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RESEARCH ARTICLE

Cloning, chromosome mapping and expression analysis of an *R2R3-MYB* gene under-expressed in maize hybrid

Chuanli Ju · Fu Zhang · Yufeng Gao · Wei Zhang · Jianbing Yan · Jingrui Dai · Jiansheng Li

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Abstract *R2R3-MYB* transcription factors play important role in transcriptional controls during higher plant metabolism and development. In this study, an *R2R3-MYB* gene was isolated from maize according to an EST, which expressed differentially between a hybrid and the two parents on a cDNA chip. The full-length cDNA, designated by *ZmMYBL1* (GenBank accession no. AY365033) consists of 1417 nucleotides and contains an open reading frame of 828 bp. The deduced amino acid sequence contained two conserved MYB domains near its N-terminus, a conserved E1 motif and an acidic Ser/Thr rich region toward its C-terminus. Southern blot analysis revealed *ZmMYBL1* could be a single copy gene belonging to a multi-gene family in the maize genome. Expression analysis showed *ZmMYBL1* transcripts accumulated in various tissues examined, with strong level in tassel and weak level in leaf. Also it was under-expressed in root, stem, and leaf of hybrid as compared with that of the two parents. *ZmMYBL1* was mapped on maize chromosome bin7.03 between two SSR markers, *bn1g339* and *umc1865* using Yuyu22 recombinant inbred line population. A QTL for root average diameter in maize seedlings was also localized on the corresponding region of chromosome 7 within the interval *ZmMYBLI-bnIg1805*. A possible role of *ZmMYBL1* and its relation to maize heterosis were discussed based on these results.

C. Ju · F. Zhang · Y. Gao · W. Zhang · J. Yan · J. Dai · J. Li (✉)
National Maize Improvement Center of China, China Agricultural University, Yuanmingyuan West Road, Haidian, 100094 Beijing, China
e-mail: lijs@163bj.com

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Introduction

The genetic basis of heterosis has been discussed for nearly a century, but the underlying mechanism remains elusive [1]. Maize is a model system for the investigation of genetic and molecular basis of heterosis, in which hybrid vigor is particularly evident. Studies indicated that when diverse genomes were brought together within a hybrid, the gene expression patterns altered with its parents which may be responsible for the observed maize heterosis [2–4]. However, the regulatory mechanism of gene differential expression between hybrid and its parents is still an area to be elucidated. The overwhelming majority of regulatory events occur at the initiation of transcription among which transcription factors play important roles. So maybe it is an important strategy to explore the differential expression of transcription factors between hybrid and its parents to understand the mechanism of crop heterosis.

Transcriptional regulators containing the MYB-homologous DNA-binding domain composed of 1–3 conserved repeat motifs were widely found in eukaryotes. Each repeat is approximately 50 amino acids in length, with regularly spaced tryptophan residues, which form a tryptophan cluster in the three-dimensional helix-turn-helix structure [5]. In contrast to animals, plants contain a MYB protein subfamily that is characterized by the R2R3-MYB domain, which constitute the largest *MYB* gene family in plants [6].

Until now, the precise functions of most plant *MYB* genes are not fully understood, although some well-studied examples suggested important roles of these genes in the regulation of secondary metabolism, the control of cellular morphogenesis, the response to disease and hormone and the regulation of cell cycle [7–9]. Previous findings led to the suggestion that the expansion of this large-sized regulatory family could have contributed to provide flexibility for plants, which require very flexible regulatory mechanism to cope with changing environmental conditions [7, 8, 10].

It harbors over 130 different *R2R3-MYB* genes in *Arabidopsis*, approximately 85 in rice, and a minimum of 200 in maize [6, 11, 12].Up to now only about 10% of the plant *MYB* genes have their functions analyzed. The aims of this investigation is to isolate the full-length sequence of a maize *R2R3-MYB* gene; to analyze its expression characterization in different tissues; to locate it on genetic linkage map in which root traits were mapped and to discuss the function *of ZmMYBL1* with regard to heterosis particular root architecture.

