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ORIGINAL ARTICLE

The maize B chromosome is capable of expressing microRNAs and altering the expression of microRNAs derived from A chromosomes

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Abstract Supernumerary B chromosomes (Bs) are nonessential chromosomes that are considered genetically inert. However, the maize B carries control elements that direct its behavior, such as that of nondisjunction, during the second pollen mitosis, and affects normal A chromosomes during cell division. Recently, the maize B has been found to contain transcriptionally active sequences and to affect the transcription of genes on A chromosomes. To better understand the regulatory mechanisms underlying the maize B, we constructed two small RNA libraries from maize B73 inbred lines with and without Bs. The sequencing results revealed that 18 known microRNAs (miRNAs) were significantly differentially expressed in response to the presence of the B, and most target mRNAs were characterized as transcription factors. Moreover, three novel B-derived miRNAs were identified via stem-loop reverse transcriptase-polymerase chain reaction (RT-PCR)-based analysis, and all showed consistent B-specific expression in almost all analyzed inbred lines and in all tissue types, including leaves, roots, and pollen grains. By the use of B-10L translocations, the three B-derived miRNAs were mapped to specific B regions. The results from this study suggest that the maize B can express miRNAs and affect the expression of A-derived miRNAs, which could regulate the expression of A-located genes.

Keywords B chromosome · microRNA · small RNA sequencing · maize · B-10L translocation

Abbreviations

CBF	CCAAT-binding factor
cDNA-AFLP	Complementary DNA-amplified fragment length polymorphism
CK	Centromeric knob
DCL	Dicer-like
DE	Distal euchromatin
DH	Distal heterochromatin
DPE	Distal portion of proximal euchromatin
hc-siRNA	Heterochromatic small interfering RNA
miRNA	MicroRNA
NAM	No apical meristem
NAT-siRNA	Natural antisense transcript small interfering RNA
NCBI	National Center for Biotechnology Information

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PE	Proximal euchromatin
Phased siRNA	Phased small interfering RNA
PPE	Proximal portion of proximal euchromatin
RISC	RNA-induced silencing complex
RT-PCR	Reverse transcriptase-polymerase chain reaction
sRNA	Small RNA
SRA	Sequence read archive

Introduction

Supernumerary B chromosomes (Bs) are nonessential chromosomes of the genome and are present in approximately 15% of eukaryotic organisms, and it is widely believed that Bs are derived from standard A chromosomes from the same species or different species (reviewed in Marques et al. 2018; Ahmad and Martins 2019). In plants, the first B was identified in maize (Kuwada 1915), and its cytological nature and genetic behaviors have since been well characterized. However, the detailed molecular organization, expression, and evolution of this chromosome are still unclear. For a long time, Bs were thought to be genetically inert (Jones et al. 2008). However, the maize B is not entirely without genetic activity. For instance, the maize B can increase the recombination frequencies of A chromosomes (Rhoades 1968), elicit breakages in A chromosomes at the second pollen mitosis (Rhoades and Dempsey 1972), and reduce fertility (Randolph 1941) and cause leaf striping (Staub 1987) when present at multiple copies.

Because the maize B is not essential for plant development, it has evolved several mechanisms to ensure its survival. There are three mechanisms that allow it to be maintained and increase in number: nondisjunction during the second pollen mitosis (Longley 1927; Roman 1947), preferential fertilization of the egg by the sperm carrying the B (Roman 1948), and prevention of lost through meiosis as a univalent (Carlson 1986). Factors that control the accumulation mechanisms are located in specific chromosomal regions. Nondisjunction requires *trans*-acting factors located in the proximal and distal euchromatic regions of the B long arm (Ward 1973; Lin 1978). The centromeric knob (CK) and short arm of the B are associated with nondisjunction in a *cis*-acting manner (Carlson 1973; Lin 1979). The element that controls preferential fertilization is a single gene located on an A chromosome (Chiavarino et al. 1998, 2001; Gonzalez-Sanchez et al. 2003). The third heterochromatic region on the B long arm is required to prevent the loss of univalent Bs during meiosis (Carlson and Roseman 1992). Although the factors required for B survival have been mapped, no related genes have been identified, and the molecular mechanism involved is still unclear.

