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Microsatellite repeats proximal to pre-miRNA are more conserved than the distal repeats among the salinity responsive miRNA genes of rice (*Oryza sativa*)

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ABSTRACT: *Micro RNAs are conserved non-coding small RNA molecules that play important role in gene regulation. In the previous study, we found mature as well as precursor-miRNAs are highly conserved when compared between two contrasting populace that differ in salinity stress response. We also found that there was variability in the primary miRNA genes that are mostly located between 250 to 500 bp from precursor miRNA. In the present study, we found that 250 bp on either side of precursor miRNA (proximal repeats) are conserved than the distal repeats among the two contrasting panels of rice that differ in salinity response. This conservation is logical from the view point of stable miRNA formation and their subsequent role in salt stress related developmental or adaptation program.*

Key words: *Genetic diversity, microRNAs, microsatellite repeat*, Oryza sativa, *SSR markers, Salinity*

INTRODUCTION

Rice (*Oryza sativa*) is the most important cereal in the world. It is important to broaden the genetic base of rice to enhance yield under adverse environments, which relies mainly on the extent of genetic variability present in the population. Molecular marker is an efficient candidate for evaluating these genetic variations and determining cultivar identities. Among the various markers, the simple sequence repeats (SSRs) are preferred over other DNA markers due to several advantages (Jeung *et al.* 2005). Estimation of the genetic diversity of a species needs the discovery and quantification of variable regions in the genome. (Chen *et al.* 2011). The genetic diversity in the miRNA genes is absorbing a significant interest. Although a huge number of SSR markers have been designed from the rice genome, yet markers from the non-coding regions such as miRNA genes are quite scanty. Quite a few studies have reported the diversity in these non-coding genetic markers in plants (Fu *et al.* 2013; Yadav *et al.* 2014). We have previously reported that while mature miRNA as well as premature miRNA are highly conserved, genetic variability as revealed by SSRs was present only in the pri-miRNA genes (Ganie and Mondal, 2014; Mondal and Ganie, 2014). We divided the pri-miRNA zone of variability into two regions, i) proximal to pre-miRNA (hairpin structure) up to 250 bp on the either side of the pre-miRNA, ii) distal to pre-miRNA (hairpin structure) from 250 to 500 bp on the either side of the pre-miRNA. While the latter region was studied earlier (Mondal and Ganie, 2014), the former region is analysed in the present study. Additionally, we demonstrated that mature miRNA sequence can be used directly as SSR marker. Therefore, in the present study, we analysed the genetic diversity in a region of 250 bps of pri-miRNA sequences flanking on the either sides of pre-miRNA, among the 24 rice genotypes and found that this region is relatively conserved than the distal region i.e. beyond 250 bps from pre-miRNA sequence.

MATERIALS AND METHODS

Twenty four rice genotypes, comprising of one salt tolerant and one salt susceptible panel with 17 genotypes in each panel were used in this study. In order to compare and understand the genetic variability of pri-miRNA sequences proximal to pre-miRNAs, we used the same panels of genotypes as in our earlier study (Mondal and Ganie 2014). DNA extraction and mining of SSR markers using *in silico* approaches were done as per our earlier report

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(Mondal and Ganie, 2014), except the following two differences, i) the search of the SSRs was limited to a maximum of 250 bp of genomic DNA from the either end of the pre-miRNA, ii) mature miRNA sequence was used directly as one SSR primer in the pair (either as forward or as reverse primer). Only SSRs with repeat number >7 were chosen for the final analysis. The PCR protocol were standardized by changing the annealing temperatures and the concentrations of different individual PCR reagents as per our previous protocol (Mondal and Ganie, 2014). The PCR reaction was performed using a C1000 Touch™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). After amplification, 1 µl of loading dye consisting of {30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF} along with 10 µl of PCR product were loaded into each well of 3% agarose SFR™ (Amresco, India) gel in 1X TAE buffer and run at 130 volts for 2.5 h. The ethidium bromide stained gel containing the resolved bands was documented using a gel documentation system (Alpha Imager HP, USA). Only the clear, unambiguous and reproducible bands present across the samples from two repeated miR-SSR reactions were used for scoring. Presence or absence of a specific allele was indicated as 1 or 0 respectively and the matrix of 1 and 0 data underwent analysis by polymorphic information content (PIC) (Botstein *et al*. 1980). The PIC value for each marker was used to justify the polymorphic information, and the mean PIC value for a group of individuals implies the genetic diversity within the group. Table 1 provides the detail information about the data generated using the studied miRNA-SSR markers.

