plants grown in plastic plots were kept in chambers programmed for a 14 h light (8000 to 10,000 lux) and 10 h dark cycle, and 80% relative humidity at 28□.

Soft-rot bacterial strains Ecc BC1 (Yuan et al., 2004) was cultured overnight at 28□C in LB medium. Bacteria were pelleted, and then washed three times with sterile ddH2O. Harvested bacteria were resuspended, and diluted with sterile ddH2O at the concentration of 2x108 cfu ml-1.

Experiments were performed with Chinese cabbage seedlings at the sixth- or seventh- leaf stage. The forth and fifth leafstalks were inoculated with 5 – 10 µl fresh bacterial suspension or mock inoculated (wound or injury), respectively. Plant materials were harvested from Ecc inoculated and mock inoculated plants at 2, 6, 12, and 24 h post inoculation (hpi). Similar parts of untreated plantlets were also harvested and used as a control.

ddH2O, 5 mM salicylic acid (SA) and 1 mM methyl jasmonic acid (MeJA) were sprayed onto the plantlets, respectively. The forth and fifth leafstalks were harvested at 2, 6, 12 and 24 h post treatments. Similar parts and weight of plantlets treated with ddH2O were used as control. All the samples harvested above were frozen immediately in liquid nitrogen and stored at -80□C.

Primer designing and gene isolation

Total RNA was extracted from harvested materials using the TE3D reagent (Robaglia et al., 1993), and treated with DNase I (RNase-free DNase set: Qiagen, USA). 3`RACE primer was designed according to the conserved sequences among several genome and/or genes sequences with the highest similarities to EST (Accession NO: DN960591) of *BrMyb*.

3`- RACE reverse transcript was obtained using BD SMART™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer’s instructions. PCR was performed using the 3`RACE primer (5`-ATGACGCGGCGATG (C or T)TCTCACTGC-3’) and the mixed primers(5`-CTAATACGACTCACTATAGGGC-3’ and 5'-CTAATACGACTCACTATAGGGCAAGCAGTGG TATCAACGCAGAGT-3') provided in the BD SMART™ RACE cDNA Amplification Kit (Clontech, USA). The reaction condition was programmed with an initial denaturation at 94□C for 4 min, 30 cycles at 94□C for 40 s, 71□C for 40 s and 72□C for 90 s and a final 72□C for 5 min. The specific PCR product was cloned into pGEM-T vector (Promega, USA) and sequenced (Sangon, China). All the experiments repeated for two times in isolating the cDNA of *BrMyb* from independed mRNA samples, and the same results were obtained.

DNA and protein sequence analyses

DNA and protein sequences were processed or deduced using Software Editseq (DNAstar. Lasergene, USA). Deduced amino acid

sequence was used to predict the protein domains with RPS-BLAST (Marchler-Bauer and Bryant, 2004) and to search against the nr database using BLAST(x/n) (Basic Local Alignment Search Tool, Altschul et al., 1997) in getting the homologues, respectively. Sequence alignment and phylogenetic tree analysis were performed by using DNAMAN (Lynnon, Corporation).

Analysis of gene expression

Reverse transcription-polymerase chain reaction (RT-PCR) was used to semi-quantitatively determine the expression profile of the *BrMyb* gene. First-strand cDNA was synthesized from 4 µg total RNA using superscript III reverse transcriptase according to the manuscripts (Invitrogen, Madison, WI, USA). Specific sense Primers 5'-TTGCTTCTAGCTCCCGTAAAG-3' and antisense primer 5'- CTGATCCGATTCTCCATTCTT-3' were designed according to the cDNA sequence. The RT- PCR was performed with an initial denaturation at 94□C for 4 min, 35 cycles of 94□C for 50 s, 60□C for 50 s, and 72□C for 2 min, and a final 72□C for 5 min. The amplified products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and quantified using the Higher Performance Ultraviolet Transilluminator (GDS-8000, Gel Documentation System, UVP, USA). β-tubulin was used as the internal standard and amplified using specific primers 5`-CTTTCCCAGGCTCCACTT-3` and 5`-CACACG CCTGAACATCTCCT-3`. The reaction was programmed at 94□C for 4 min, 35 cycles of 94□C for 50 s, 55□C for 50 s, and 72□C for 2 min, and a final 72□C for 5min. Relative fold inductions were determined by compare ratio of the target band intensity to the Beta-tubulin with the ratio from control band intensity to the control Beta-tubulin. The experiments were repeated three times with the similar results and one of them is presented.