Understanding the molecular nature of the maize B is restrained by the extremely high levels of similarity between the DNA sequences of B and A chromosomes. For several decades, various approaches have been applied to overcome this hindrance, and numerous B-derived sequences have been identified. Almost all of these sequences are highly homologous to their A-derived homologs (Alfenito and Birchler 1993; Stark et al. 1996; Cheng and Lin 2003; Lamb et al. 2005; Peng et al. 2005; Lo et al. 2009; Kao et al. 2015). This homology suggests that a high degree of similarity should also exist between the B and A chromosomes at the RNA level. However, relatively few studies have investigated the transcriptional activity of the maize B, and the current evidence suggests that the maize B is transcriptionally active and that the expression of A-located genes is affected by the presence of this chromosome (Lamb et al. 2007; Lin et al. 2014; Kao et al. 2015; Huang et al. 2016). A comparison of the complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) profiles of various maize inbred lines with and without Bs revealed that the maize B has a negative impact on A-located gene transcription (Lin et al. 2014). Moreover, RNA sequencing analysis was applied to analyze the transcriptome of maize B73 lines carrying various copies of Bs, and 115 upregulated and 15 downregulated A-located genes were detected in response to the presence of the B (Huang et al. 2016). The maize B is therefore obviously capable of regulating gene expression at the RNA level.

Small RNA (sRNA)-guided posttranscriptional regulatory mechanisms have been shown to play important roles in many aspects of plant biology, including metabolism, hormone responses, and epigenetic control of transposons (Liu and Chen 2009; Nag and Jack 2010; Lisch 2013). In plants, sRNAs can be categorized into several major classes, including microRNAs (miRNAs), heterochromatic small interfering RNAs (hc-siRNAs), phased small interfering RNAs (phased siRNAs), and natural antisense transcript small interfering RNAs

(NAT-siRNAs), all of which regulate gene expression at the posttranscriptional level (Lu et al. 2005; Axtell 2013). Typically, miRNAs are approximately 22- to 24-nucleotide-long siRNA sequences that are generated from precursor miRNAs with a hairpin structure and then further processed into miRNA/miRNA* duplexes by Dicer-like (DCL) enzymes in the nucleus (Mallory and Vaucheret 2006). Mature miRNAs are then exported to the cytoplasm and incorporated into an RNA-induced silencing complex (RISC), which controls how complementary mRNAs undergo cleavage or translational repression (Li and Mao 2007). To date, the regulatory role of plant miRNAs has been exemplified by the critical regulatory behavior of miRNAs at key positions in a variety of pathways, such as root (Wang et al. 2005), shoot (Goiz 2006), leaf (Kidner and Martienssen 2004), and flower development (Mallory et al. 2004) and cell fate (Carraro et al. 2006). Heterochromatin has long been considered inert, but it is now believed to give rise to sRNAs, which, via RNA interference, direct the modification of proteins, DNA in heterochromatic repeats and transposable elements (Lippman and Martienssen 2004). Considering that the maize B contains a massive amount of heterochromatin and that the expression of some A-located genes is affected by the presence of the B, this chromosome should generate miRNAs or affect the expression of A-located miRNA genes.

The aims of this study were to verify whether the presence of the maize B can affect the expression of A-located miRNA genes and whether the B can generate miRNAs. We achieved these aims by constructing two sRNA libraries of maize B73 inbred lines with and without Bs, followed by sRNA sequencing. Comparison of known miRNAs in the two libraries revealed that the presence of the B affected the expression level of several classes of miRNAs. Using stem-loop reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, we identified three novel B-derived miRNAs, and all showed consistent B-specific expression in different tissue types, including leaves, roots, and pollen grains. Furthermore, the B-specific expression of the three novel B-derived miRNAs was further confirmed in other inbred lines. By the use of B-10L translocations, the three B-derived miRNAs were mapped to specific B regions. The results from this study suggest that the maize B can generate miRNAs and affect the expression of A-located miRNA-encoding genes.