Materials and methods

Plant materials

An elite maize hybrid (Yuyu22), the two parents, Zong3 and 87-1, and a set of F7 recombinant inbred lines (RILs) derived from the hybrid Yuyu22, were used as plant materials. Yuyu22 is a widely extended hybrid with highly heterotic performance in grain yield and root related traits recently. A total of 155 individual lines were randomly selected from Yuyu22 RIL population for root QTL analysis and 92 individual lines from the 155 lines used for QTL analysis were only employed to map the *ZmMYBL1* gene.

Roots and leaves of the hybrid Yuyu22 and the two parents, Zong3 and 87-1 were collected from seedlings cultured in quartz sands with Hogland nutritional solution and grown with 12 h/12 h light/dark cycles at 28°C incubation for 8 days and stems (without knots) and tassels were harvested during the period of tassel differentiation for RNA extraction.

Cloning of the full-length cDNA

Total RNA isolated from root using TRIZOL reagent (Invitrogen, USA) was reverse transcribed using cDNA synthesis Kit (TaKaRa, Japan) with negative control not adding reverse transcriptase to monitor genomic DNA contamination. Two nested gene specific primers: 5′-GCTTTGCCTCTGCTGCTGCTCG-3′ and 5′-ACCGCAAGCTCATCAACTTCATCC-3′, which were designed from a differential expressed EST sequence (GenBank accession no. AI770808), and Oligo dT-Adapter primer were used to obtain the 3′ end fragment of the gene. The PCR products were purified and cloned into pMDI8-T Vector (TaKaRa, Japan) for sequencing, According to the sequencing result, a pair of specific primers, 5′-CGATAGCCGCAACCGTGAGA-3′ (forward) and 5′-ATTGCATCCCAGCCGTTAGT-3′ (reverse), were used to get the full-length cDNA. Sequence analysis was performed using SMART [13] and PROSITE [14] programs.

Southern blot analysis

Genomic DNA, extracted from the hybrid Yuyu22 and the two parents, Zong3 and 87-1, was completely digested with *Bam*HI, *Eco*RI, *Hind*III, and *Xho*I, respectively. The 10 μg digests were separated by 1.0% agarose gel and blotted onto Hybond-N^+ membrane (Amersham, UK). The blot was hybridized to [α-^{32}P]-dCTP labeled PCR amplicons of full-length *ZmMYBL1* cDNA and a 410 bp specific probe derived from non-coding C-terminal sequence of *ZmMYBL1*, respectively, at 65°C for 16 h. After washing the membrane with 2 × SSC,signals were detected by exposure to X-ray film at −70°C.

Semi-quantitative RT-PCR analysis

Total RNA from maize root, stem, leaf, and tassel digested with DNasel (invitrogen, USA) were used for first strand cDNA synthesis with negative control not adding reverse transcriptase. The specific Primers for amplifying the full-length open reading frame of *ZmMYBLI* were 5′-CGATAGCCGCAACCGTGAGA-3′(forward) and 5′-ATTGCATCCCAGCCGTTAGT-3′(reverse). The gene *efl*α was used as an internal control, amplified with primers 5′-GGTATGGTTGTTACTTTTGGGCCTACTGGT-3′ (forward) and 5′-CGAGGTAACCAACAAGCAAGCACAGC-3′(reverse). Various PCR cycles were tested to ensure that the reaction did not reach the plateau. Finally the three RT-PCR replications were conducted with the following amplification profile: a 2-min denaturation step at 94°C was followed by 36 cycles of 45 s at 94°C, 45 s at 53°C, 1.5 min at 72°C, plus a final extension for 10 min at 72°C.

