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Exploring the evolutionary differences of SBP-box genes targeted by *miR156* and *miR529* in plants

Li-Zhen Ling · Shu-Dong Zhang

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Abstract The combinatorial control of one target by multiple miRNAs brings big challenges to elucidate its precise evolutionary mechanism. Squamosa promoter binding protein-like (SBP) gene family exhibits the different regulatory patterns, in which some members are only regulated by *miR156* and others by *miR156* and *miR529*. Here, we explored the different evolutionary patterns and rates between *miR156* targets and *miR529* ones in three species (moss, rice, and maize). Our work found that the *miR529* targets were members of *miR156* target dataset, indicative of cooperative control. Further phylogenetic analyses as well as gene structure features demonstrated that *miR529* targets derived from a monophyletic branch of *miR156* targets which evolved into two independent branches duo to the ancient gene duplication. Moreover, inspection of evolutionary rate parameters (d_N/d_S, d_N and d_S) for *miR156* targets and *miR529* ones revealed they were under different selection strength. *MiR529* targets were more constraint by strong purifying selection and evolved conservatively with a slow rate. By contrast, *miR156* targets evolved more rapidly and experienced more relaxed purifying selection, which may contribute to their functional diversification. Our results will enhance the understanding of different evolutionary fates of SBP-box genes regulated by the different numbers of miRNA families before functional studies.

Keywords miRNA · SBP-box genes · Evolutionary rate · Combinatorial control

Introduction

MicroRNAs (miRNAs) are endogenous and small non-coding RNAs that regulate the expression of protein-coding genes at the post-transcriptional level. In animals, one target transcript is typically bound by more than one miRNA with limited complementarity (Chen et al. 2006; Rajewsky 2006). Plant miRNAs, by contrast, are thought to largely regulate transcripts by single, highly complementary target sites (Voinnet 2009). Consequently, plant miRNAs are predicted to have only a limited number of messenger RNA (mRNA) targets. Recent high-throughput methods, such as Parallel Analysis of RNA Ends (PARE) or degradome profiling followed by computational analysis, have defined tens of thousands of miRNA binding sites in plants (German et al. 2008; Addo-Quaye et al. 2008). Moreover, these studies have yielded invaluable information that the combinatorial control of the same mRNA target by multiple miRNA families is also pervasive in plants (Zheng et al. 2011). In particular, the members of the transcription factors (TF) family, such as *MYB*, *APETALA2* (*AP2*) and MADS-box gene families, can be targeted by

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L.-Z. Ling · S.-D. Zhang (✉)
Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China
e-mail: sdchang@mail.kib.ac.cn

L.-Z. Ling · S.-D. Zhang
Plant Germplasm and Genomics Center, Germplasm Bank of Wild Species, Kunming 650201, China

L.-Z. Ling
Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

distinct miRNA families. Why some members are regulated by multiple miRNA families and others are targeted by only a single miRNA family is largely unknown in plants. Understanding the evolutionary patterns of these genes will help to frame the question.

Squamosa promoter binding protein-like (SBP) genes encode transcription factors that share a common DNA-binding domain (the SBP-box) and recognize similar target DNA sequences. In plants, most members of this family are targeted by *miR156* family (Rhoades et al. 2002; Xing et al. 2010; Xie et al. 2006). Their interplay provides the paradigms for how these SBP-box genes exert their functions in development. For example, the low-level expression of SBP-box genes in *miR156*-overexpress mutant prolonged the juvenile phase in both maize (Chuck et al. 2007) and *Arabidopsis* (Wu and Poethig 2006). Furthermore, this regulatory relationship is conserved among all land plants (Guo et al. 2008). Recently, we revealed that the evolution patterns of *miR156*-targeted SBP-box genes were significantly different from those of non-targeted ones in plants (Ling and Zhang 2012). In previous work, a similar miRNA gene, *miR529*, was described that shares 14-16 nt of homology with *miR156* and targets the same SBP-box gene in plants (Cuperus et al. 2011). The best known example is the *tasselsheath4* (*TSH4*) gene, which is involved in the bract development and the establishment of meristem boundaries in maize (Chuck et al. 2010). Unlike *miR156*, *miR529* displays more restricted taxonomic distributions. It was present in the common ancestor of embryophytes but were lost in the common ancestor of eudicots (Cuperus et al. 2011). These data suggested that the regulatory relationship between *miR529* and its targets seems to rewire during plant evolution. Nevertheless, the SBP-box genes targeted by *miR156* and *miR529* might provide us useful information for tracing the ancient evolutionary pattern. Do the SBP-box genes targeted by the single *miR156* family and those by *miR156* and *miR529* families evolve in the same manner and at the same rate? Until now, such question has yet to be tackled seriously.