Materials and methods

Plant materials

Maize inbred lines carrying Bs were generated from a B-containing L289 plant via continuous backcrossing to four inbred lines, B73, W22, W23, and A619, for at least 15 generations. Four B-10L translocations (TB-10L18, TB-10L7, TB-10L26, and TB-10L36) in the W23 background were used to generate heterozygous translocation, tertiary trisomic and hypoploid plants. The procedure for producing the three plants of each translocation was performed as previously described (Lin et al. 2014). Chromosomal constitutions were determined in Feulgen-stained root tip cells (Lin 1977). Each translocation has a breakpoint at various positions along the B and a second breakpoint within the long arm of chromosome 10 (Lin 1979; Chien et al. 2014).

RNA isolation, library preparation, and sequencing

Total RNA was extracted by TRIzol® Reagent (Invitrogen, USA) according to the instruction manual. The RNA purity was quantified at OD260 nm using an ND-1000 spectrophotometer (Nanodrop Technology, USA) and purified using a Bioanalyzer 2100 (Agilent Technology, USA) with an RNA 6000 labchip kit (Agilent Technologies, USA). The sRNA library construction and deep sequencing were carried out at Biotechnology Company (Welgene, Taiwan). Samples were prepared using an Illumina sample preparation kit according to the TruSeq Small RNA Sample Preparation Guide. The 3′ and 5′ adapters were ligated to the total RNA, which was used to perform reverse transcription followed by PCR amplification. The enriched cDNAs were size fractionated on a 6% polyacrylamide gel, and the bands containing 18–40 nucleotide RNA fragments (140–155 nucleotides in length with both adapters) were purified. The libraries were sequenced on an Illumina instrument (75 cycle single read), and the resulting sequencing data were processed by Illumina software. The sRNA raw sequencing data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession PRJNA482490.

Table 1 Sequences of three novel B-derived miRNAs and their corresponding stem-loop RT-PCR primers

miRmB1	miRNA sequence	AUAACUGAACAGGCGACCCGUG
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACGGG
	Forward primer	GGCTCGATAACTGAACAGGCGA
miRmB2	miRNA sequence	UUCUGUAGAUCAAUGGUUGUG
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAC
	Forward primer	GGCTCGTTCTGTAGATCAATG
miRmB3	miRNA sequence	AAGUACAGCGUAGAAGUAGGGCCU
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGCCC
	Forward primer	GGCTCGAAGTACAGCGTAGAAGTA
miR156	miRNA sequence	UGACAGAAGAGAGUGAGCAC
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
	Forward primer	GCGGCGGTGACAGAAGAGAGT
Universal	Reverse primer	GTGCAGGGTCCGAGGT

sRNA sequencing analysis

The sequences generated were initially filtered to obtain qualified reads. Trimmomatic was implemented to trim or remove the reads according to the quality score. After the low-quality (> 80% of Q20) data were filtered, the qualified reads were analyzed using MiRDeep2 to remove the 3′ adapter sequence and discard reads shorter than 18 nt and longer than 28 nt before the reads were aligned to the *Zea mays* genome from the NCBI database. Only reads that mapped perfectly to the genome five or fewer times were used for miRNA detection, since miRNAs usually map to few genomic locations. MiRDeep2 was then used to estimate the expression levels of known miRNAs and identify novel miRNAs (Friedländer et al. 2012).

Stem-loop RT-PCR analysis

Validation of potential B-derived miRNAs was carried out by stem-loop RT-PCR as described by Varkonyi-Gasic et al. (2007), with some modifications. Total RNA (0.2 μg) was treated via a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) with RT primers (Table 1). The reactions were incubated at 65 °C for 10 min, followed by 50 °C for 30 min. The reactions were then terminated by incubation at 85 °C for 5 min. PCR was carried out at a volume of 20 μl consisting of 1× Super Run EX-*Taq* buffer, 0.2 mM dNTPs, forward and reverse primers (at 0.2 μM each) (Table 1), and 5 μl of diluted cDNA under the following conditions: 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s and 58 °C for 1 min. miR156 was selected as an internal control. The PCR products were visualized on 4% agarose gels by ethidium bromide staining.