RESULTS AND DISCUSSION

Microsatellites or SSRs are highly competent and effective molecular markers that are used for a wide range of population, genetic and evolutionary studies (Garland *et al.* 1999; Tu *et al.* 2007). Due to their conserved nature, miRNAs are efficient candidates to study the genetic diversity in closely related genotypes (Ganie and Mondal 2014). The genetic diversity in miRNA genes is attracting the interest of researchers significantly. Baring few recent studies (Fu *et al.* 2013; Ganie and Mondal 2014; Mondal and Ganie 2014; Yadav *et al.* 2014), the genetic diversity in miRNA genes is quite unexplored.

In our previous studies (Mondal and Ganie 2014; Ganie and Mondal 2014), we showed that SSR markers (designed from 250-500 bps each on either sides of pre-miRNA, designated as distal repeats here) from the pri-miRNA gene sequences are very much variable among the rice genotypes having different genetic background. The variability as revealed by SSR markers was higher in susceptible rice panel than the tolerant panel. The present study mainly aimed at analysing the diversity in a region of 250 bps of pri-miRNA genes flanking immediately on either sides of pre-miRNA, among the rice genotypes using mature miRNA either as forward or reverse primer. Twenty eight SSRs were found on 3´-end (miRNA was used as forward primer) of pre-miRNA sequences (Table 1A) and 33 SSRs were found at 5´-end (miRNA was used as reverse primer) of pre-miRNA sequences (Table 1B). Interestingly, it was found that the SSR markers designed from this region of pri-miRNA genes registered a much less variability than the markers designed from the 250-500 bps on either sides from pre-miRNAs (Ganie and Mondal 2014) as indicated by PIC value (Table 2). Figure 1 shows the amplification of genomic DNA of 24 diverse

Table 1A
SSR markers developed from the pri-miRNA sequences on the 3′ end of the pre-miRNA

	miRNA as Forward primer	
miRNA	*Repeat motif*	*Chr. No.*
miR156g	$(CT)_9$	9
miR160c	$(TC)_7$	8
miR171a	$(AT)_{14}$	6
miR171i	$(TA)_{27}$	3
miR408	$(CT)_{12}$	1
miR156h	$(TC)_8$	8
miR1428f	$(AT)_{37}$	7
miR169n	$(CT)_9$	11
miR159f	$(CT)_{19}$	1
miR156a	$(CT)_6$	1
miR156f	$(AG)_9$, $(TC)_8$	8
miR156j	$(TC)_6$	6
miR159e	$(AG)_9$	1
miR164f	$(CCG_{)8}$	5
miR169i	$(AT)_6$	8
miR171b	$(CT)_{10}$, $(TA)_{26}$	8
miR172d	$(AT)_6$, $(AG)_6$	2
miR396c	$(TC)_{21}$	2
miR396g	$(CGC)_7$	6
miR439d	$(AT)_{22}$	3
miR1861k	$(AT)_{11}$	8
miR2100	(CT)6	11
miR2873a	$(AT)_{21}$, $(AT)_9$, $(AT)_{10}$, $(AT)_{10}$	11
miR5808	$(TCT)_6$	9
miR5836	$(TA)_{20}$, $(AT)_6$	6
miR1319b	$(TC)_5(TC)_4$	3
miR167d	$(TA)_5$	7
miR394	$(AG)_5$	2