In present study, we focused on 27 SBP-box genes targeted by *miR156* and/or *miR529* families from three distinct species (moss, rice, and maize). 17 out of 27 genes were targeted only by *miR156* family (termed *miR156* targets) and the remaining 10 ones were targeted by both *miR529* and *miR156* families (termed *miR529* targets). We first analyzed the evolutionary relationships of two classes of targeted SBP-box genes through the phylogeny and gene structure information. Next, the evolutionary rate parameters (d_N/d_S, d_N, d_S) were calculated and compared. Finally, we also discussed the association between the different evolutionary rates of *miR156* targets and *miR529* ones and their expression patterns.

Methods

Data collection

Considering that the appearance of SBP-box genes span a wider evolutionary distance than that of *miR156* or *miR529* family in plants, we first used all the species in which SBP-box genes were identified as the species index. A total of 15 plant species from unicellular algae to flowering plants are available on PlnTFDB v3.0 (Riano-Pachon et al. 2007). And then, the copy number of SBP-box gene family, *miR156* and *miR529* family was respectively counted in each plant species (see Table S1). As a result, we found only four plant species (including *Physcomitrella patens*, *Oryza sativa* subsp. *japonica*, *Sorghum bicolor*, *Zea mays*) contained these three gene families that the copy number was more than zero (Table S1). However, we noticed that the *miR529* from *S. bicolor* was obtained by similarity search and not assessed with sufficient stringent criteria prior to the addition to the miRBase database. To eliminate the potential inaccuracy, *miR529* from sorghum was excluded from the following analyses. Therefore, only three out of fifteen plant species fit the criteria and were used in this study. Detailed information about all SBP-box genes in these three plant species can be found in our previous study (Ling and Zhang 2012).

The sequences of protein, coding sequence (CDS), mRNA and SBP-box genes were downloaded from genome annotation databases. The moss gene set is available through Phyzogome v7.0 (http://www.phytozome.net/physcomitrella). The rice and maize genes of SBP-box family were downloaded from The Institute info Genomic Research (TIGR) rice genome annotation database (release 7.0, http://rice.plantbiology.msu.edu/) and maize sequence genome database (release 5b.60, http://www.maizesequence.org/index.html), respectively. The sequences of *miR156* and *miR529* of these three plant species were obtained from miRBase database (Release 18.0) (Kozomara and Griffiths-Jones 2011).

Prediction of *miR156* and *miR529* binding sites on SBP-box genes

Binding sites of *miR156* and *miR529* on SBP-box gene transcripts of moss, rice and maize were identified by using psRNATarget server (http://bioinfo3.noble.org.psRNATarget/) with default settings (Dai and Zhao 2011). PsRNATarget is a popular and convenient tool for plant miRNA target analysis; and users may submit their miRNAs against their transcripts. To further increase the stringency of prediction, we used empirical parameters as a second filter (Schwab et al. 2005). The empirical parameters in this study were used as previously described (Ling and Zhang 2012).

