Gene 471 (2011) 13–18

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Expression divergence of *TaMBD2* homoeologous genes encoding methyl CpG-binding domain proteins in wheat (*Triticum aestivum* L.)

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ARTICLE INFO

Article history:
Accepted 5 October 2010
Available online 14 October 2010

Received by G. Theissen

Keywords:
Expression divergence
Triticum aestivum L.
Methyl CpG-binding domain proteins
Homoeologs
Abiotic stress

ABSTRACT

Most hexaploid wheat genes are present as triplicate homoeologs derived from the ancestral species. Previously, we isolated six wheat cDNAs with open reading frame, encoding methyl CpG-binding domain proteins (MBDs). In this study, the genomic and cDNA sequences of three *TaMBD2* homoeologous genes were obtained and mapped on chromosomes 5A, 5B and 5D, respectively. These sequences showed a very high conservation in the coding region and the exon/intron structure, but the cDNA sequences are distinguishable by a 9-bp insertion in coding region and a size polymorphism in the 3′-untranslated region (UTR). The expression patterns of each homeologous gene in different tissues of various developmental stages and in response to abiotic stress were analyzed by using real-time PCR. Relative mRNA abundance of the three homoeologs varied considerably in different developmental stages from seedling to developing seeds. Most notably, *TaMBD2-5B* and TaMBD2-5D were highly responsive to salt stress and *TaMBD2-5B* was specifically upregulated by low temperature in the seedling leaves. These results provide further evidence for the expression variation of genes duplicated in allopolyploids. Moreover, the variation of *TaMBD2* homoeologous gene expression in response to environmental stress may enable plants to better cope with stresses in their natural environments.

1. Introduction

One of the most common and well-characterized epigenetic phenomena is DNA methylation, which frequently occurs as 5-methylcytosine (m^5C) (Gruenbaum et al., 1981; Matzke et al., 1989). The presence of extensive 5-methylcytosine is strongly correlated with the reduced transcriptional activity, for which two molecular mechanisms have been suggested (Bird, 2002; Bird and Wolffe, 1999). One mechanism is the direct inhibition of gene transcription by methylated promoter regions, which blocks the binding of transcription factors to their cognate *cis*-elements in promoters. Another mechanism involves the recruitment of transcriptional repressors by DNA methylation, and the best evidence comes from the discovery and characterization of a group of mammalian CpG-binding domain (MBD)-containing proteins (MeCP2, MBD1, MBD2 and MBD3) (Ballestar and Wolffe, 2001). These mammalian MBD proteins, except for MBD3, have been shown to selectively bind methylated CpG through their MBD, and physically recruit distinct transcription repressor complexes, which contain histone deacetylases, chromatin remodeling proteins and transcription repressors, to cause global silencing of gene expression (Springer and Kaeppler, 2005).

Plant MBD proteins can be divided into eight classes based on sequence similarity and phylogenetic analyses of sequences obtained from rice, *Arabidopsis*, and maize (Springer and Kaeppler, 2005). Functional analysis revealed that decreased expression of *AtMBD11* gene caused a number of aberrant morphological and developmental traits, including extra rosettes, serrated leaves, low fertility, reduced apical dominance, and delayed flowering (Berg et al., 2003). Recently, three mutant alleles resulting from T-DNA insertions in *AtMBD9* gene were isolated and each exhibited early flowering and enhanced shoot branching phenotype (Peng et al., 2006). These results indicate that some MBD genes play an important role in Arabidopsis development by epigenetic control of gene expression.

Wheat is a hexaploid species with the genome constitution of AABBDD that originated from three diploid ancestral species, *Triticum urartu*, *Aegilops speltoides* and *Aegilops tauschii* (Feldman and Levy, 2005). Allopolyploidization leads to the generation of duplicated homoeologous genes (homoeologs), as opposed to paralogous genes

Abbreviations: MBD, methyl CpG-binding domain; UTR, untranslated region; DAP, days after pollination; CTAB, cetyltrimethylammonium bromide; RACE, rapid amplification of cDNA ends; ORF, open reading frame; MEGA, Molecular Evolutionary Genetics Analysis; PHYLIP, Phylogeny Inference Package.

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doi:10.1016/j.gene.2010.10.001

(paralogs). There are three possible evolutionary fates for homoeologous genes in polyploids: functional diversification, gene silencing, and retention of original or similar function (Wendel, 2000). Functional diversification of homoeologs is one of the important factors in the evolutionary success of polyploidy species. Furthermore, the evolutionary success of allopolyploids is also due to the retention of function of all homoeologs in many loci and to the gene silencing in other loci (Shitsukawa et al., 2007). In spite of the paramount role of polyploidy in the evolution of higher plants, there are only a few studies on the extent of nucleotide divergence between homoeologous genes, but this knowledge would be necessary for a better understanding of the molecular evolution of duplicated genes (Wendel, 2000).

Previously, we isolated six wheat cDNAs with open reading frame encoding putative methyl-binding domain proteins, designated *TaMBD1* to *TaMBD6*, respectively (Li et al., 2008). In this study, the genomic and cDNA sequences of three *TaMBD2* homoeologous genes (*TaMBD2-5A, TaMBD2-5B* and *TaMBD2-5D*) were obtained, and their spatial expression patterns were analyzed. In addition, transcript changes of the three *TaMBD2* homoeologous genes under abiotic stress were also investigated.

