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Full Length Research Paper

Genetic mapping of the 'eui' gene in rice using microsatellite markers

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Development of cytoplasmic male sterile (CMS) lines with elongated uppermost internode (EUI) trait is a desirable alternative to eliminate the use of GA_3 in hybrid rice production. In this study, a simple and reliable PCR based, simple sequence repeats (SSR) markers was identified, for the eui gene to facilitate marker assisted selection (MAS). A total of 28 SSR markers, mapped in the vicinity of the genomic clone AC130605 (PD0010D04), were chosen for assessing the polymorphism between IR58025B and IR91-1591-3. A set of 151 F_2 plants derived from the cross IR58025B/IR91-1591-3 were surveyed using 15 polymorphic SSR markers. Analysis using MAPMAKER 3.0 revealed that seven SSR markers *viz*., RM6054, RM3870, RM3476, RM5970, RM7801, RM7446 and RM3620 were linked with *eui* locus at the genetic distance ranging from 1.0 to 7.1 cM with EUI phenotype. Furthermore, these markers were validated in – F_2 (IR58025A/IR91-1591-3) and backcross (IR58025B/IR91-1591-2//IR58025B) population segregating for *eui* gene. The SSR markers reported in this study might be useful for the incorporation of *eui* gene into CMS lines of rice through marker-assisted backcrossing.

Key words: Rice, cytoplasmic male sterility, elongated uppermost internode, microsatellites, marker assisted selection, panicle exsertion.

INTRODUCTION

Hybrid rice technology offers great potential to increase rice production and productivity on a sustainable basis. Presently cytoplasmic genetic male sterility (CMS) system is being commercially used to develop hybrids in rice. Among various CMS sources available in rice, wild - abortive (WA) type is the most widely exploited in developing male sterile lines (Sheeba et al., 2009).

However, CMS lines with this cytoplasm have a problem of incomplete panicle exsertion wherein 30 - 40% of the spikelets remain enclosed in flag leaf sheath (Gangashetti et al., 2004). Incomplete exsertion, known to be caused by reduced level of endogenous GA_3 synthesis, is a major bottleneck in obtaining higher seed yield in hybrid rice seed production plots. This problem is usually overcome by application of GA_3 (50-100 g/ha) which in turn increases the seed production cost and adversely affect the quality of hybrid seed through reduced dormancy and storage life (Honnaiah, 2003). Hybrid rice breeders therefore, have been in constant search for a genetic alternative to GA_3 application. Internode length of rice plant plays an important role in deciding the extent of panicle exsertion (Wang et al, 2005). The first recessive

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Abbreviations: CMS; cytoplasmic male sterile, **EUI;** elongated uppermost internode, **MAS;** marker assisted selection, **TGMS;** thermosensitive genic male sterile.

rice internode elongation mutant was isolated from the Japanese rice cultivar Norin 8 by gamma ray treatment (Okuno and Kawai, 1978). Subsequently, Rutger and Carnahan (1981) identified a recessive gene causing elongation of the uppermost internode (EUI phenotype) in a japonica rice line 76:4512. Later the mutant rice line is characterized by a near doubling of uppermost internode thus enhancing panicle exsertion and panicle length with almost no effect on other internode or plant characters. Due to its ability to cause complete panicle exsertion the EUI phenotype in CMS line controlled by recessive genes prove a very useful trait in hybrid seed production and hence was called a fourth genetic element after the three genetic tools viz. male sterile (A), maintainer (B) and restorer (R) lines (Rutger and Carnahan, 1981). During the past two decades hybrid rice breeders in china have therefore been instrumental in transferring *eui* gene into a number of female parents (cytoplasmic male sterile and thermosensitive genic male sterile lines) to improve the panicle exsertion (He and Shen, 1991; Yang et al., 2000a, Li and Pandey, 2002; Yang et al., 2002; Zhou et al., 2002; Zhang et al., 2002). Further, CMS/TGMS lines incorporated with *eui* gene are likely to be available for commercial hybrid seed production in China (Fang, personal communication). So far two *eui* loci controlling the elongated internode have been identified in rice. The gene conditioning the *EUI* phenotype in the mutant 76:4512 has been localized in chromosome 5 through trisomic analysis and named as *'eui-1'* (Librojo and Khush, 1986). Subsequent studies on inheritance of internode elongation trait also confirmed it to be controlled by single recessive gene (Gangashetti et al., 2004; Ma et al., 2004). Yang et al. (1999) identified another recessive gene (*eui-2*) in a gamma rays irradiated maintainer line, Xinquing ZhaoB which was mapped on chromosome 10 using SSR markers (Yang et al., 2000b; Yang et al., 2001). Gangashetti et al. (2004) identified a recessive gene controlling *EUI* in the rice breeding line IR91-1591-3 and later Gangashetti et al. (2006) tagged the gene by RAPD markers and mapped on chromosome 5 using a Sequence Tagged Site (STS) marker. In view of the fact that the elongated uppermost internode gene is recessive in nature, hence its transfer is laborious and time consuming as one generation of selfing is required after every cycle of backcrossing. This problem could be overcome with the availability of