Chromosome mapping of *ZmMYBL1*

Cleaved amplified polymorphic sequences (CAPS) described by Konieczny and Ausubel [15] was employed to develop the marker for mapping *ZmMYBL1* using genomic DNA isolated from the two parents, Zong3 and 87-1, and the individual family lines of Yuyu22 RIL population. According to the sequence information of *ZmMYBL1*, the two primers, 5′-CCGAGGAAACAGTATAAAAGCG-3′(forward) and 5′-CGTGGAGGTCGATGACGA-3′ (reverse), were designed to amplify partial region of the genomic sequence of *ZmMYBL1*, where the *Msp*I polymorphic site was located in the non-coding region of the gene between Zong3 and 87-1. All the PCR products were restricted with *Msp*I (TaKaRa, Japan) and separated on 6% polyacrylamide gel electrophoresis (PAGE) to count segregating information among RILs. The map position was estimated using the computer program ''MAPERMAKER'' (version 3.0; LOD threshold = 3.0).

Quantitative trait loci mapping of root average diameter

In total, 115 individual lines randomly selected from Yuyu22 RIL population were grown using the sand culture with Hogland nutritional solution. Root trait data of each line after 4 days of germination was evaluated with WinRHIZo, an optical-scanner-based image analysis system (Regent instrument Inc., Quebec, Canada). With the genetic linkage map constructed in our lab, quantitative trait loci (QTL) mapping and the genetic effect evaluation were performed based on composite interval mapping method [16, 17] using soft QTL cartographer (version 2.0).

Results

Cloning and characterization *of ZmMYBL1* gene from maize

Using a maize cDNA chip (Unigene-1-02-01) from Arizona containing 8464 unigenes, gene expression patterns of maize seedling roots were investigated between the hybrid Yuyu22 and the two parents, Zong3 and 87-1. An EST coding for a MYB domain expressed as 0.66 and 0.36 times in the hybrid Yuyu22 as in the two parents, respectively, showing this gene was underexpressed in maize hybrid compared with the two parents. The 3′ region fragment was obtained by RT-PCR with a pair of specific primers designed on the basis of the whole sequences cloned from the two parents.

When sequences were compared between two parents, Zong3 and 87-1 (Fig. 1), it was observed that there were only two nucleotide differences, which caused one amino acid variation between them. The full-length cDNA of *ZmMYBL1* consists of 1417 nucleotides and contains an 828 bp open reading frame plus a 184 bp 5′-untranslated region and a 389 bp 3′-untranslated region, respectively. The putative *ZmMYBL1* protein contains 275 amino acids and has a calculated molecular weight of 30 kD with a pI of 5.44. Two structurally conserved MYB domains were identified in *ZmMYBL1* (Fig. 1). The MYB DNA-binding domains with regularly spaced tryptophan residues were conserved among different species, where *ZmMYBL1* shared 96% identity with rice *MYB15* domain (Fig. 2). Compared with amino acid sequences of full-length, ZmMYBL1 showed 79%, 55% and 52% identity with MYB15 and MYB13 from rice, ODORANT1 from Petunia and MYB85 from *Arabidopsis*, respectively. So *ZmMYBL1* could code for a typical R2R3-MYB protein. Another worthy feature is that a short fragment immediately downstream the R3 domain designated E1 motif (LXXMGIDPVTHK/RP), which is highly conserved in some subgroups of R2R3-MYB proteins [11], was found in the ZmMYBL1 sequence. Also an acidic Ser/Thr rich area were observed at the C-terminal region (Fig. 1),which might act as an activation domain for transcription and four casein kinase II phosphorylation motifs (T/S-X-X-D/E).

ZmMYBL1 gene in the maize genome

From Fig. 3, only one strong band was seen when the DNA was digested with *Bam*HI, *Eco*RI or *Hind*III and hybridized with 3′-end specific probe (Fig. 3**b**), indicating that *ZmMYBL1* may be a single-copy gene in maize genome. Hybridization with the whole cDNA probe revealed the presence of more than one bands (Fig. 3**a**), showing that *ZmMYBL1* could belong to a multiple-gene family in maize. Previous studies also demonstrated that many MYB genes existed in maize [12, 18]. While when one parent, 87-1, was digested with *Xho*I (Fig. 3**b**), 87-1 and Zong3 were digested with *Hind*III (Fig. 3**a**), all the results showed two intense bands. The reason may be that, the enzymes cut the corresponding genome sequences into two fragments although there were no restriction sites in the cDNA probes. When digested with *Hind*III a co-dominant polymorphism between the two parents was detected (Fig. 3**b**).