Table 1 The detail information of predicted targets for *miR156* and *miR529* in three species

Species	Target	Position	Length[a]	Target sequence[b] (5′-3′)
Moss	Pp1s50_125V6*	CDS	716	(GGUC)**GUGCUCUCUCUCUUCU**(GUCA)
	Pp1s194_53V6*	CDS	760	(GGCC)**GUGCUCUCUCUCUUCU**(GUCA)
	Pp1s194_57V6*	CDS	700	(GGCC)**GUGCUCUCUCUCUUCU**(GUCA)
Rice	LOC_Os01g69830*	CDS	413	(GAUU)**GUGCUCUCUCUCUUCU**(GUCA)
	LOC_Os08g39890*	CDS	418	(GAUU)**GUGCUCUCUCUCUUCU**(GUCA)
	LOC_Os09g32944*	CDS	473	(AGCU)**GUGCUCUCUCUCUUCU**(GUCA)
	LOC_Os09g31438*	CDS	401	(AGCU)**GUGCUCUCUCUCUUCU**(GUCA)
	LOC_Os02g04680	CDS	470	AUGCUCUCUCUCUUCUGUCA
	LOC_Os02g07780	UTR	252	GUGCUCUCUCUCUUCUGUCA
	LOC_Os04g46580	CDS	361	GUGCUCUCUCUCUUCUGUCA
	LOC_Os06g45310	CDS	344	GUGCUCUCUCUCUUCUGUCA
	LOC_Os06g49010	CDS	476	GUGCUCUCUCUCUUCUGUCA
	LOC_Os07g32170	UTR	217	AUGCUCCCUCUCUUCUGUCA
	LOC_Os08g41940	CDS	456	GUGCUCUCUCUCUUCUGUCA
	LOC_Os11g30370	CDS	353	GUGCUCUCUCUCUUCUGUCA
Maize	GRMZM2G126018*	CDS	384	(AGCU)**GUGCUCUCUCUCUUCU**(GUCA)
	GRMZM2G307588*	CDS	379	(AGCU)**GUGCUCUCUCUCUUCU**(GUCA)
	GRMZM2G460544*	CDS	409	(AGCU)**GUGCUCUCUCUCUUCU**(GUCA)
	GRMZM2G061734	CDS	430	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G065451	CDS	483	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G097275	CDS	480	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G101511	CDS	465	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G106798	CDS	218	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G148467	CDS	451	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G163813	CDS	332	GUGCUCUCUCUCUUCUGUCA
	GRMZM2G414805	CDS	440	GUGCUCUCUCUCUUCUGUCA
	GRMZM5G878561	CDS	414	GUGCUCUCUCUCUUCUGUCA

The genes with asterisk indicate the *miR529* targets

[a] The length indicates the protein length of each gene

[b] The sequence is mixture of targets under combinatorial control by *miR156* and *miR529*, the first four nucleotide acids in the bracket indicate the target sequence for *miR529* and the last four in the bracket indicate the target sequence for *miR156*, and the bold segment indicates the overlapped target sequence for *miR156* and *miR529*

Finally, our analyses led to the prediction of 27 and 10 SBP-box genes as the putative targets for *miR156* and *miR529* family, respectively (Table 1).

Alignment, phylogenetic analyses, and rate evaluation

The amino acid sequences were first aligned using ClustalX (Thompson et al. 1997) and refined manually. The leading and trailing edges of each amino acid sequence were trimmed to generate consensus edges. Next, the alignment result was used to guide the alignment of their corresponding nucleotide sequences.

Phylogenetic analyses of the SBP-box genes based on the nucleotide consensus sequences were carried out using neighbor-joining (NJ) and maximum-likelihood (ML) methods in MEGA version 5.05 (Fig. S1) (Tamura et al. 2011). NJ analysis was done with the following parameters: Kimura 2-parameter model, pairwise deletion, and bootstrap (1,000 replicated; random seed). For ML analysis, the Jukes-Cantor model was used and the bootstrap consensus tree was inferred from 1,000 replicates. In addition, the outgroup sequence (CRR1) was chosen from green algae homologs according to previous phylogenetic analysis (Ling and Zhang 2012; Guo et al. 2008). The sequence alignment and ML tree were used to calculate the ratio of nonsynonymous (d_N) to synonymous (d_S) substitution rates (d_N/d_S) along each group/subgroup branch through the online tool (http://services.cbu.uib.no/tools/kaks). The ratio of d_N/d_S provides a sensitive test of natural selection. A statistically significant d_N/d_S ratio lower than,

equal to, or greater than 1.0 can indicate purifying selection, neutral evolution and positive selection, respectively.

