EVALUATION AND DNA MARKER-ANALYSIS OF STIGMA CHARACTERISTICS IN RICE (*ORYZA SATIVA L.*)

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ABSTRACT

Stigma characteristics are important traits, which contribute to the efficient improvement of commercial seed production in hybrid rice. Two cytoplasmic male sterile (CMS) lines of rice from different male sterility sources were evaluated for stigma characters and other various floral traits. Spikelet and stigma traits were recorded in the CMS lines, F1 hybrids, and F2 population of the crosses. Considerable variations existed between CMS lines for these traits. Molecular marker analysis was screened to identify a specific DNA marker linked to stigma characters. Results identified one DNA marker named T86, which was associated with stigma traits.

Key words: Floral traits, Stigma characters; Molecular Markers; CMS lines; Oryza sativa

INTRODUCTION

Production of hybrid seed plays a key role in successful implementation of hybrid rice. However, it is difficult to produce enough amount of hybrid rice seeds, because rice is strictly self-pollinating crop (Azzini and Rutger 1982). Therefore, development of an efficient seed-production system is an essential factor for the commercialization of hybrid rice. Floral characteristics, such as flowering time, spikelet opining angle, stigma dimensions and stigma exsertion rate (SER) are associated with the rate of out-crossing and hybrid seed efficiency (Jayamani *et al.*, 1995). Stigma with large size is known to facilitate better exsertion and hence effective interception of air borne pollen. Large stigmatic surface is desirable for effective cross-pollination. It has been reported that high rate of stigma exsertion promotes out-crossing (Tian, 1996). Synchronization of flowering results in many spikelets is not available for cross-pollination. Spikelets that have stigmas remain exserted after flowering has an extended opportunity to be cross-pollinated. Exserted

stigmas remain viable up to six days with a decrease of 20% of seed set from cross-pollination per day (Xu and Shen 1988). As a result, stigma exsertion including single and dual stigmas and other stigma traits play important roles in hybrid seed production and receive considrable attention from rice researchers (Li *et al.* 2001; Xu 2003; Miyata *et al.* 2007).

Stigma exsertion in rice species is controlled by multi-genes (Virmani and Athwal, 1974). Recent progress in the DNA marker techniques has been providing genetic information about stigma exsertion applicable to actual breeding. For example, nine quantitative trait loci (QTLs) for the frequency of stigma exsertion were detected in the recombinant inbred lines (RILs) derived from the cross between a *Japonica* (Asominori) variety and an *Indica* (IR24) variety (Yamamoto *et al.* 2003). Using a segregating population derived from *Indica* (IR24) and *Japonica* (Koshihikari), a major QTL qES3 on chromosome 3 was identified using restriction fragment length polymorphism (RFLP) markers (Miyata *et al.* 2007). In order to develop superior rice hybrids, it is a pre-requisite to develop CMS lines with good floral characters especially with large stigma size and high SER. This investigation was conducted to evaluate the stigma characters and screen for DNA molecular markers for providing marker-assisted selection for stigma characters in cytoplasmic male sterile line.

MATERIALS AND METHODS

Plant materials

In order to study the floral characteristics, two CMS lines (G46A and IR69625A) and restorer variety (Giza 178R) were used to make two crosses; the fist cross was G46A X Giza 178R, and the second one was IR69625A X Giza 178R. The parents, F1 and F2 populations were grown in the experimental farm of Rice Research and Training Center (RRTC), Sakha, Kafrelsheikh, during the cropping seasons in 2006 and 2007, respectively. Single row plot including 10 plants, 20 cm square in spacing by transplanting for parents, F1 and F2 was arranged by a randomized complete block design with three replications. At the peak flowering time (12 a.m. to 1 p.m.), a single panicle from five different plants was selected from each plot, resulting in 15 panicles sampled for each germplasm. All the spikelets with open lemma and palea, indicative of flowering on the day were tagged. Five spikelets from each panicle whose lemma and palea

stayed open were removed and kept in a tube containing FAA (70% of ethanol: acetic acid: formalin by 90: 5: 5, respectively) for 24 h. Spikelet length and width, and stigma length and width were then measured with a micrometer under a stereomicroscope. Exserted stigma was defined as the rate (%) of the number of exserted stigmas to the total number of stigmas on the flowered glumes. In addition, heading date, distance between opening spikelet tips, opening angle of spikelet, seed set rate, and spikelet fertility % were measured.