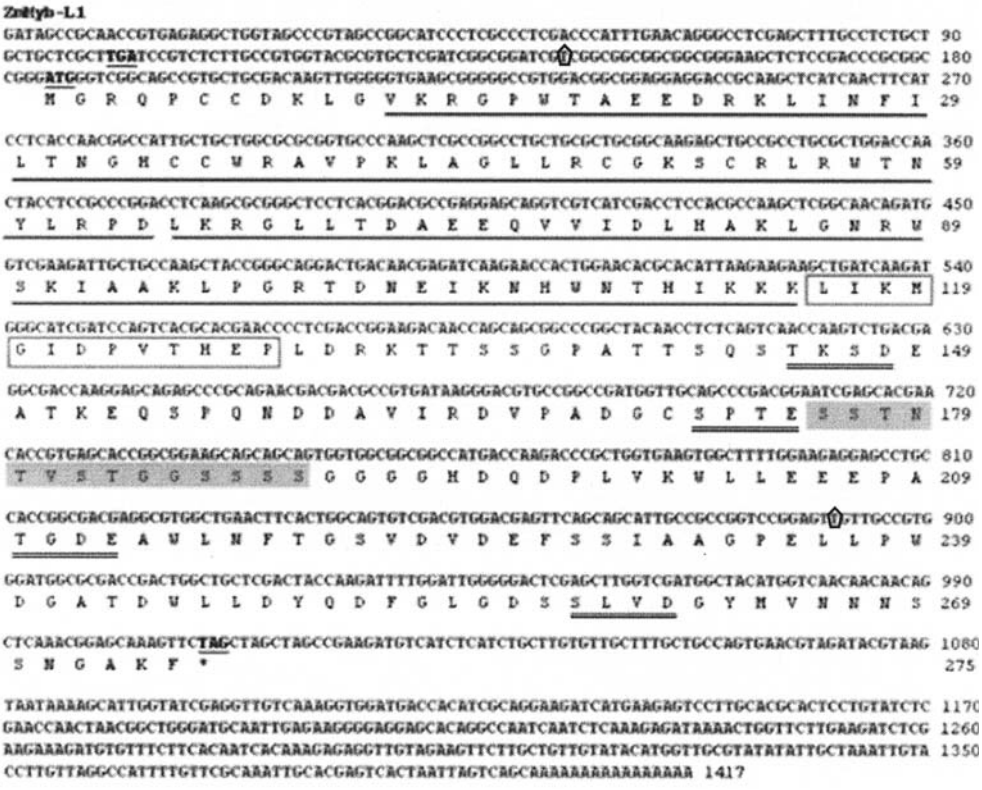

Fig. 1 Nuleotide and deduced amino acid sequence of *ZmMYBL1*. *The initiation and stop code were underlined and the stop codon was marked by an asterisk. Bold black bars marked R2 and R3 MYB domains. A conserved E1 motif was indicated with boxes, a Ser/Thr rich area was showed with a shaded rectangle, four casein kinase II phosphorylation sites were marked with double underlines in the carboxy-terminal of the protein. The two pentagons denoted the allelic nucleotide variation sites between the two parents, 87-1 and Zong3, which were G and C, respectively in Zong3. The latter nucleotide variation changed amino acid from L to S