Exon/intron structure analysis

The CDS and genomic sequences of SBP-box genes were used to derive exon/intron structure with Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/).

Results and discussion

The predicted targets for *miR156* and *miR529* within SBP-box genes

High-confidence prediction of miRNA targets was performed by psRNATarget based on sequence complementarity and evolution conservation (Dai and Zhao 2011). To further increase the stringency of predicted targets, we used empirical parameters as a second filter (Schwab et al. 2005). These algorithms are designed to reflect molecular target recognition mechanisms that are assumed to apply to miRNA target recognition. By applying these rules, our analysis led to the prediction of 27 and 10 SBP-box genes as the putative targets for *miR156* and *miR529* family, respectively (Table 1). To estimate the accuracy of putative targets, we compared the prediction results with those reported in the literatures. For example, all the putative targets for *miR156* and *miR529* in rice have been experimentally validated by several independent laboratories (Li et al. 2010; Xie et al. 2006). These results suggested that psRNATarget, combined with the empirical parameters, is an effective method for finding miRNA targets in plants.

Table 1 summarized 27 targets for *miR156* with 3 from moss, 12 from rice and maize, respectively. Similarly, 10 *miR529* targets were predicted in these three species, including 3 from moss and maize as well as 4 from rice. These 10 *miR529* targets were also predicted as the *miR156* targets, indicative of cooperative control. For the sake of simplicity, 10 targets by cooperative control were referred to as *miR529* targets, while the remaining seventeen targets as *miR156* targets. Among these species, the *miR529* targets from moss were the same as those of *miR156*. However, the number of *miR529* targets from rice and maize was far lower than that of *miR156* ones (Table 1). The number comparison of *miR529* targets and *miR156* ones has been considered as an equivalent of comparison of correspondence between miRNA families to describe co-evolution of interacting partners during monocot evolution. Studies on SBP-box gene family have revealed that duplication events have contributed to increasing their numbers in monocots (Guo et al. 2008; Ling and Zhang 2012; Xie et al. 2006). Moreover, various studies have demonstrated that *miR156* family tends to expand by the same processes of tandem, segmental and whole genome duplication (Wang et al. 2007; Maher et al. 2006). However, at the same processes, the number of *miR529* family tends to be smaller. For example, in rice, *miR156* family has 12 members and only 2 members of *miR529* family were detected (Table S1). These digits suggested that *miR529* copies have undergone different fates during gene duplication, which affects the existence and extent of miRNA-target interactions. Conceivably, the members of *miR529* family might originate through duplication. Next, some of the originating loci were lost or rapidly diverged due to the accumulation of mutations within mature sequences. Thus, the affinity of the de novo-generated *miR529* for a target will be altered and affect their regulatory relationship. On the other hand, the evolvability of the targets themselves seems to be different after duplication events. For example, the binding sites of *miR156* resided in the coding regions as well as untranslated regions. By contrast, the *miR529* binding sites were only located in coding regions and overlapped with those of *miR156* on these genes (Table 1). These data provide the evidence for the more evolutionary diversification of *miR156* targets as compared to *miR529* ones. Here, we are interested in the evolutionary differences between *miR156* targets and *miR529* ones, which will be further studied in the following sections.