Results

Overview of sRNA libraries sequencing

To investigate sRNAs related to the maize B, we constructed two sRNA libraries from the third leaf of single B73 inbred plants containing no (B73+0B) and two Bs (B73+2B) at the four-leaf stage for deep sequencing. Total reads of 21,385,975 (B73+0B) and 22,871,438 (B73+2B) were generated by Illumina HiSeq in the two libraries. After removing the low-quality tags, null 3′-adapters, null inserts, 5′-adapter contaminants, polyA reads, and reads shorter than 18 nt and longer than 28 nt, 2,816,673 (B73+0B) and 2,806,967 (B73+2B) clean reads were obtained. Of these sRNAs, the 24 nt category was the most abundant, followed by 21 nt and 22 nt categories (Fig. 1). These results were consistent with the lengths of sRNAs in plants.

Differentially expressed known miRNA in the presence of the B

To identify known miRNAs, sRNA sequences were aligned against published maize miRNAs registered

in miRBase (http://www.mirbase.org/). A total of 44 known miRNAs belonging to 18 miRNA families were identified (Suppl. Table S1). The expression of the known miRNAs in B73+0B and B73+2B was compared to identify changes in miRNA expression, and the results revealed that 18 miRNAs were significantly differentially expressed. Compared with the levels of miRNA expression between the B73 +0B and B73+2B libraries, the expression levels of 9 miRNAs (miR528a/b, miR408b, miR164b/c/g, miR169m, miR171l, and miR396g) were upregulated (fold change greater than 2), whereas those of the other 9 miRNAs (miR164e, miR169a/b/o/r, and miR395a/h/j/p) were downregulated (fold change less than − 2) in the presence of the B (Fig. 2). These results suggested that the expression levels of some miRNAs changed significantly in response to the presence of the B in maize.

Identification of novel B-derived miRNAs

To identify the novel miRNAs derived from the maize, we mapped unannotated sequences to the genome sequences of maize and removed the reference genome reads from the sRNA sequencing results. As a result, B73+0B contained 107,665 nonreference sRNA reads, while B73+2B contained 133,354 reads. There were 76,785 common reads among the nonreference reads of B73+0B and B73+2B. Therefore, 30,880 nonreference reads were unique to B73+0B, and 56,569 nonreference reads were unique to B73+2B. The B73+2B unique nonreference reads were analyzed via MiRDeep2 to identify putative novel miRNAs, and stem-loop RT-PCR experiments further confirmed which novel miRNAs were derived from the B. We selected the top 14 B73+2B unique novel miRNAs on the basis of their expression levels and designed stem-loop RT primers to analyze the total RNA from the leaves of B73+0B, B73+1B, and B73+4B. Among these, the primers of three novel miRNAs of the maize B (miRmB1, miRmB2, and miRmB3) amplified B-specific PCR products from B73+1B and B73+4B but not from B73+0B (Table 1; Fig. 3). Moreover, the B-specific expression of the three B-derived miRNAs was consistent in all tested tissue types, including mature leaves, roots, and pollen grains (Fig. 4).

We further verified the B-specificity of the three novel B-derived miRNAs in four other inbred backgrounds (W22, W23, A619, and L289; Fig. 3). The results revealed that the miRmB1 and miRmB2 primers amplified B-specific PCR products from all four inbred lines with Bs, with the exception that the miRmB2 primers amplified PCR products from L289 without Bs. The miRmB3 primers amplified B-specific PCR products from W22 and W23 but not from A619 and L289. The results suggested that these novel miRNAs are derived from the maize B but are also expressed from the A chromosomes of particular inbred lines.