RESULTS

Isolation and analysis of *BrMyb* from Ecc diseased Chinese cabbage

In order to get the coding sequence (CDS) of *BrMyb*, one of its EST (Genbank accession No: DN960591) was used as a “bait” to search against the nr database, and many genomic or cDNA sequences (accession No: AC155347.1, AC155343.1, AC183493.1, AC183495.1 and AT5G47390) with identities >83% and E-value< e-49 were obtained and used to design primers. Then, 3`RACE was performed, and PCR product from soft-rot diseased Chinese cabbage was cloned into pGEM-T vector and sequenced. As a result, the presumably CDS sequence of *BrMYB* cDNA (Figure 1) obtained was 1047 bp in length between the first in-frame initiation codon ATG at the 5’-end and stop codon TAA in downstream. The sequence showed high identities to CDS sequences of MYB transcription factor gene from B. oleracea (96.1%) and A. thaliana (87.8%), respectively, and considered as complete CDS of *BrMYB*.

Nucleotide sequence alignment with CDS and genomic sequence from *BrMYB* and homologues in B. oleracea and A. thaliana (Figure 1) revealed that there were two introns and three extrons in *BrMYB*; splice sites of this gene were conserved among B. rapa, B. oleracea and A. thaliana; there were insertion or deletion mutations in the extrons and higher divergence in the introns among three genes. In addition, alignment also showed the CDS of *BrMYB* did not match with its corresponding sequence in genomic sequence completely (Figure 1).

Comparing of the deduced protein *BrMyb* with its homologues in other organisms

The protein deduced from *BrMyb* cDNA had 349 amino acids with a theoretical protein size of 48 kDa and a predicted isoelectric point of 7.57. SANT/MYB DNA binding domain (Figure 2) located at near the N-terminal of deduced *BrMyb* protein. The SANT domain was identified based on a ~50 amino acid motif (Figure 2) with a high degree of sequence similarity to some Myb DNA binding domain (Aasland et al., 1996). Comparison of the deduced amino acid sequences of *BrMyb* with representative *BrMyb* homologues from other species (Figure 2) showed that SANT/DNA-binding domains and N-terminal sequences had higher conservation among Myb-related transcriptions factors (Figure 3).

In order to analyze the sequence evolution among *BrMyb* and homologues in other organisms, phylogenetic tree was (Figure 3) generated based on the alignment of *BrMyb* and 31 *BrMyb* homologues (E-value< e-25), including eight from Glycine max, six from Arabidopsis, seven from Oryza sativa, three from B. oleracea and closely related *BrMybs* in other plants. As a result, two main clusters generated and *BrMyb* was grouped into cluster I that contained *BrMyb* homologues from dicotyledon and monocotyledon with higher identities.

Expression of *BrMyb* under different stresses

In this study, changes in relative fold induction by Ecc inoculation were investigated and showed that expression of *BrMyb* was enhanced in inoculated site at all the time points as compared with untreated plants (Figure 4A). As shown in Figure 4 (B, C and D), the expression of *BrMyb* was also up-regulated by the induction of wound, SA and MeJA, respectively. The expression levels of *BrMyb* varied among the four different treatments. For example, *BrMyb* transcripts were up-regulated in first six hours and then down-regulated at 12 and 24 h at wounded site (Figure 4B) and varied significantly at different time points in MeJA sprayed plants, but it was much stable and highly expressed in both Ecc infected and SA treated plants.