Evolutionary relationship of the targets for *miR156* and *miR529*

To explore the evolutionary relationship of *miR156* targets and *miR529* ones, phylogenetic analyses of 27 SBP-box genes were estimated using neighbor-joining (NJ) and maximum-likelihood (ML) approaches, which resulted in generally similar topologies. In both cases, the *miR156* targets evolved into two big branches (group A and group B) through an early duplication event (Fig. 1a). These two ancestral branches then gave rise to additional homologs through several rounds of duplication and formed various distinct subgroups. Such topology provides a more powerful explanation that gene duplications were a main resource to diversify these *miR156* targets. Group A was further classified into three subgroups (subgroup A-1, A-2 and A-3). For *miR529* targets, most of them were located in subgroup A-1 and the remaining two were grouped together with some *miR156* targets, which formed another clade (subgroup A-2). Sister to these two clades was three *miR156* targets (subgroup A-3), which formed reciprocally a monophyletic branch—group A. Besides, our data indicated that *miR529* targets were not distributed in the branch of group B. Such clustering of *miR529* targets revealed that they evolved from a single origin. However, *miR529*

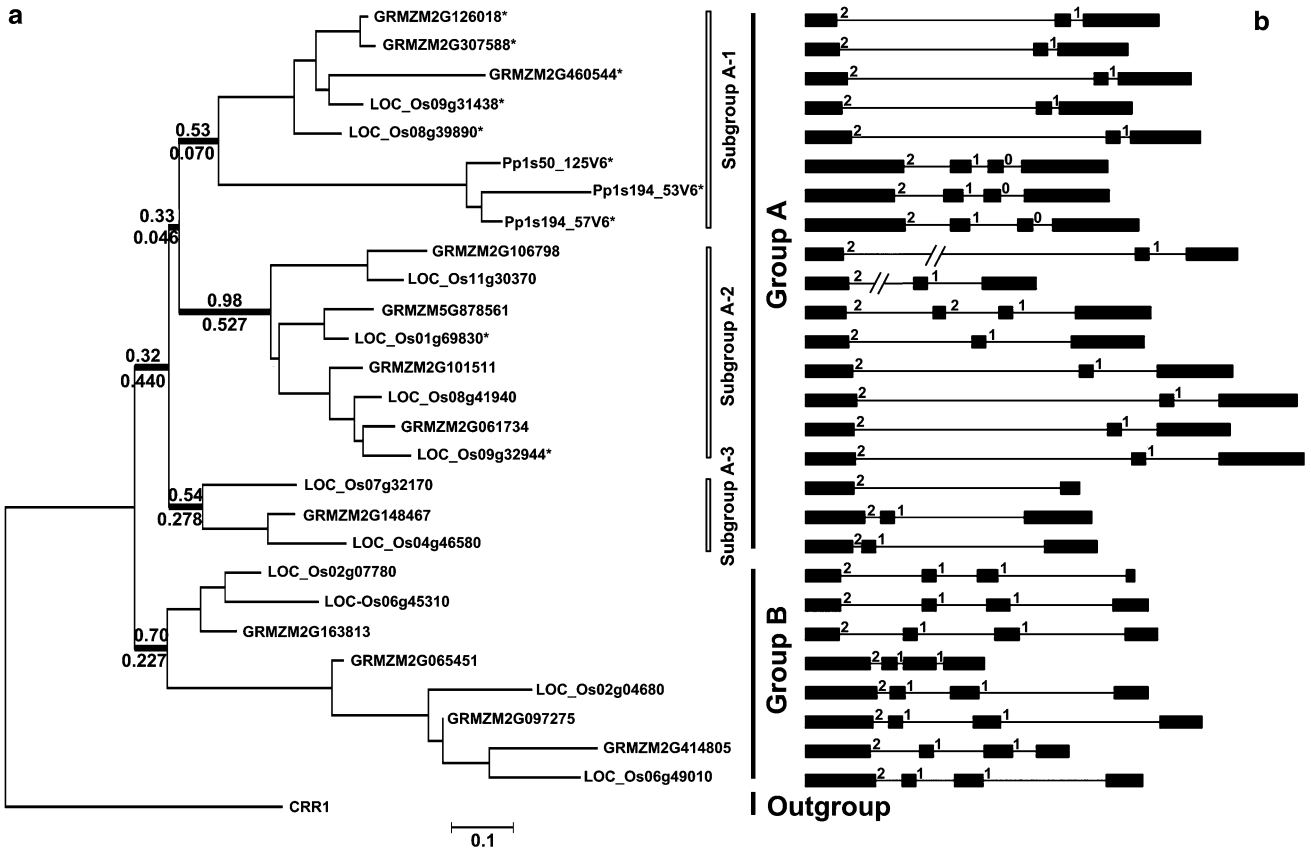

Fig. 1 Phylogenetic analysis based on the *miR156* and *miR529* targets and their respective gene structure in moss, rice, and maize. **a** The ML tree of *miR156* and *miR529* targets and estimates of d_N/d_S for key branches (in *bold*) alongside the relevant branches. **b** Exon and intron structure. *Green boxes* the exon regions, *line* introns. *Number 0, 1, and 2* intron phases. The length of the *boxes* and *lines* are scaled based on the length of genes except for LOC_Os11g30370 and GRMZM2G106798 with long introns denoted by "//". The percentage of support for each clade is given. *Top numbers* indicate ML bootstrap values; *numbers below* indicated the d_N/d_S values. Branches are drawn in proportion to their lengths, defined as the expected numbers of nucleotide substitutions per site. The genes marked by the *asterisk* in the phylogenetic tree were cooperatively regulated by *miR529* and *miR156*

targets and partial *miR156* targets embedded within the same monophyletic branch, such as, the *miR529* targets from subgroup A-1 and those from subgroup A-2. This arrangement suggests that *miR529* binding sites could be lost due to the accumulation of mutations during evolution. In addition, our result demonstrated that the *miR529* targets from moss were clustered together with those from monocots in group A-1. Thus, there might be extensive conservation of *miR529* targets in this group.