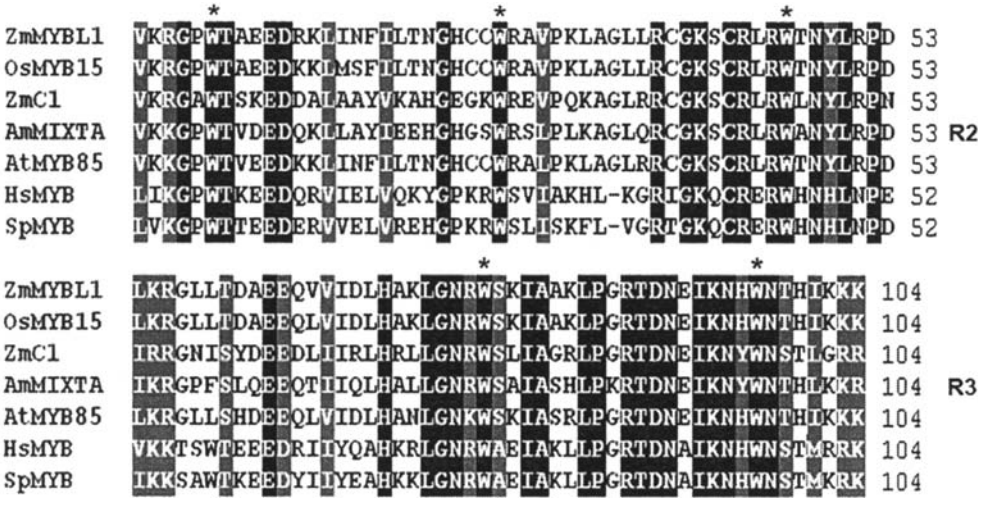

Fig. 2 Alignment of amino acid sequences of the R2R3 domains of ZmYBL1 and C1, OsMYB15 from *O. sativa*, AmMIXTA from *A. majus*, AtMYB85 from *Arabidopsis*, HsMYB from *H. sapiens*, SpMYB from *S. purpuratus*. *The asterisks mean the conserved tryptophan residues. Shading represents residues identical (black) or similar (gray) among different MYB domains

Expression pattern of *ZmMYBL1* in maize

Expression analysis of *ZmMYBL1* in the hybrid Yuyu22 and the two parents revealed that there was a tissue-specific expression pattern in root, leaf, stem and tassel (Fig. 4). The results indicated that *ZmMYBL1* transcripts accumulated in various tissues examined, with strong level in tassel and weak level in leaf not only in the hybrid but also in the two parents. Also the gene is expressed relatively weaker in the root, stem, and leaf of the hybrid compared with that of the two parents. The differential expression pattern of the gene in root detected by RT-PCR confirmed the cDNA chip hybridization experiment. The results demonstrate that *ZmMYBL1* was an under-expressed gene in maize hybrid.

Chromosome mapping of *ZmMYBL1* with maize RIL population

By alignment of part of *ZmMYBL1* cDNA sequence and relative genomic sequences of the two parents 87-1

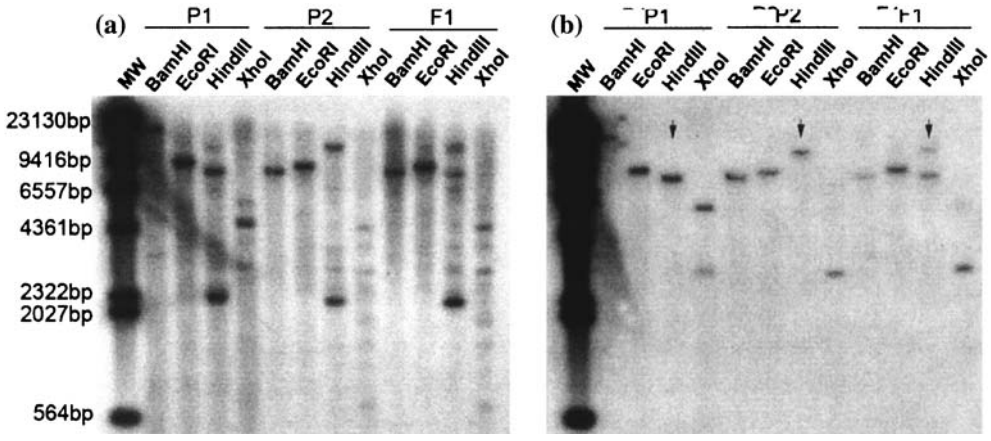

Fig. 3 Southern blot results with probes of full-length sequence (**a**)and 3′-end gene-specific fragment (**b**) in the hybrid Yuyu22 (F1) and the parents, 87-1 (P1) and Zong3 (P2). *The same membrane was used twice, allowing the comparison of the hybridization pattern. Genomic DNA was completely digested with four restriction enzymes, among which only *Xho*I cut inside the *ZmMYBL1* cDNA. Arrows showed the co-dominant marker between the two parents