DISCUSSION

MYB super family comprises a large number of transcription factors in plants, and many have been extensively studied (Riechmann and Ratcliffe, 2000). Up

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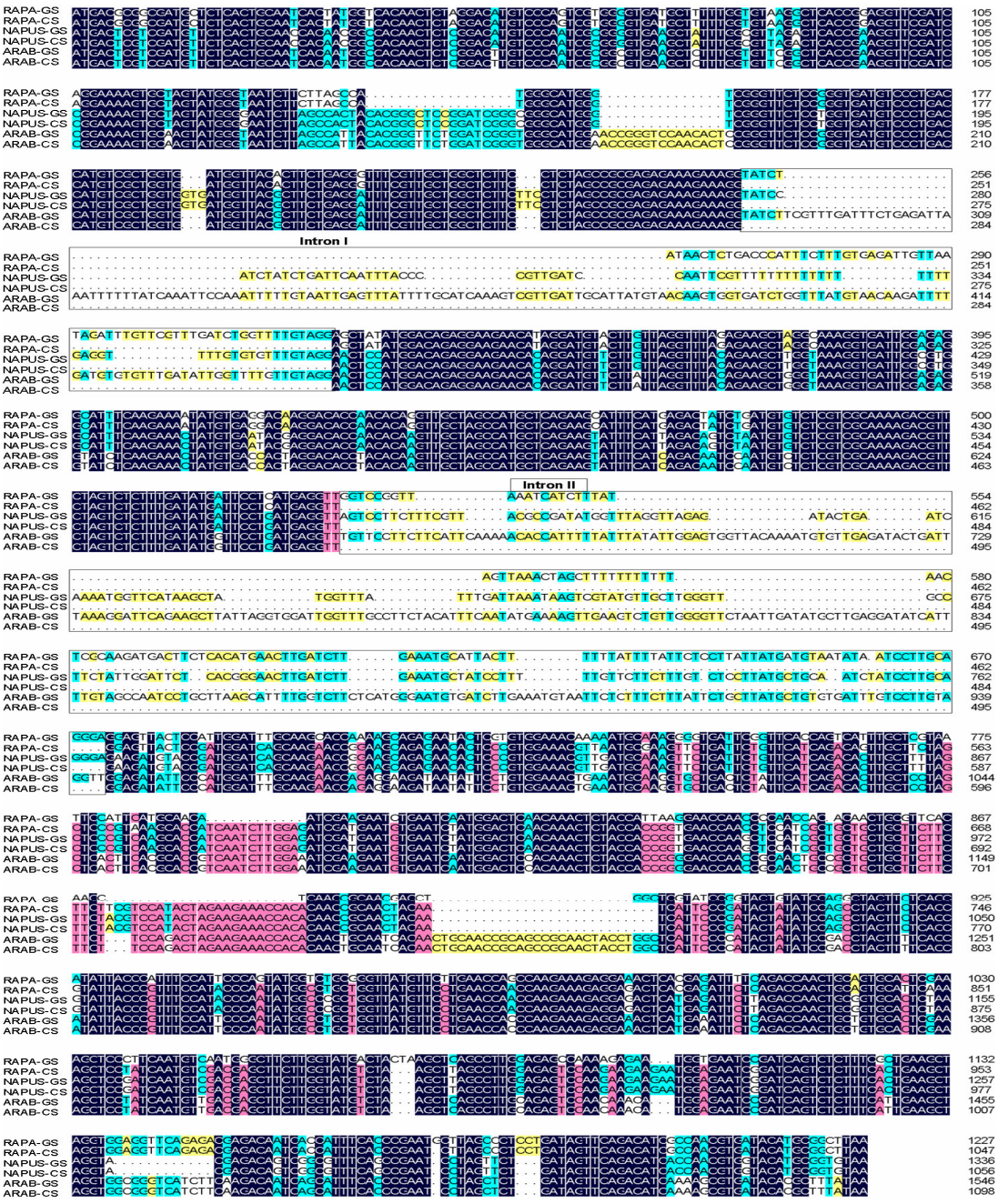