2. Materials and methods

2.1. Plant materials

The wheat genotype Nongda3338 was used for gene cloning and expression analysis. Seeds were allowed to germinate on moist filter paper and transplanted into pots to grow in the greenhouse. Light was provided by cool white fluorescent and incandescent lights (intensity ≥ 3000 lx) for a 12-h photoperiod, and the plants were watered with a 1/10-strength Hoagland's solution or water when necessary. Dry seed embryos, primary roots and shoots of 1 day after germination, roots and fully expanded leaves of 10 days after germination, leaves in tillering stage, stems in jointing stage, flag leaves, immature ears and basal 1.5-cm-long portion of the first internode in heading stage, and seeds at 6 and 12 days after pollination (DAP) were harvested, respectively. For abiotic stress treatment, seedlings at trefoil stage were transferred into 100-ml beakers containing NaCl (250 mM). Low-temperature treatments were performed by transferring seedlings to a growth chamber set for 4 °C. Leaf tissues were harvested at 2 h after treatment and immediately frozen in liquid nitrogen. In addition, special care was taken to characterize these materials used and three replicates were harvested.

2.2. Isolation of DNA and total RNA

Total DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984) with minor modifications, and total RNA was extracted using a standard Triazol RNA isolation protocol (Life Technologies, USA). The amount and quality of the total RNA were checked by electrophoresis on 1.0% formamide agarose gel.

2.3. Rapid amplification of cDNA ends (RACE)

The mRNA was purified through oligotex chromatography (Clontech) from the total RNA, and 5′-RACE and 3′-RACE were performed using the SMART-RACE cDNA amplification kit (Clontech) according to the manufacturer's protocol. Gene specific primers used for the first and second round PCR were 3′-GSP1 and 3′-GSP2 (3′-RACE), 5′-GSP1 and 5′-GSP2 (5′-RACE), respectively (Table 1).

Table 1
Primer sequences for gene cloning, RACE and real-time PCR.

Name	Sequence (5′-3′)	
TaMBD2-aL	GGCCTCGATCTCTCCTCA	Genomic sequence cloning
TaMBD2-aR	CCAGAGTCTGCTTCCATCC	
TaMBD2-bL	CACTATCCAATGTGCTAGGTG	
TaMBD2-bR	GTACGTCAGACTGCAGCTT	
TaMBD2-1L	TGTGTGCTTGGCTGCTTGCCTATG	Chromosome location
TaMBD2-1R	GAGGTACCTGCAAAGTATGGCATAG	
TaMBD2-2L	GTCCAAGTGATGAAGCAACAACT	
TaMBD2-2R	GCAACCGTTACCAATTTCACGAAT	
3′-GSP1	TCCAAGTGATGAAGCAACAACT	3′-RACE
3′-GSP2	GCTAAGGCAAGTGAACCAGC	
5′-GSP1	TGGCGATCCACGGTATGACACGT	5′-RACE
5′-GSP2	GGGAACTGTCTTCTCCAAATTCATCTTG	
TaMBD2-5A-L	CTCCTACTTCGAGGAAGCTGAGAT	Real-time PCR
TaMBD2-5A-R	TTGCCTCTCCTGGTTCTGCTT	
TaMBD2-5B-L	GAAGAGGAAAGCAGAAGGCTCT	
TaMBD2-5B-R	TTCCACGATACTACAACATTCAGTC	
TaMBD2-5D-L	CCTACTGCGAGGAAACTGAGG	
TaMBD2-5D-R	ATCTGACTCTCCTGGTTCTGCTT	
β-action-L	GGAATCCATGAGACCACCTAC	
β-action-R	GACCCAGACAACTCGCAAC	

2.4. PCR amplification, cloning and sequencing

PCR amplification conditions were optimized according to those described by Plaschke et al. (1995), and the primer information was listed in Table 1. For gene cloning, PCR products, separated in 1.0% agarose gel and purified by Sephaglas BandPrep kit (Amersham Pharmacia), were cloned into pGEM-T plasmid vector (Promega) and sequenced by an ABI PRISM 377 capillary sequencer (PE Applied Biosystem) using an ABI Prism Dye Terminator sequencing kit (PE Applied Biosystem) and either vector or sequence specific primers.

The PCR products of Chinese Spring (CS) and its nulli-tetrasomic lines were separated in vertical non-denaturing 8% (silver staining) polyacrylamide gels (Bio-Rad protein cell II, 0.75 mm thick and 20 cm long) in 1× TBE (90 mM Tris-borate (pH 8.3), 2 mM EDTA) at 300 V for 4 h.

2.5. Reverse-transcription

Equal amounts of 2 μg RNA were transcribed to cDNA in 20 μl reactions containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 μM dNTPs, 200 U MMLV reverse transcriptase (Promega4) and 1 μg T_{15} anchor primer. Reverse transcription was performed for 60 min at 37 °C with a final denaturation step at 95 °C for 5 min. Aliquots of 2 μl of the obtained cDNA each were subjected to real-time PCR analysis.

2.6. Quantitative real-time PCR

TaMBD2 homeologous gene specific primers were used for quantitative real-time PCR analysis. For normalization, *β-actin* gene was amplified as an endogenous control (Table 1). For quantitative real-time PCR, cDNAs from three biological samples were used for analysis and all the reactions were run in triplicate. PCR conditions were performed as follows: 94 °C for 4 min, followed by 40 cycles: 94 °C for 30 s, 66 °C/58 °C 30 s, 72 °C 30 s, and then 72 °C for 5 min. Quantification results were expressed in terms of the cycle threshold (CT) value determined according to the manually adjusted baseline. Relative gene expressions in different samples were determined using the method as described previously. Briefly, differences between the CT values of target gene and *β-actin* were calculated as $\Delta CT = CT_{target} - CT_{\beta\text{-}actin}$, and expression levels of target genes relative to *β-actin* were determined as $2^{-\Delta CT}$. For each sample, PCR was