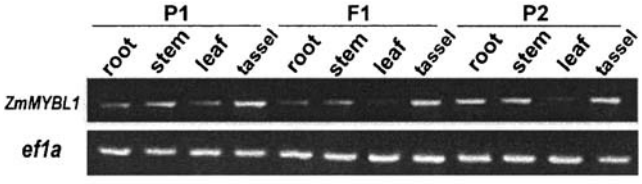

Fig. 4 Expression analysis of *ZmMY3L1* in different tissues of the hybrid Yuyu22 (F1), and the two parents, 87-1 (P1) and Zong3 (P2). *The *efl*α gene was used as an internal control

and Zong3, an *Msp*I site in the non-coding region of *ZmMYBL1* revealed polymorphism between Zong3 and 87-1 (Supplementary data Fig. 1), which was used for developing CAPS marker. From Fig. 5, a clear polymorphism between the two parents was detected after PCR products were digested by *Msp*I. With the PCR-based CAPS primers, 92 individual family lines of RIL population were screened, among which 45 individuals showed the same bands as 87-1 and in 47 individuals the same bands as Zong3 existed. The marker segregation was in accordance with an expected ration, 1:1 ($P < 0.05$). Therefore, *ZmMYBL1* was mapped on maize chromosome bin7.03 based on the genetic linkage map constructed with SSR markers in our lab (Fig. 6). It was linked with SSR markers, *bn1g339* and *umc*1865, having the genetic distance 18.6 and 10.7 cM, respectively. It should be noted that *ZmMYBL1* was located in the same region with EST AI770808 in Maize Database, according to which, the *ZmMYBL1* gene was isolated in this investigation. Also homologous genes were located on rice chromosome 8 (20.46 Mb) and chromosome 9 (20.56 Mb) according to BLASTN searching result of the rice genome database (E value < e-50).

QTL mapping for root average diameter using *ZmMYBL1* as a marker

The names, locations, genetic effects and phenotypic variation of QTL associated with root total length (RTL), root surface area (RSA), root average diameter (RAD) and root total volume (RTV) were listed in Table 1. Three QTL for root average diameters were detected with the criterion of LOD > 2.5. They were located on chromosome 5 (interval *bnIg1870-phi0918*, LOD = 3.0, the proportion of phenotypic variance explained, $R^2 = 6.9\%$), chromosome 7 (interval *ZmMYBL1-bnlg1805*, LOD = 3.4, $R^2 = 13.6\%$) and chromosome 10 (interval *phi32315-bnlgl815*, LOD = 4.2, $R^2 = 10.8\%$), respectively. It was interesting to note that a QTL for root average diameter was found near CAPS marker *ZmMYBL1*. More interestingly, when marker *ZmMYBL1* was added in the genetic linkage map, the sharper QTL interval and increased LOD value (from 2.6 to 3.4) were observed

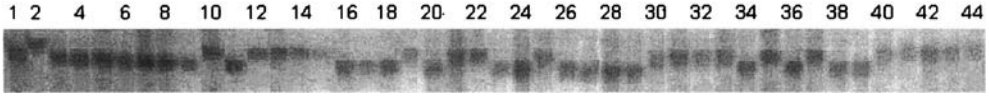

Fig. 5 Marker segregation of Cleaved amplified polymorphic sequences (CAPS) for *ZmMYBL1* in Yuyu22 RIL population. *Lane 1 and 2 were the co-dominant bands in 87-1 and Zong3, respectively. Lanes 3–44 were different individuals in RIL population