Mapping of novel B-derived miRNA genes on the B

A systematic B deletion derived from four B-10L translocations (TB-10L18, TB-10L7, TB-10L26, and TB-10L36) in the W23 background was applied to map the positions of novel B-derived miRNA genes on the B. As shown in Fig. 5, the breakpoints of the four B-10L translocations are as follows: TB-10L18 is located within the short arm, TB-10L7 is located in the central region of the proximal euchromatin (PE), TB-10L26 is located near the junction of distal heterochromatin (DH) 2 and 3, and TB-10L36 is located between DH3 and DH4. The rationale of this mapping was as follows: when a B-specific RT-PCR product was amplified from the tertiary trisomic RNA of a B-10L translocation, its gene was mapped to the B portion proximal to the breakpoint. In contrast, when a B-specific RT-PCR product was amplified from the hypoploid RNA of a B-10L translocation, its gene was mapped to the B section distal to the breakpoint.

Stem-loop RT-PCR primers of the three B-derived miRNAs were used to analyze the total RNA of B-10L translocations. As shown in Fig. 6, the B-specific PCR product of miRmB3 was present in the tertiary trisomic RNA of TB-10L18 and TB-10L7 but was absent in hypoploid RNA of both TB-10L18 and TB-10L7, suggesting that the position of miRmB3 gene was between the breakpoints of the two translocations, which contains the CK and the proximal half of PE (PPE). The map position of the miRmB1 gene could be assigned to DH3 because it could be

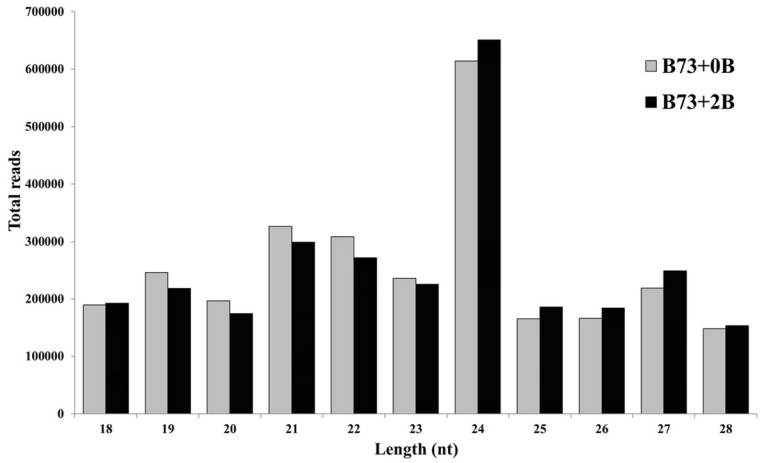

Fig. 1 Length distribution and abundance of sRNAs in B73+0B and B73+2B. The sRNA libraries were constructed from the third leaf of B73+0B and B73+2B plants at the four-leaf stage. The most abundant sRNAs were 24 nt in length, followed by those that were 21 nt and 22 nt in length

amplified from the tertiary trisomic RNA of TB-10L36 and the hypoploid RNA of TB-10L26 but not from the tertiary trisomic RNA of TB-10L26 and the hypoploid RNA of TB-10L36. The B-specific PCR product of miRmB2 was present in the hypoploid RNA of TB-10L36 but was absent in the tertiary trisomic RNA of TB-10L36, suggesting that miRmB2 gene located in the region containing both DH4 and distal euchromatin (DE). The map positions of the three novel B-derived miRNA genes in relation to the B breakpoints of the four B-10L translocations are summarized in Fig. 5.

Discussion

The presence of the B affects the expression of A-located genes via miRNAs

Although the maize B is not essential for plant growth and development, its presence affects A chromosomes in some way (Rhoades 1968; Rhoades and Dempsey 1972; Randolph 1941; Staub 1987). Current evidence has further revealed that the maize B is transcriptionally active and can affect the expression of A-located genes at the transcription level (Lin et al. 2014; Huang et al. 2016).