molecular markers associated with this trait. PCR based markers such as simple sequence repeats (SSR), which exhibit co-dominant inheritance can be effectively used in marker based screening of *eui* gene and phenotype. A highly saturated SSR map of rice with over 18000 markers covering almost the entire rice genome is now available in public database (http//:www.gramene.org/), which greatly facilitates easy identification of SSR markers closely linked to gene of interest to enable MAS. In this study, SSR markers linked to *eui* gene in the donor line IR91-1591-3 was identified to enable MAS for developing CMS lines with *EUI*.

MATERIALS AND METHODS

Plant material and mapping populations

In the present study, a breeding line, IR91-1591-3 from the International Rice Research Institute (IRRI), Philippines was used as the donor parent for elongated uppermost internode (*EUI*) trait. The maintainer line, IR58025B with normal internode (*Eui/Eui*) was crossed with IR91-1591-3 (*eui/eui*) to develop F_2 population (151 individuals) segregating for the trait of internode elongation at the Barwale Foundation Research Farm, Hyderabad, India (Coordinates: 17° 24' 22''N and 78° 12' 40''E). The *EUI* and non-*EUI* phenotypes were scored based on internode elongation following the procedure described by Yang et al. (2002). Two more populations, F_2 (IR58025A/IR91-1591-3) and backcross (IR58025B/IR91-1591-3//IR58025B) consisting of 123 and 115 plants respectively, were also used for validating the markers.

Selection of microsatellite markers

In the earlier study, Gangashetti et al. (2004) reported a RAPD marker, $OPAG01_{1000}$ linked to *eui* gene in the same F_2 population derived from the cross IR58025B/IR91-1591-3. Subsequently, the RAPD marker was converted into a sequence tagged site (STS) marker, $SAG01_{1051}$ through cloning and sequencing. This STS marker was mapped on chromosome 5 through linkage analysis (Gangashetti et al., 2006). The sequence homology search using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) showed that the sequence of RAPD fragment showed 99.62% homology with a portion of nucleotide sequence in the clone P0010D04 (AC130605) on chromosome 5 of rice available in the database. In this study, a total of 28 rice SSR markers with map position near P0010D04 (AC130605) and other adjacent clones (OJ1118_C04, OJ1579_G03, OSJNBa0088I06 and OJ1119_H02) located on chromosome 5 (http//: www.gramene.org/) were selected. These SSR markers were expected to cover a map distance of approximately 10 cM flanking the *eui* locus, which has been previously described with the STS marker $SAG01_{1051}$.

DNA extraction, bulked segregant analysis and PCR amplification

Genomic DNA was extracted from freshly harvested young leaves of parents and individual F_2 plants derived from the crosses, IR58025B/IR91-1591-3 and IR58025A/IR91-1591-3, and backcross IR58025B/ IR91-1591-3// IR58015B using the protocol described by Dellaporta et al. (1983). The DNA from 10 each of *EUI* and non-*EU*I F2 plants was bulked in equal quantity to form *EUI* and non-*EUI* bulks respectively. PCR was carried out using selected rice SSR markers as per the protocol of Gangashetti et al. (2006). PCR amplified products were resolved in 3% agarose gel (Sigma, Molecular Biology Grade, Cat. No. A9539) followed by ethidium bromide staining or 6% denaturing polyacrylamide gel electrophoresis followed by silver staining (Panaud et al., 1996). Further, Utility Alphaease® (Alphainnotech, USA) software was used to determine the fragment sizes of the amplions with 100 bp ladder molecular weight marker (MBI Fermentas, Lithuania) as size standard.