However, the bootstrap values are not good to give a strong support in this phylogenetic tree. Therefore, we sought other evidence to support the reliability of the subgroup/group designations. The exon–intron structures of 27 SBP-box genes by subgroups/groups were investigated. It is evident from the Fig. 1b that there is a relation between branches and gene structures. In group A, all the SBP-box genes from monocots except LOC_Os07g32170 and GRMZM5G878561 share a conserved splicing pattern of three exons and two introns. Nevertheless, the lack of gene structure conservation of LOC_Os07g32170 and GRMZM5G878561 within group A broadly supports suggestions that exon shuffling might rise and thus the recombinatorial fusion and exchange of protein domains could rapidly occur (Patthy 1999; Roy 2003). The exon–intron structure is also surprisingly similar in the branch of group B, which is comprised of four core exons and significantly different from that of group A. These results support the subgroup designations of *miR156* and *miR529* targets through the independent criterion of splicing pattern. A similar approach to gene classification using exon–intron structure has been applied in the kinesin family, *MYB* family and the bHLH family (Lawrence et al. 2002; Toledo-Ortiz et al. 2003; Jiang et al. 2004). In addition, we also examined the position of splicing with respect to the open reading frame (ORF)—the intron phase. Fig. 1b shows the conserved phase of introns within the same subgroup. From the above analyses, it can be seen that *miR156* targets were diversified during monocot evolution. However, the gene structure of *miR529* targets showed the same exon numbers and intron phase, which seems be more

conserved than that of *miR156* targets. It is worth noting that the targets for *miR156* and *miR529* in moss, an early branching species, show the different exon–intron structures. Although the sequence analyses can be assigned them to subgroup A-1, they exhibits different evolutionary patterns with the exceptional exon numbers and intron phase. Thus, it is remarkable that the targets of *miR156* and *miR529* in moss originated from a common ancestor with those of monocots but diverged after split of moss and monocots. Hence, we separately analyzed the targets for *miR156* and *miR529* from moss and monocots in subsequent sections. In summary, *miR529* targets derived from a monophyletic branch of *miR156* ones which developed into two big branches through an ancient duplication event. Meanwhile, the loss of the *miR529* binding sites continually occurred, which resulted in the decreased number of the extant *miR529* targets during evolution of monocots.