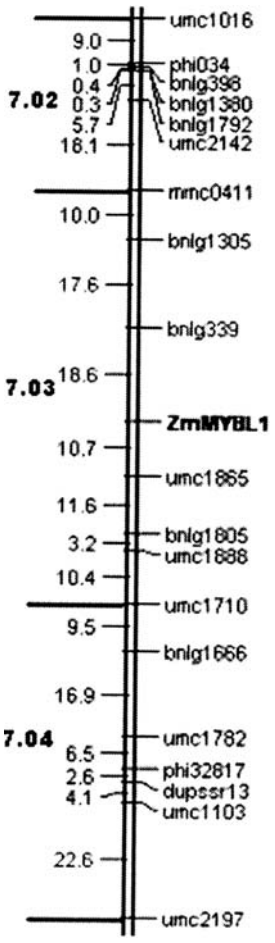

Fig. 6 Chromosome position of *ZmMYBL1* on maize genetic linkage map. *Markers were listed to the right of the map and genetic distances (cM) were on the left. Large numbers to the left indicated the chromosome number and bins

(Fig. 7); also the explained phenotypic variations increased from 10.2% to 13.6%.

Discussion

A large percentage of transcription factors were discovered in plants. For example, *Arabidopsis* dedicates 5.9% of its genome to code for more than 1500 transcription factors [6]. The numerous *trans*-acting factors that modulate gene expression were hypothesized to be an important mechanism for quantitative variation [20, 21]. Birchler et al. [1] envisioned that regulatory gene allelic interactions in hybrids cause gene expression to deviate relative to the mid-parent predictions. Tsaftaris and Kafka [22] reported that ABA transcription factor Rab2l expressed at higher level in one parent than in the hybrid and another parent. In this study, a differential expressed transcription factor was observed, whose transcript was weaker in the root, stem, and leaf of the hybrid than that of the parents. Allelic expression variation occurred frequently in maize hybrids, which could result from various regulatory mechanisms [21]. As for *ZmMYBL1* which had nearly no changes in the coding region of the two parents, the reason caused its differential expression between the hybrid and the two parents may be because of different regulatory elements in the promoter regions.

The challenge in the development of a molecular model to understand heterosis is to make the correct association between phenotype and any causative molecular events that occur in hybrids [1]. Herein it is interesting to note that in the same location of *ZmMYBL1* gene, bin7.03, a QTL controlling average diameter of seedling roots was mapped using the RIL population, which associated with flanking markers, *bn1g339* and *umc1865*. More importantly with marker *ZmMYBL1* added to the map, the QTL interval *(ZmMYBL1BL-bnlg18O5)* became sharper and the LOD value showed upward trend. A QTL for maize primary root diameter in the hydroponics' culture was also located on bin7.03 [23]. The identification and mapping of differentially expressed *ZmMYBL1* could provide a candidate gene for the root trait QTL dissection, as well as a combination analysis for understanding molecular and genetic basis of heterosis.

In plants, MYB proteins are involved in the control of form and metabolism. *WER*, a *MYB* gene in *Ara-*

Table 1 Names, locations, genetic effects and phenotypic variation of QTL influencing root traits in maize seedlings (LOD > 2.5)

QTL	Chr.	Interval	Position	LOD	R^2 (%)	A
RTL-1	1	*phi011-bnlg1597*	353	4.45	10.85	−2.00
RSA-1	1	*phi011-bnlg1597*	353	5.58	13.24	−0.67
RSA-2	5	*umc1260-phi024*	49	3.13	9.11	−0.57
RAD-1	5	*bnlg1870-phi0918*	114	2.98	6.93	2.57
RAD-2	7	*ZmMYBL1-bnlg1805*	160	3.43	13.61	−3.52
RAD-3	10	*phi32315-bnlg1815*	196	4.24	10.79	3.13
RTV-1	1	*umc2151-umc1122*	249	2.69	6.66	1.31
RTV-2	1	*phi011-bnlg1597*	353	4.11	9.48	−1.51
RTV-3	5	*bnlg1006-umc1260*	45	2.82	8.38	−1.43

*RTL, RSA, RAD, RTV represented root total length, root surface area, root average diameter, and root total volume, respectively