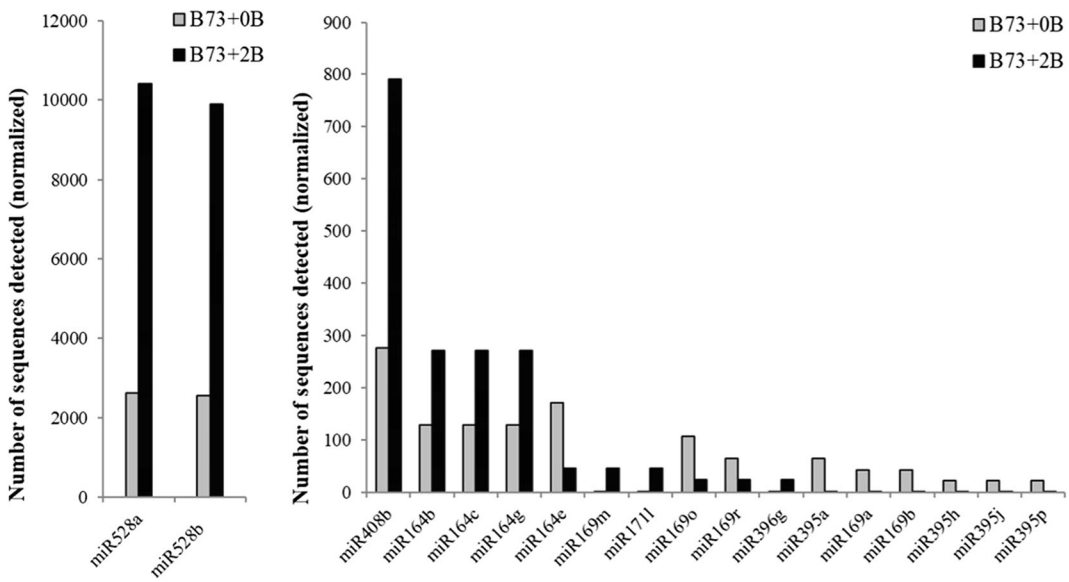

Fig. 2 Differential expression of 18 known miRNAs in B73+0B and B73+2B sRNA libraries

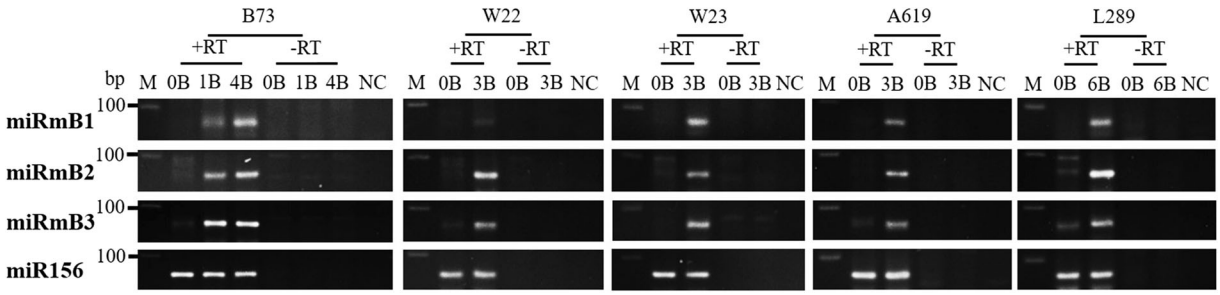

Fig. 3 Stem-loop RT-PCR of B-derived miRNAs in different maize inbred lines. Stem-loop RT-PCR primers of three B-derived miRNAs, miRmB1, miRmB2, and miRmB3 were used to analyze the total RNA from the leaves of five maize inbred lines, B73, W22, W23, A679, and L289, with and without Bs. miR156 was used to confirm equal amounts of RNA. M 100 bp marker, NC water as a negative control

How the B regulates the expression of A-located genes is unclear; however, it is known that sRNAs play important roles in gene regulation in plants (Jones-Rhoades et al. 2006). In this study, we identified 18 known miRNAs that were significantly differentially expressed in the presence of the B (Fig. 2). These known miRNAs were members of seven miRNA families, miR164, miR169, miR171, miR395, miR396, miR408 and miR528, and their target genes have previously been predicted in maize (Zhang et al. 2009). The members of four of these miRNA families, miR164, miR169, miR171 and miR396, target various families of transcription factors, such as no apical meristem (NAM) proteins, CCAAT-binding factors (CBFs), GRAS transcription factors, and zinc finger proteins, respectively. miR395 regulates sulfate metabolism by targeting sulfate transporter genes and ATP sulfurylase proteins. Both miR408 and miR528 target the copper-containing proteins cupredoxin and multicopper oxidase and lassase genes and thus might play a critical role in regulating physiological processes and stress responses. These results suggest that the maize B might affect the expression of A-located genes by miRNA-directed regulation in posttranscriptional regulation and transcription networks.