DNA marker analysis

To identify specific DNA markers associated with stigma characters in CMS lines, ten promising lines that have a good sigma characters from F2 population of each cross were selected to isolate their genomic DNA for molecular markers analysis. The total DNA was extracted from the green leaves of the selected lines according to the method of Guillemaut and Marechal-Drouard (1992). Five PCR-based specific markers were used in this study. They consisted of the markers described on the website of the Rice Genome Project in Japan (http://www.rgp.dna.aVrc.go.jp) and the sequences of the markers are shown in Table (1).

Table (1): SSR Primers used in this study

Primer	Forward sequence	Reverse sequence
T86	CTCGCCGTCGAATCCGCCAT	CACTCTCCTCTCCTGCCCCC
RM 20775	AGGATACAGCGCACCAGATTAGC	GGTGTTCATCTTTGTTGAGTCAG
RM 21242	GAACAGGCATGGTGAAGAGTGC	GAGAGGAATGGAATGGAATGAG
RM 27400	GGTGAAGTACTCACATTCCGATGG	CTCGTTCCTCCTTCCTCATCACC
RM 1986	CATCTCTCCTCTAGGCGGATTGG	TCTGTGGAGAAGAAATGGATCTC

PCR Reaction Program

Plant genomic DNA was used as templates for polymerase chain reaction (PCR) amplification. The PCR amplification is perform in 25-µl volumes containing 25ng of template DNA, 10mM Tris HCl pH 8.3, 50mM KCl, 2.0mM $MgCl_2$, 0.001% (w/v) gelatin, 100µM of each dNTPs. 15 ng of primers and 1 unit of Taq DNA polymerase. Amplification reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 35 cycles after pre-denaturing for 5

min at 95 ^{0}C. Each cycle consists of 1 min at 95 ^{0}C, 1 min at 55 ^{0}C, and 2 min at 72 ^{0}C. The 35 cycles are following by a 10 min final extension at 72 ^{0}C.

Gel electrophoresis photography

Aliquots of 5 µl of PCR products were loaded in 1% Agarose gels for electrophoresis in lx TAE (lx TAF: 4 mM Tris-acetate plus 1 mM EDTA). Gels were stained with ethidium bromide and photographed under UV light using Gel Documentation unit.

RESULTS

Characterization of floral traits is essential for enhancing hybrid rice seed production. Therefore, the objectives of this study were to characterize the floral traits of hybrid rice parental lines and identification of DNA marker associated with floral traits. In this investigation, two CMS lines (G46A, IR69625A) were crossed with Giza 178R to produce two hybrids.

Heading date

Heading date for the first hybrid (G46A/Giza 178R), its parental lines and F2 population derived from this cross are shown in Table (2). Results showed that all the lines showed some differences in heading date among them. G46A headed after 97 days, while Giza 178R headed after 111 days. F1 hybrid progeny headed after 113 days. The selected lines from F2 population of this cross showed shortest time for heading date comparing with parental lines and F1 progeny. Heading date for the second hybrid (IR69625A/Giza 178R), its parental lines and F2 population derived from this hybrid are shown in Table (3). The results indicated that IR69625A headed after 112 days similar to Giza 178R. While F1 hybrid progeny heading was shorter than its parents, which recorded 91 days for heading. But selected lines from F2 population of this cross ranged from 90 days to 97 days for heading, which were shorter than the parents.

Spikelets Characters

The most important trait of spikelets is opening angle (OAS), which related to spikelet length and the distance between opening spikelet tips (DOST).Results in Table(2) revealed that the OAS was over 29.5 ° for G46A, whereas it was 23.3 ° for Giza 178R. But the OAS for F1 (G46A/Giza 178R) was almost intermediate (26.7 °) between the parents. For the selected lines of F2 from the cross (G46A/Giza

178R), the OAS ranged from 29.5° to 23.6° (Table 2). Regarding the F1 of the second cross (IR69625A/Giza 178R), the OAS data is shown in Table (3). Results indicated that the IR69625A recorded 26.7° of OAS, while Giza 178R recorded 23.3°. But F1 progeny of this cross recorded the highest degree of OAS similar with that IR69625A line. Whereas the selected lines of F2 population of this cross recorded OAS ranged from 26.7° to 20.7° (Table 3).