Linkage analysis

Linkage relationships and map construction for SSR markers and *eui* locus were performed using Mapmaker 3.0 software (Lander et al., 1987). Linkage group was obtained using two-point analysis with a default logarithm of odds (LOD) score of command. Linked markers within the linkage groups were ordered using multipoint

Table 1. List of SSR markers which were found to be polymorphic around *eui* locus on chr 5 between the parents IR58025B and IR91-1591-3.

SSR primers	Forward sequence	Reverse sequence	Start (bp)	Stop (bp)	Size (bp)	
					IR91-1591-3	IR58025B
RM163	CGCCTTTATGAGGAGGAGATGG	AAACTCTTCGACACGCCTTGC	19,107,762	19,107,791	120	100
RM164	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC	19,175,845	19,176,109	250	280
RM440	GGTAGGCACCAAAGAGTTTGACG	GGCATCACCTTATCCAATCACC	19,830,882	19,830,947	200	190
RM3351	GTCGAAACGTAGCCAGGCAATGG	CCATGGAAGGAATGGAGGTGAGG	20,615,059	20,615,088	150	130
RM173	CCTACCTCGCGATCCCCCCCTC	CCATGAGGAGGAGGCGGCGATC	21,623,805	21,623,990	190	180
RM3295	AGACACGGCAAGGACAAAGC	CGTTCGGACTCCTTTGGATAGC	22,187,443	22,187,470	220	210
RM6054	CCCTCCGTACGGATACACAC	CTCTTCGGCTTCATCTCCTC	22,758,636	22,758,825	150	130
RM3870	TACATCTCCGGCGTTTACAC	CCAAGGTTGAAACAGGAAGC	22,879,699	22,879,891	160	150
RM3476	GATTCTCGTCGTAATCAAGA	GATTCTCGTCGTAATCAAGA	23,823,303	23,823,590	150	130
RM5970	CCCATCTGGTTCACCTTCAC	AGGAGCAGCCTTTTGTCTTC	23,925,644	23,925,763	120	110
RM7081	CCGCACTACACTGCACTCC	AACTTGCTCATGGAGTTGGG	24,503,829	24,503,925	90	85
RM7446	TGAAGGCAGTTTCACTGACG	AGCCAAGAAGAAGAAAGGGG	24,932,863	24,933,057	190	130
RM3620	TCCTCTCCACCTTCAAATCC	ACCATCCTCTGCTGCTGC	25,183,014	25,183,125	110	130
RM6972	CATGGTGCTCCTACTGGTTGTACC	CCCATCCATAATCACAACTCAGC	25,208,346	25,208,384	180	200
RM233B	CCAAATGAACCTACATGTTG	GCATTGCAGACAGCTATTGA	NA	NA	160	155

analysis with 'compare' command. The map distances were calculated in centiMorgan (cM) using Kosambi function (Gangashetti at al, 2006).