The maize B is capable of expressing miRNAs

Because nine known upregulated miRNAs were identified in the presence of the B, we were interested in whether any upregulated miRNAs were also expressed from the maize B. However, it is unable to discriminate between miRNA genes that are located on both the B and A chromosomes. To verify whether the B is capable of expressing miRNAs, we used MiRDeep2 to identify putative novel miRNAs in B73+2B unique nonreference reads and obtained three novel miRNAs that exhibited amplification from B73+Bs but not from B73+0B by stem-loop

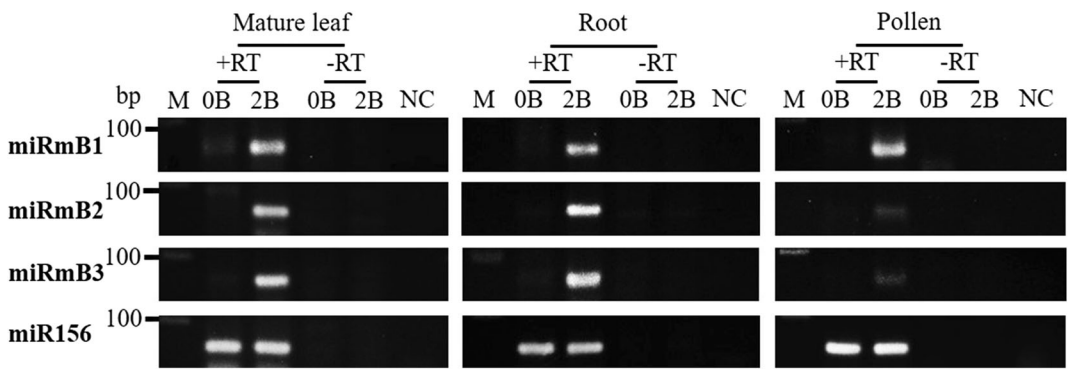

Fig. 4 Stem-loop RT-PCR of B-derived miRNAs in different tissue types. Stem-loop RT-PCR primers of three B-derived miRNAs, miRmB1, miRmB2, and miRmB3, were used to analyze the total RNA from the mature leaves, roots, and pollen of B73+0B and B73+2B. miR156 was used to confirm equal amounts of RNA. M 100 bp marker, NC water as a negative control

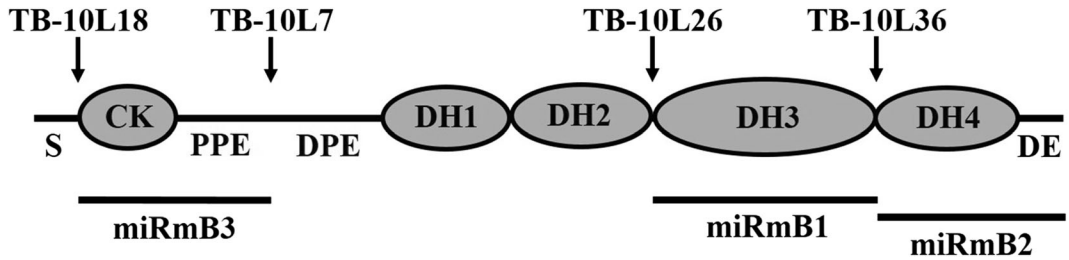

Fig. 5 Map positions of B-derived miRNA genes (black lines) in relation to the breakpoints (arrows) of four B-10L translocations. S short arm, CK centromeric knob, PPE proximal half of the proximal euchromatin, DPE distal half of the proximal euchromatin, DH1–DH4 distal heterochromatin 1–4, DE distal euchromatin