Elevated constraint on *miR529* targets

According to the above analyses, we know *miR529* targets are under combinatorial control and have more conserved gene structure. The next question to ask is whether these genes are slower in evolutionary rate and under more selection pressures than *miR156* targets. To elucidate the evolutionary fates of these duplicate homologs, we first calculated the d_N/d_S ratio using the tree topologies suggested by ML analysis of the consensus sequences. Our result indicated that d_N/d_S values were much lower than 1.0 (suggesting purifying selection) for all the branches (Fig. 1a). d_N/d_S was estimated as 0.440 for group A and 0.227 for group B (Fig. 1a). Though our dataset spans a large evolutionary distance, the d_N/d_S estimates seem unlikely to be affected by sequence saturation; d_S, the synonymous substitution rate, was less than 2.0 for all branches within the ingroup. The d_N/d_S estimate along each subgroup branches ranged from 0.070 to 0.527 (Fig. 1a). These results suggested the different purifying selection pressures acted on all *miR156* targets from different subgroups/groups, which may represent co-evolution of the interacting these targets in association with the diversification and evolution of monocots. In addition, the subgroup A-1 has the lowest d_N/d_S estimate among key subgroup branches (Fig. 1a). This result suggested that the *miR529* targets of this branch are subjected to the strong selection pressures and have evolved slowly since its origin via gene duplication. Under this scenario, their protein sequences should be more conserved due to the low protein evolutionary rates. With this in mind, we examined the full alignment of their protein sequences. Fig. S2 shows the alignments for members of *miR529* targets in subgroup A-1 from monocots and moss, respectively. It is obvious that the sequence similarity in monocots is high, though the evolutionary conserved sites are non-contiguous (Fig. S2a). A similar observation is also presented in three genes of moss (Fig. S2b). Compared to subgroup A-1, the alignments of sequences from other subgroups and group B were highly variable and few consensus sequences exist when SBP-box domains were masked (Fig. S2c, d, and e)), though these genes also show a high similarity in gene structures (Fig. 1b). On the contrary, the subgroup A-2 exhibits the highest d_N/d_S value, which suggested that the relaxed selection pressures acted on these genes (Fig. 1a). This selection force allows for the more mutations occurred at the sequence level and thus these mutations might be responsible for the loss of *miR529* target sites.

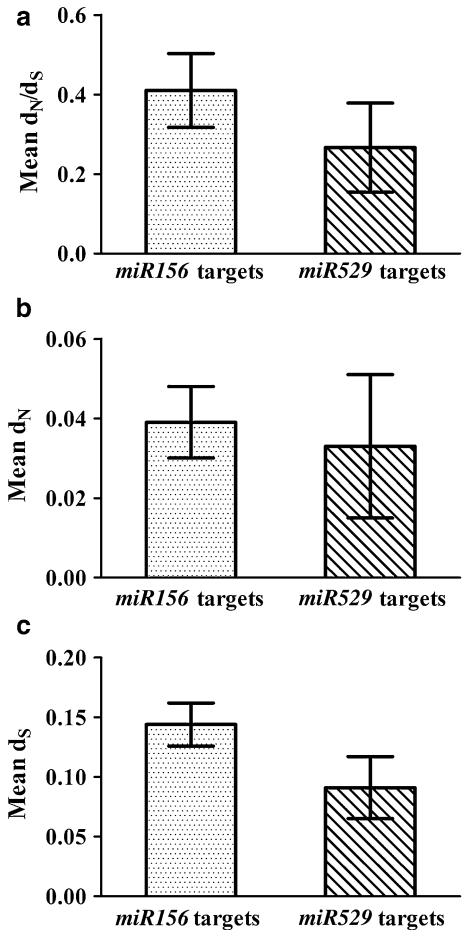

Fig. 2 Comparison of the mean d_N/d_S ratios (**a**), the mean d_N value (**b**) and the mean d_S value (**c**) for *miR156* targets and *miR529* ones in monocots. *Error bars* indicate the standard error of the mean