RESULTS

In this study, an attempt was made to further map the *eui* locus derived from the donor IR91-1591-3 with closely linked SSR markers. The F_2 population, consisting of 151 individuals, derived from the cross IR58025B/IR91-1591-3 was phenotyped for non-EUI and EUI. Out of the 151 plants, 109 and 42 were found to exhibit normal and elongated internode respectively and segregated in 3:1 ratio for non-EUI and EUI trait (^{2} = 1.73 and P > 0.5). The 3:1 ratio for normal and elongated internode plants in the population is expected for a single recessive gene controlled trait. The selected set of 28 SSR markers was first surveyed between parents to assess their polymorphism. Out of 28, 15 SSR markers were found to be polymorphic between parents, IR58025B and IR91-1591-3. Further, bulked segregant analysis was done on EUI and non- EUI bulks, all the 15 polymorphic markers were found to be associated to the respective bulks hence, 151 F_2 plants derived from the cross IR58025B/IR91-1591-3 were genotyped with all the SSR markers. Marker-trait association indicated that only seven markers co-segregated with normal and elongated internode traits. Sequence of these primers along with their approximate fragment size as amplified in the parents: IR91-1591 -3 and IR58025B are given in Table 1. Genotype of F_2 individuals based on some of these SSR primers has been furnished in Table 2. All the seven marker genotypes were found to be in agreement with 1 (homozygous, non-EUI): 2 (heterozygous, non-EUI): 1 (homozygous, EUI) ratio, further confirming the monogenic recessive inheritance of the trait of internode elongation in the breeding line IR91-1591-3. The amplification pattern of four SSR markers in F_2 population derived from the cross IR58025B/IR91-1591-3 is shown in Figure 1. Linkage analysis using MAPMAKER (version 3.0b) software revealed RM3870 and RM3476 flanking *eui* locus at a genetic distance of 3.0 and 1.0 cM respectively (Figure 2). These results indicated that RM3870 and RM3476 are the closest flanking markers linked to the *eui* locus. The recombination frequency and LOD score of each locus is given in Table 3. This observation suggests the *eui* locus could be defined within a physical interval between the clones AC108503 (RM3870) and AC093952 (RM3476). Association of these seven SSR markers, closely linked to *eui*

Table 2. Segregation of SSR markers in F_2 population of the cross IR58025B/IR91-1591-3.

SSR locus	Phenotype and genotype of F_2 plants			Total	^{2} value
	Non-EUI plants		EUI plants		
	Eui/Eui	*Eui/eui*	*eui/eui*		
RM6054	41	72	38	151	0.4
RM3870	41	73	37	151	0.4
RM3476	37	74	40	151	0.2
RM5970	38	73	40	151	0.2
RM7081	32	78	41	151	1.2
RM7446	32	79	40	151	1.2
RM3620	33	79	39	151	0.8