Figure 1. Comparing the genomic sequence and CDS of *BrMyb* with those of homologues from *B. rapa* (RAPA-GS and RAPA-CS), *B. oleracea* (OLER-GS and OLER-CS) and *A .thaliana* (ARAB-GS and ARAB-CS). Position of introns in box was marked with I and II. GS: genomic sequence; CS: coding sequence.

till date, the majority of Myb proteins described belong to the R2R3-Myb protein family (Martin and Paz-Ares, 1997; Chen et al., 2006), while only a few MYB-related genes have been studied functionally involving in the maintenance of circadian rhythms (Wang and Tobin, 1998; Green and Tobin, 1999; Kuno et al., 2003) and in response to stress (Nagaoka and Takano, 2003). In this study, we first isolated and characterized a gene *BrMyb* encoding the Myb family proteins in Chinese cabbage.

Recent (Jiang et al., 2004) report showed that a majority of R2R3 Myb genes had conserved three extrons and two introns in Arabidopsis and rice. In this study, (Figure 1) *BrMyb* and its homologues also had three extrons and two introns, and showed high conservation in extrons

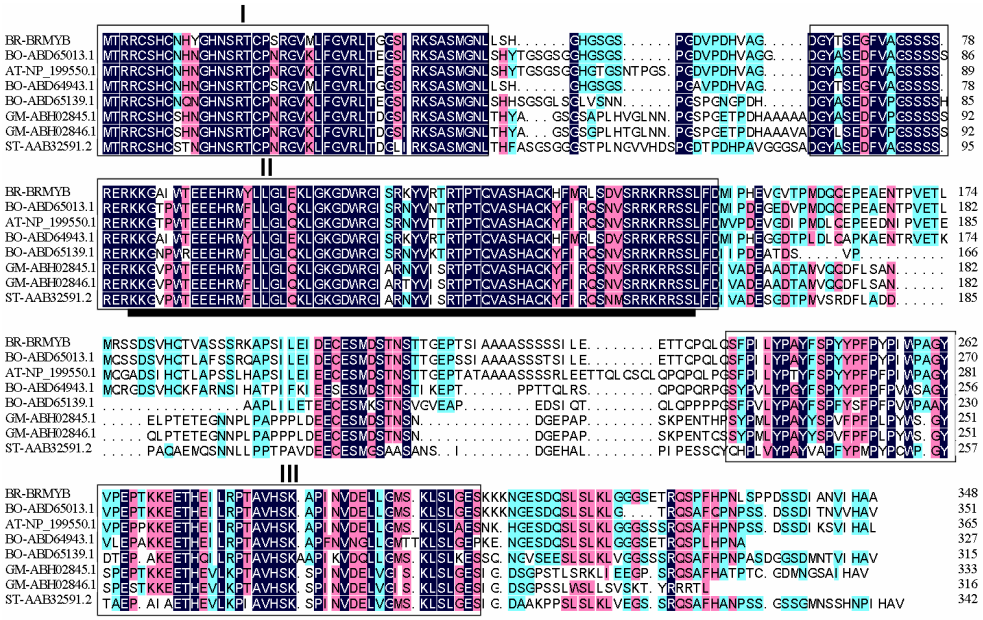

Figure 2. Alignment of deduced amino acid sequence of BrMyb with that of other closely related sequences from plant: *B. oleracea* (BO), *A. thaliana* (AT), *Glycine max* (GM) and *S. tuberosum* (ST). Higher conserved regions were marked in box with I, II and III. Black bar indicated the SANT/MYB-DNA binding domain under sequence.