To further seek the evidence that *miR529* targets were under more selection constraints than *miR156* ones, we incorporated the full datasets for all *miR156* and *miR529* targets and estimated and compared their mean d_N/d_S ratios. The result showed that a strong difference between mean d_N/d_S ratios for these two groups. The *miR156* targets have elevated d_N/d_S ratio, whereas the *miR529* ones have lower d_N/d_S ratio (Fig. 2a), which is consistent with the

results of branch analysis. Moreover, for each collective dataset, the estimates of the nonsynonymous (d_N) and synonymous (d_S) substitution rates were obtained, respectively (Fig. 2b, c). Within the monocots the overall mean d_N and d_S values for *miR529* targets were 0.033 and 0.091, respectively (Fig. 2b, c). In contrast, *miR156* targets have higher mean d_N and d_S values as compared to *miR529* ones (Fig. 2b, c). When considering the *miR156* and *miR529* targets of moss, a similar trend was also observed. These results indicated that the difference of evolutionary rates exhibited by d_N or d_S values of these two groups precisely matched those of their respective d_N/d_S values, which provided additional evidence that *miR156* targets and *miR529* ones were under different selection strength. With regards to the *miR156* targets, they showed a significantly higher rate of both synonymous and nonsynonymous substitutions, suggesting a relaxed or low purifying selection on these genes. This pressure might endow *miR156* targets with the functional diversification, which can be explained by two scenarios. The first one is the accommodation of the subtle changes (in particular the nonsynonymous) within critical functional domains of the protein. Secondly, gene expression pattern is perhaps changed while keeping the protein activity domains conserved through the course of evolution. Take rice as an example, the *miR156* targets showed a wider range of expression patterns than that of *miR529* ones (Table S2). In terms of the *miR529* targets, they are under combinatorial control by distinct miRNAs and correspondingly tend to have slower evolutionary rates at the protein level. The similar findings have also been reported in human and mouse (Cheng et al. 2009). In addition, the expression levels seem to be strongest predictor of evolutionary rates (Kong et al. 2004), However, in our study the evolution rate of *miR529* targets is independently of the intensity of their expression level (Table S2). Perhaps, the protein sequence conservation and multiple miRNA regulations shape the evolution of *miR529* targets. Therefore, these genes might be stringently regulated by miRNAs in specific spatially or temporally expression pattern due to the strong purifying selection. A recent study has validated such specific expression patterns of *miR156* and *miR529* and their common target (Os*SPL14*) in different tissues (Jeong et al. 2011). In seeding, Os*SPL14* is predominantly targeted by both *miR156* and *miR529*, whereas in panicle it is predominantly targeted by *miR529*. Thus, this study gave a good example that an agriculturally significant phenotype may be regulated by one gene under the control of the unique or multiple miRNA families in specific expression patterns. Besides, the other factors, such as protein structure, the length of protein or UTRs and so on can affect the heterogeneous evolutionary rates (Cheng et al. 2009; Bloom et al. 2006; Toth-Petroczy and Tawfik 2011). In the same way, we didn't observe the relationship between the evolutionary rate and protein length (Table 1). Whether protein structure and other factors impose the influence on different evolutionary rates of *miR156* and *miR529* targets requires further investigation.

Conclusions

These results can be regarded as the supporting evidence for the differences in evolutionary fate between the *miR156* targets and *miR529* ones. This novel study will help us to understand how the mechanisms of pattern formation evolve and provide a foundation for analyzing the diversification of the members in the same gene family regulated by the single or multiple miRNA families at the post-transcriptional level.

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