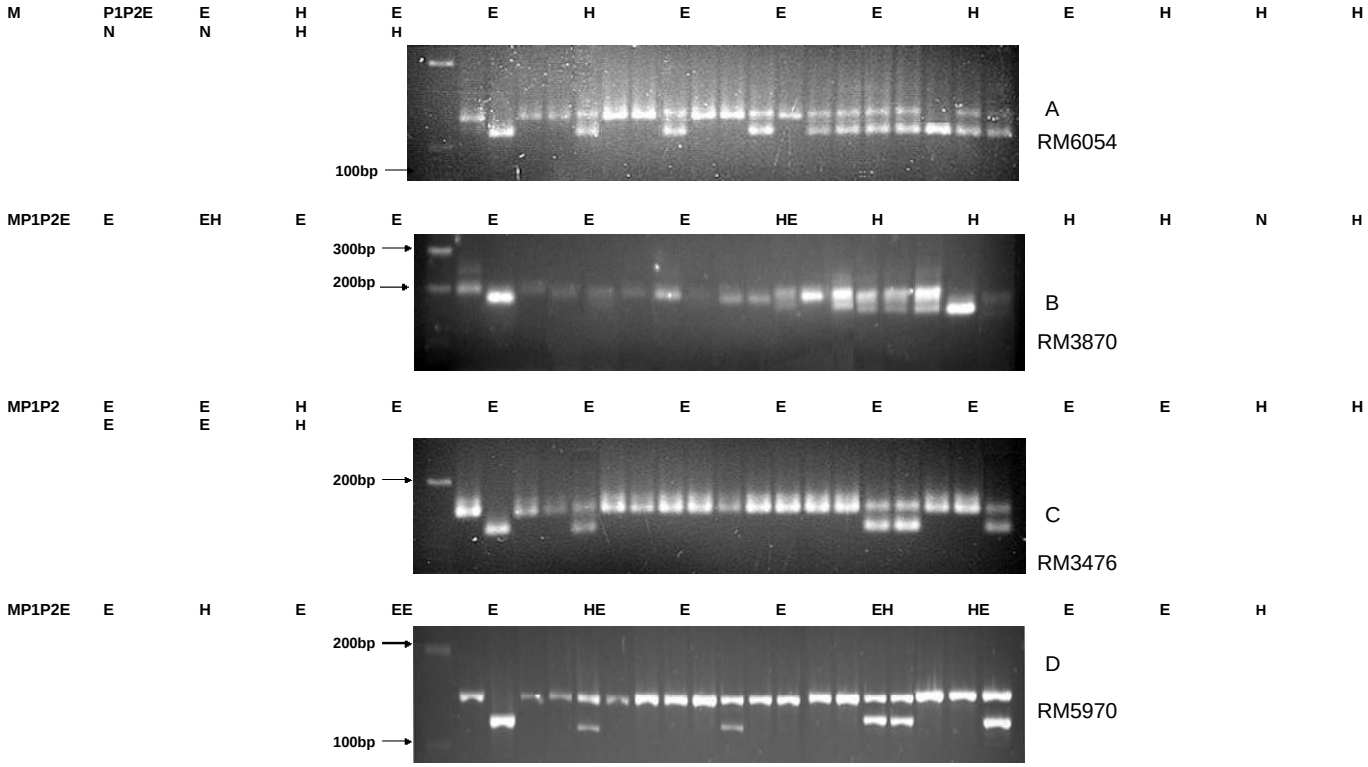

Figure 1. Microsatellite marker genotyping of F_2 population derived from the cross IR58025B/IR91-1591-3. P1: IR58025B; P2: IR91-1591-3 E: Homozygote for *eui* allele; N: Homozygote for *Eui* allele; H: heterozygote (*Eui/eui).* A: Segregation of *eui* and *Eui* alleles for the marker RM6054 in 6% denaturing poly acrylamide gel stained with silver nitrate. B: Segregation of *eui* and *Eui* alleles for the marker RM3870 in 3% agarose gel stained with ethidium bromide. C: Segregation of *eui* and *Eui* alleles for the marker RM3476 in 3% agarose gel stained with ethidium bromide. D: Segregation of *eui* and *Eui* alleles for the marker RM5970 in 3% agarose gel stained with ethidium bromide.

locus, with EUI phenotype were further validated in two more segregating populations – F_2, derived from the cross from(IR58025B/ IR91 -1591-3// IR91-1591-3).

DISCUSSION

Incorporation of *eui* gene can help minimize the cost of commercial hybrid seed production in rice. The gene can be deployed in two ways either through male parent (restorer line) for better pollen transfer from tall males to short females or by obtaining better panicle exsertion of female line (CMS or TGMS) panicles from the flag leaf boot. However, presence of *eui* gene in an *indica* background is considered advantageous as it overcomes various problems likely to be encountered in crossing and

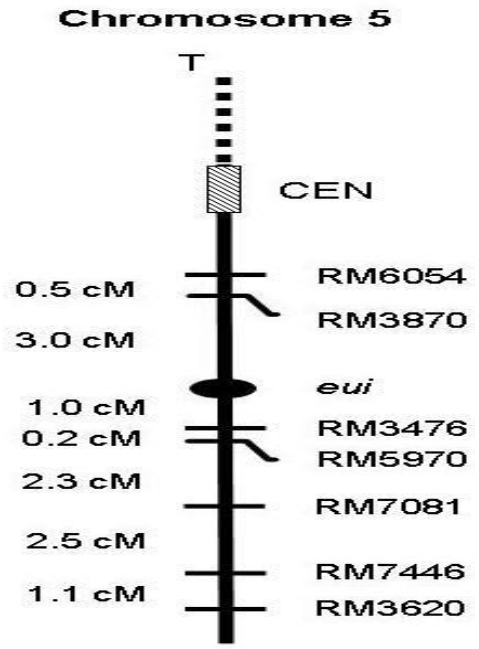

Figure 2. Linkage map of *eui* gene locus as inferred from co segregation analysis using SSR markers in F_2 population derived from the cross IR58025/IR91-1591-3. Map distances are given in centiMorgan (cM). CEN – Centromere and T – Telomeric region.