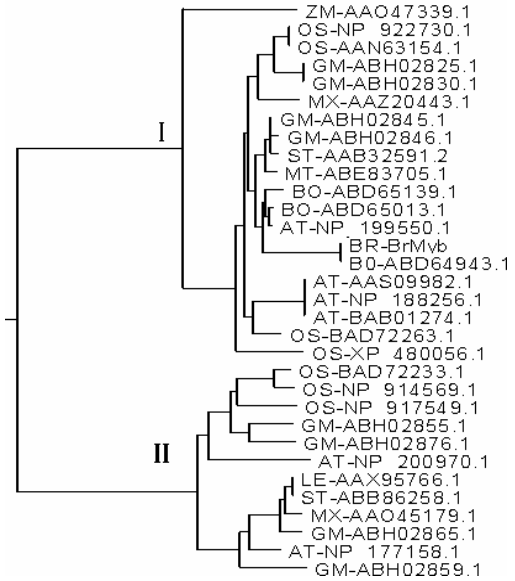

Figure 3. Phylogenetic tree based on an alignment of Chinese cabbage BrMyb with eight Glycine max, six *Arabidopsis*, three *B. oleracea* and closely related MYBs in other plants : *Glycine max* (GM), *Arabidopsis thaliana* (AT), *Brassica oleracea* (BO), *Lycopersicon esculentum* (LE), *Malus xiaojinensis* (MX), *Medicago truncatula* (MT), *Oryza sativa* (OS), *S. tuberosum* (ST), *Zea mays* (ZM). Two clusters were generated and marked with I and II, respectively.

while higher divergence in introns, suggesting the splicing sites were conserved and the length and identities among introns varied significantly during evolution. Both CDS and genomic sequences showed high similarities among three organisms (Figure 1), indicating that they might evolve from the same ancestor. While the CDS of *BrMyb* did not match with the putative CDS regions in *BrMyb* genomic sequence perfectly suggesting that these might be caused by a few mutations in plants or sequencing error in genomics sequence deposited in Genbank and should be further studied.

SANT (SWI3, ADA2, N-COR, and TFIIIB BW) domain was identified based on its homology to the DNA binding domain of c-myb (Ogata et al., 1994; Boyer et al., 2002) and exists in a number of eukaryotic transcriptional regulatory proteins. Recent research found that the SANT domain plays essential role in the functioning of multiple chromatin remodeling enzymes and participate in the regulation of a plant-specific developmental program (Barg et al., 2005; Boyer et al., 2002). In this study, the deduced amino acid sequence of *BrMyb* contained a SANT/*MYB* DNA binding domain and showed higher identities with MYB transcription factors from B. oleracea (89.2, 81.9 and 69.7% identities with ABD65013.1, ABD64943.1 and ABD65139.1, respectively), A. thaliana (81.2% identity with NP_199550.1), G. max (58.0 and 60.3% identities with ABH02845.1 and ABH02846.1, respectively), and Solanum tuberosum (59.6% identity with AAB32591.2). Extensive protein BLAST(x) analyses using SANT domain of *BrMyb* as a probe showed that orthologous sequences could be only found in many plant species (data not shown), suggesting it might be an old plant specific MYB gene. In addition, alignment of the