Table 1B
SSR markers developed from the pri-miRNA sequences on the 5′ end of the pre-miRNA

	miRNA as Reverse primer	
miRNA	*Repeat motif*	*Chr. No.*
miR156g	$(CT)_{12}$	9
miR169n	$(AG)_{17}$	11
miR167a	$(TCT)_{8}$	12
miR167b	$(CCT)_{8}$	3
miR167d	$(TC)_{22}$, $(GT)_{10}$	7
miR169j	$(AGA)_{23}$	9
miR169o	$(GA)_{8}$	11
miR393b	$(GA)_{10}$	4
miR396f	$(TG)_{6}$, $(GA)_{7}$	2
miR399b	$(CT)_{16}$, $(TTCT)_{11}$	2
miR160c	$(TC)_{7}$, $(TC)_{7}$	8
miR160d	$(TG)_{6}$, $(TCT)_{8}$	3
miR164b	$(AT)_{5}$, $(AG)_{6}$	5
miR166a	$(AG)_{6}$	10
miR166b	$(CAAG)_{5}$	6
miR166d	$(CT)_{8}$	2
miR166g	$(TG)_{6}$	12
miR167h	$(TC)_{6}$	12
miR171a	$(AT)_{5}$	6
miR171d	$(TTC)_{7}$	10
miR172d	$(AT)_{6}$, $(AG)_{6}$	2
miR395a	$(CA)_{6}$	4
miR396e	$(TC)_{7}$, $(GGT)_{6}$	4
miR397b	(AC)7	2
miR818c	$(TA)_{6}$	2
miR1425	$(CCG)_{8}$	5
miR1435	$(AT)_{6}$	1
miR2907c	$(CT)_{8}$	11
miR5509	$(AT)_{8}$	11
miR5796	$(AT)_{7}$, $(AT)_{24}$	8
miR5826	$(CT)_{9}$	11
miR390	$(TTC)_{6}$	3
miR394	$(CA)_{6}$	2

genotypes with miR393b-SSR. A total of 28 alleles were scored with 10 amplified SSRs from 24 salt tolerant and sensitive genotypes, which is virtually 1/3rd of the number of alleles (88) obtained by 12 miRNA-SSRs designed from a region beyond 250 bps of pre-miRNA sequences (Mondal and Ganie 2014), hence making an average of <3 alleles/marker. This further confirms that the region of pri-miRNAs studied here are less polymorphic than the region studied in our previous report (Mondal and Ganie, 2014). While SSRs from pri-miR393b gene sequence showed the maximum number of 5 alleles, SSR from pri-miR169n sequence produced the lowest number (1) of alleles. The differences in amplicon size between the smallest and the largest allele for a given SSR locus varied from a minimum of 10 bp (miR169) to maximum of 85 bp (miR393b) which is again lesser than the difference observed in our previous study (Mondal and Ganie 2014) that was 14 and 98 bps respectively. The amplicon size ranged from 190 bp (SSR for miR396f) to 425 bp (SSRs for miR393b) which is inaccordance with the size of SSR amplification products reported previously (Temnykh et al. 2000; Kar *et al.* 2013). These results indicate that the regions of miRNA genes up to 250 bps flanking on the either side of pre-miRNAs are more conserved than the pri-miRNA sequences beyond 250 bps at least from the view point of variable number of microsatellite repeat units. The reduced variability and greater conservation of the proximal region of pri-miRNAs is much likely due to the fact that pre-miRNA sequences (which have the most conserved miRNA region) are relatively more conserved (Bartel *et al.* 2004). Since this pri-miRNA region of significant conservation is very close to the pre-miRNA (within