Table 3. Result of two-point analysis for segregation of the SSR markers and the 'eui' gene in F2 population of 151 individuals from the cross IR58025B/IR91-1591-3.

Locus	Expected ratio				´ value	Recombination frequency	LOD score
	9	3	3	1			
	Observed class						
eui/RM6054	105	8	4	34	164.08**	0.0650	41.28
eui/RM3870	116	8	3	34	112.45**	0.0615	42.27
eui/RM3476	108	3	1	39	147.80**	0.0234	54.19
eui/RM5970	108	1	3	39	147.80**	0.0268	53.18
eui/RM7081	106	4	3	38	135.13**	0.0580	40.40
eui/RM7446	105	6	3	37	73.61**	0.0686	37.08
eui/RM3620	105	7	4	35	110.86**	0.0758	35.26

backcrossing the *japonica* donor with *indica* during its transfer to other *indica* restorer or CMS lines. Subsequently, *eui* gene was transferred into a number of *indica* hybrid rice parental lines by scientists at IRRI and in China (He and Shen, 1991; Virmani, 1996; Yang et al., 2000a). So far two *eui* genes namely *eui-1* and *eui-2* on chromosome 5 and 10 respectively have been identified in rice.

Earlier studies have showed that the donor IR91-1591-3 carried a recessive *eui* gene and located on chromosome 5 (Gangashetti et al., 2006) . The *eui* gene in the donor line IR91-1591-3 might be allelic to *eui1* gene reported by Ma et al. (2006) as they share the same genomic locations. Molecular markers closely linked to *eui* gene can be useful for marker assisted transfer of *eui* gene into desired hybrid rice parental lines.

In recent years, genes that contribute to important agronomic traits in rice have been tagged with tightly linked SSR markers to enable MAS breeding (Biradar et al., 2004; Jena et al., 2006; Singh et al., 2006). SSR markers are considered as the markers of choice for gene mapping and MAS especially because of their abundance, simplicity, reliability and ability to distinguish heterozygotes. The availability of rice genome sequences (Yu et al., 2002; Goff et al., 2002; IRGSP, 2005) coupled with the ability to develop large collection of new SSR

markers (McCouch et al., 2002) has greatly facilitated the physical map based identification of SSR markers for fine-mapping and MAS for the genes of interest.

Considerable progress has been made by China in exploiting *eui* genes for hybrid rice parental line development (Yang et al., 1999; Yang et al., 2001; Ma et al., 2004) but in India this is the first time *eui* gene have been identified and mapped (Gangashetti et al., 2004, 2006). The donor line (IR91-1591-3) of this gene is basically a partial restorer and hence poses difficulties while transferring this gene in to maintainer lines. Currently, RM3870 and RM3476 are being used for introgression of *eui* gene from IR91-1591- 3 into commercially usable CMS line through backcross breeding.

CONCLUSION

The *EUI* trait is an important agronomic trait that has the potential to overcome the problem of incomplete panicle exsertion in CMS lines which in turn help produce good quality hybrid seeds in rice. In the present study seven markers viz., RM6054, RM3870, RM3476, RM5970, RM7801, RM7446 and RM3620 linked with *eui* locus at the genetic distance from 1.0 to 7.1 cM were identified. These markers were further validated in F_2 (IR58025A/IR91-1591-3) and backcross (IR58025B/IR91-1591-2//IR58025B) population. The *eui* gene in donor line, IR91-1591-3 is basically in a restorer background which poses difficulties in transferring this gene in maintainer line. With the help of linked SSR markers RM3870 and RM3476 we have successfully transferred the *eui* gene from IR91-1591-3 in IR58025B, a maintainer of most popular CMS line being commercially exploited in India for development of hybrids. Further work is underway to transfer the *eui* gene from IR58025B (*eui*) in CMS line IR58025A. The SSR markers reported in this study should be very useful to the breeders interested in transferring *eui* gene into their promising parental lines of hybrid rice.

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