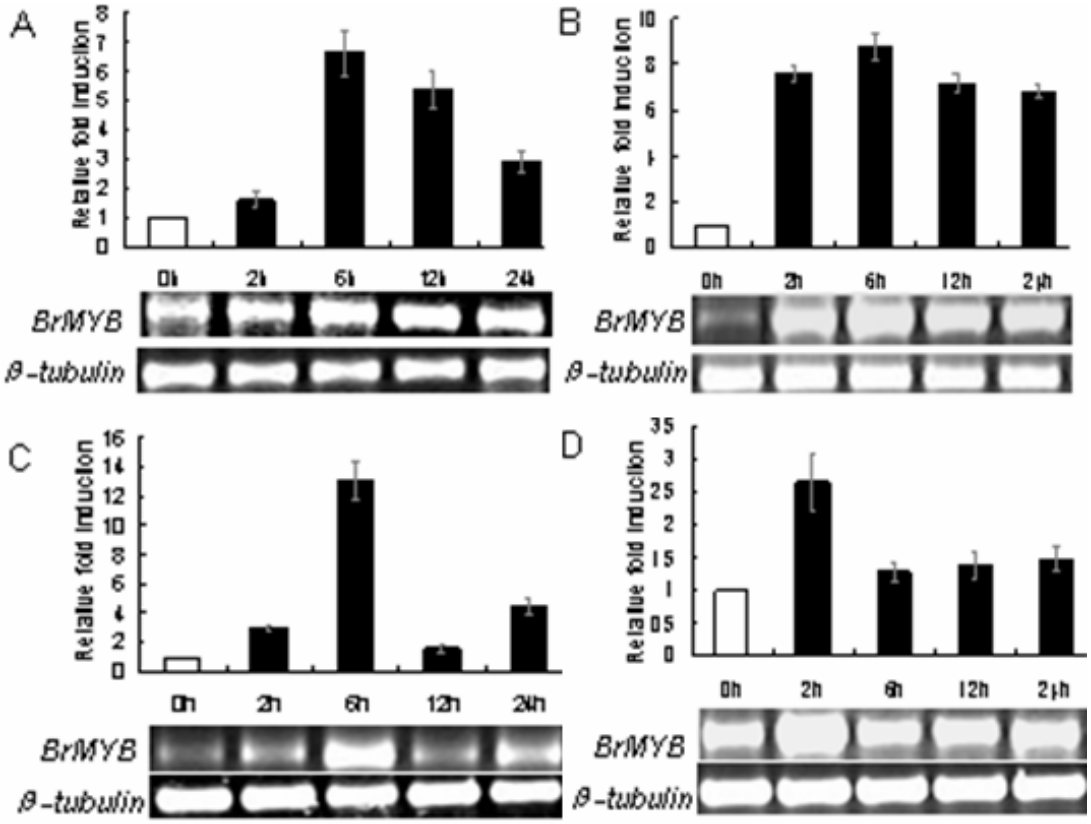

Figure 4. BrMyb gene expression in Chinese cabbage in response to different stimuli and hormones. A, wound; B, *Ecc* infection; C, Methyl jasmonate acid (JA); D, salicylic acid (SA). Total RNA from five time points (0, 2, 6, 12, 24 h) of every treatment were separately amplified by RT–PCR and analyzed as described in Methods. The average fold induction and standard deviations (error bars) for three independent RT–PCR amplifications are shown in the histograms; an autoradiogram of a representative gel electrophoresis is shown below.

BrMyb and its 31 homologues showed similar results with those in Figure 2B, i.e. regions containing the N-terminal and SANT/MYB DNA binding domain showed much higher conservation among the *BrMyb* homologues (data not shown), and the sequences outside the two regions were always divergent (Jiang et al., 2004), suggesting that the two conserved regions are important for their function, such as in chromatin remodeling or DNA binding and/or regulating the expression of downstream gene.

Recent research showed that many Myb-related genes were responsive to one or multiple types of hormones and stress treatments in Arabidopsis (Chen et al., 2006). Expression of *BrMyb* was detected in leafstalks of untreated Chinese cabbage plants indicating it may be involved in the developmental process (Figure 4). As compared with injured plants, *BrMyb* could be regulated rapidly and highly expressed in Ecc infected plants, suggesting that expression of *BrMyb* could be induced significantly in response to Ecc infection. MeJA and SA are plant-produced defense related signals that activate plant defense genes after herbivory (Moran and Thompson, 2001) or pathogen attack (Seskar et al., 1998). Both MeJA and SA could also enhance the expression of *BrMyb* in this study. Recent report showed that *BrMyb* homologues (Chen et al., 2006) also expressed in response to various hormones, including MeJA and SA, and stress treatments in Arabidopsis. These results suggested that there was a cross-talking between the signal transduction pathways (Stout et al., 1999; Li et al., 2002), although the JA and SA signal cascades activate many different sets of plant defense genes (Thomma et al., 1998) or even act antagonistically (Preston et al., 1999; Felton et al., 1999). These results indicated that *BrMyb* involved not only in development but also in response to injury, pathogen infection and hormones.

In conclusion, we cloned a new gene *BrMyb* from Chinese cabbage with high similarities to MYB transcriptiton factors, and its expression could be induced or enhanced by different stress or hormones in this study. These results provided the important information in further studying the precise role of *BrMyb*, including whether and how it binds to the target DNA and activate the genes expression and its expression in other development or stress process.

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