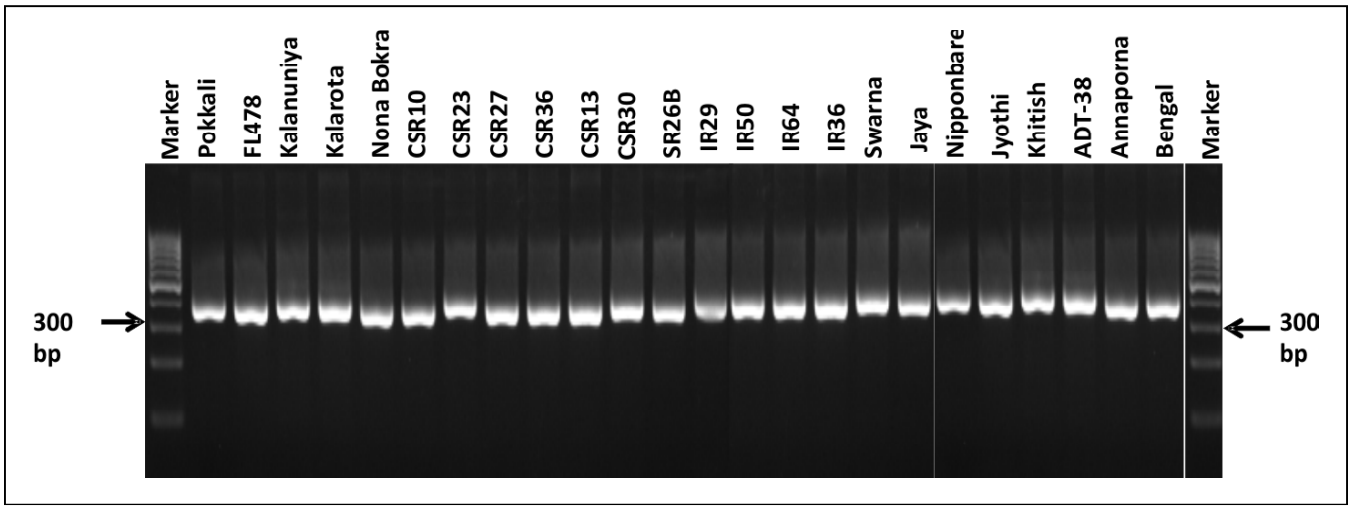

Figure 1: Amplification of genomic DNA from different rice genotypes with miR393b-SSR.

Table 2
Details of polymorphism of 24 rice genotypes as detected by 10 miR-SSRs

miRNA	*Forward primer*	*Reverse primer*	*miRNA as primer*	*No. of alleles*	*PIC value*	*Amplicon size range*	*Chr. No.*	T_m (^0C)
mi169j	AATCCAATTTCTCTTTAATTCTGCAACTG	CAGGCAAGTCATCCTTGGCTACC	Reverse	3	0.41	251-291	9	55
mi396f	ATATGGGTCCAATTACGTCTGTCCAC	CAGTTCAAGAAAGCCTGTGGAGAGC	Reverse	3	0.33	190-222	2	55
mi393b	CCTCTTCCAGCTAGCGTTGCTTG	AGATCAATGCGATCCCTTTGGA	Reverse	5	0.25	330-425	4	55
miR160d	CGAACGCCATTGTTCATCTTGTACTAC	TGGCATACAGGGAGCCAGGCA	Reverse	2	0.00	330-350	3	55
miR408	CAGGGATGAGGCAGAGCATGG	CCTTGAAGTGTCACATCACTCTAG	Forward	4	0.32	280-310	1	55
miR169n	TAGCCAAGAATGACTTGCCTA	GAGATCAGGATGAACATTACC	Forward	1	0.00	250	11	55
miR159f	CTTGGATTGAAGGGAGCTCTA	ATGCAACCTTCATTTGGGCAG	Forward	3	0.44	200-233	1	55
miR156g	GAGGAAGAAGACAATAGGATAGC	GTGCTCACTCTCTTCTGTCA	Reverse	2	0.37	280-300	9	55
miR169n	CACAATTCACACACCAGAGAG	TAGGCAAGTCATTCTTGGCTA	Reverse	2	0.44	250-260	11	55
miR1428f	AATTCACAGGCCCTATCTTGTG	GGTGCGGTTTAGCGCTGCCAT	Forward	3	0.48	200-262	7	55

250 bp on both sides of pre-miRNA) any contraction or expansion in this region due to SSR instability will lead to the instability of the pre-miRNA and eventually will culminate in the defects in the mature miRNA formation. This is the first genetic diversity study in miRNA genes of plants in which miRNA itself has been used as marker.

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