RT-PCR analysis (Table 1; Fig. 3). Moreover, the stem-loop RT-PCR primers of the three novel miRNAs amplified B-specific PCR products from at least three inbred lines carrying Bs (Fig. 3), and from the leaves, roots and pollen grains of B73+2B (Fig. 4). Via B-10L translocations, the three novel B-derived miRNA genes were mapped to specific B regions (Figs. 5 and 6). The results provide strong evidence that the B can express miRNAs. Those B-derived novel miRNAs might target B-located genes to direct the B's behaviors during cell division, such as nondisjunction during the second pollen mitosis and prevention of a univalent B lost during meiosis.

In this study, we confirmed that the maize B is capable of affecting the expression of A-derived miRNAs. A total of 18 known miRNAs were differentially expressed in the presence of the B. We also identified three novel B-derived miRNAs and

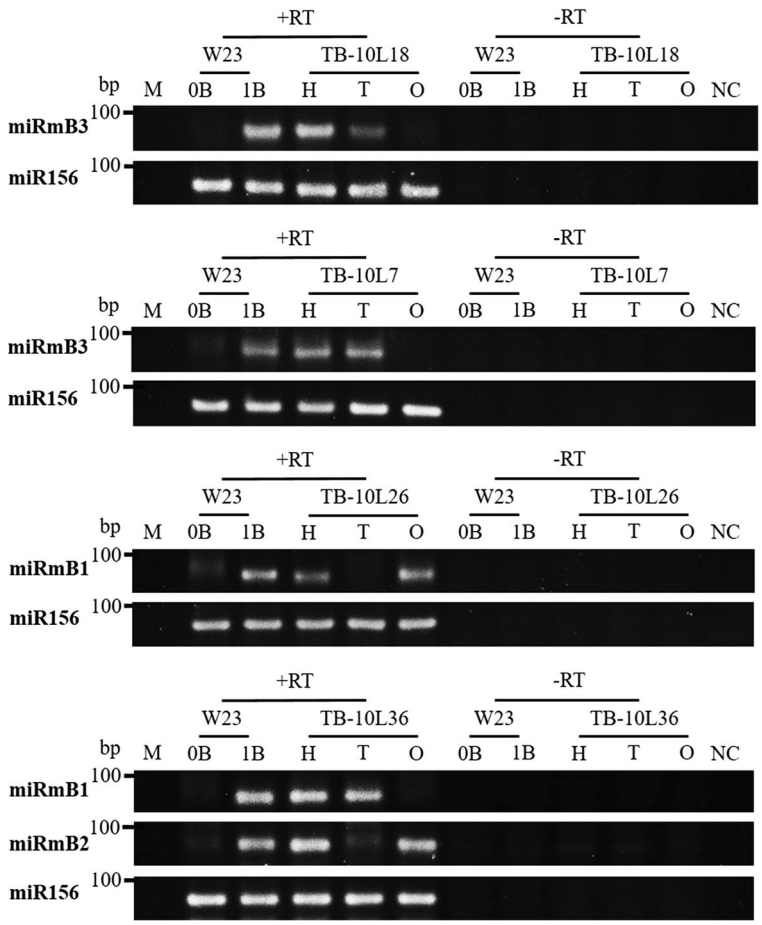

Fig. 6 Mapping of B-derived miRNA genes by B-10L translocations. Stem-loop RT-PCR primers of three B-derived miRNAs, miRmB1, miRmB2, and miRmB3, were used to analyze the total RNA from W23+0B, W23+1B, and four B-10L translocations (TB-10L18, TB-10L7, TB-10L26, and TB-10L36). miR156 was used to confirm equal amounts of RNA. H translocational heterozygote, T tertiary trisome, O hypoploid, M 100 bp marker, NC water as a negative control

mapped their genes on specific B regions via B-10L translocations. Our results may improve the understanding of the molecular mechanisms that control the genetic behaviors and activities of the B in maize.

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