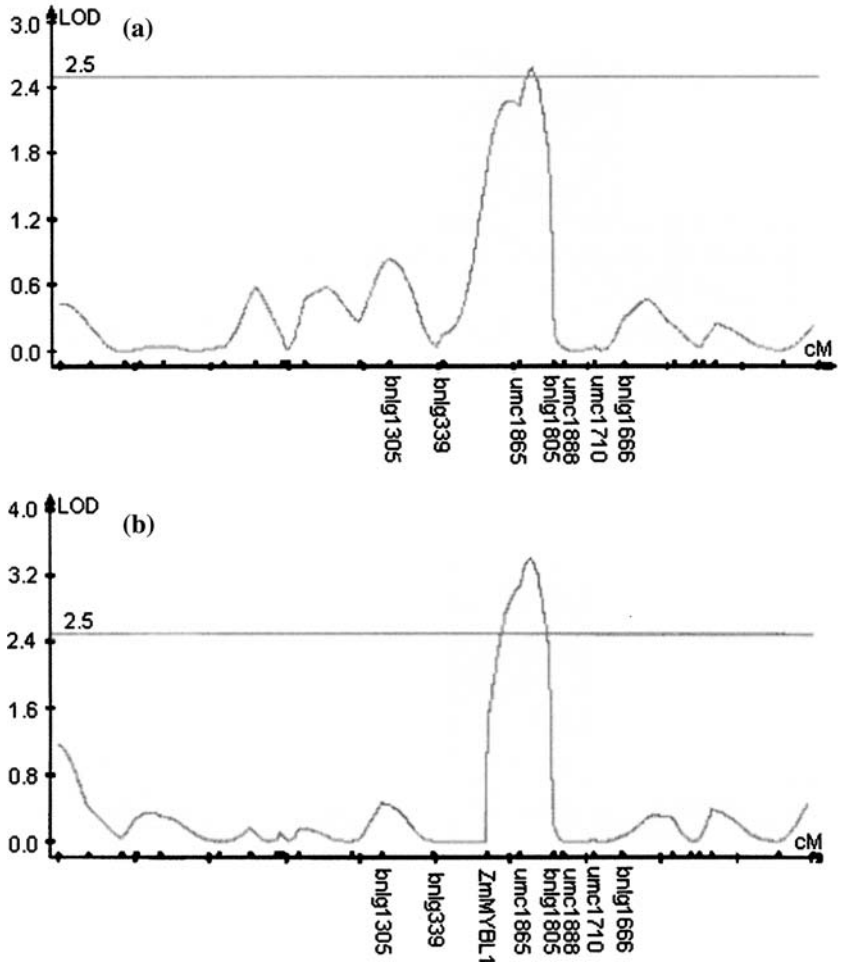

Fig. 7 LOD values for QTL associated with root average diameter on chromosome 7 in maize. *A and B referred to the LOD curve without and with *ZmMYBL1* marker, respectively

bidopsis, participates in the differentiation of epidermal cells to trichomes by regulating *CPC* and *GL2* transcription through binding to their promoter regions [24, 25]. The *mixta* controls cell shape by activating the directional synthesis of specialized epidermal cell-wall materials, which affect flower color intensity [26]. Another *MYB*-related transcription factor, *MYB308*, can change the shape of leaf palisade cells through modification of cell-wall production [27]. The complex control of phenylpropanoid and flavonoid biosynthesis by MYB proteins demonstrates a key function played by this family of regulatory factors [7, 8]. Kranz et al. [19] determined the expression and genetic map positions of more than 60 *MYB* genes in *Arabidopsis*. A strategy of reverse genetics was applied to investigate further functional information on *Arabidopsis MYB* genes [28]. These systematic studies especially the expression. analyses of *R2R3-MYB* genes indicated their potential involvement in various regulatory processes. The differential expression analysis of the *MYB* transcription factor together with its mapping position in this study provide a substantial basis for its further function of analysis and investigating regulatory mechanism of the differential gene expression between the maize hybrids and their parents.

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