Stigma Characters

Out crossing in rice depends on the capacity of stigma to receive alien pollen. Therefore, out cross rate in CMS lines is largely influenced by opening angle of spikelet (OAS), and stigma exertion rate (SER). Stigma characters mainly depend on stigma area that related to length and width of stigma and SER. Results in Table (2) revealed that the stigma area was the highest (0.55 mm) for G46A, whereas it was 0.33 mm for Giza 178R. But the stigma area for F1 (G46A/Giza 178R) was almost intermediate (0.41 mm) between the parents. The selected lines of F2 from the cross (G46A/Giza 178R), showed considerable variation in the stigma area, which ranged from 0.51 mm to 0.28 mm Table (2). Regarding to SER, the data presented in Table (2) showed that G46A recorded the highest SER (52.3%), whereas it was 36.6% for Giza 178R. For F1 (G46A/Giza 178R) recorded SER almost intermediate (43.6%) between the parents. The selected lines of F2 from the cross (G46A/Giza 178R), showed that SER ranged from 51.2% to 32.1% (Table 2). Stigma characters of the second cross (IR69625A/Giza 178R) is shown in Table (3). Results revealed that the IR69625A was the highest for stigma area (0.56 mm) and SER (54.5%), while Giza 178R recorded 0.30 mm for stigma area and 37.3% for SER. F1 progeny of this cross recorded 0.42 mm for stigma area and 45.5% for SER. The selected lines of F2 population of the cross (IR69625A/Giza 178R) were varied for stigma area (ranged from 0.50 mm to 0.34 mm) and SER (ranged from 55.1% to 37.2%).

Molecular markers Analysis

Stigma exsertion is one of the important traits which contribute to the efficient improvement of commercial seed production in hybrid rice. In order to identify the genetic factors related to stigma exsertion of an Egyptian CMS lines in Molecular marker analysis was conducted

using the F_2 population between the two CMS lines with Giza 178 restorer variety.

In this study using the F_2 population from the two crosses, the first cross was between G46A/Giza 178R and the second cross was between IR69625A/Giza 178R to identify a molecular marker associating with stigma traits of CMS lines. Ten plants were chosen from 250 individuals of F2 population of each crosses according to their floral and stigma characters (Table 2 and 3), to be a source of DNA to use as a template for DNA marker analysis. The DNA was extracted from G46A, IR69625A, Giza 178R, and ten selected plants from F2 of each cross and was employed in PCR analysis to identify DNA markers associated with the stigma traits of CMS lines. A total of five random primers were screened for their polymorphism between the F_2 individual plants and parental lines. Of these, only one DNA primer is T86 (forward 5'-**CTCGCCGTCGAATCCGCCAT-3'** reverse 5'-**CACTCTCCTCTCCTGCCCCC-3'**) was identified and detected polymorphism (Fig. 1 and 2). This primer T86 amplified polymorphic products that were present in G46A (lane 1), and some lines (lane 2,3,4,7) from F2 selected lines of the cross (G46A/Giza 178R) (Fig. 1). But no amplification product was observed in Giza 178R (lane 8) and other selected lines (lane 5,6) of F2 (Fig. 1). In addition, the same primer (T86) showed amplified band present in IR69625A (lane 1) and some lines (lane 2,3,4,5,6,8,9) of F2 of the cross (IR69625A/Giza 178R) (Fig. 2). Whereas, there was no amplification product in Giza 178R and other selected F2 lines (lane 7) (Fig 2). Also molecular analysis showed that the genotypes that exhibited DNA band with the T86 marker are the same lines that recorded the highest values of stigma traits (Table 2). On the other hand, the genotypes that did not exhibited amplification product with the T86 marker showed the lowest values of stigma traits Table (3). From these results we suggested that the T86 primer may be associated with stigma traits in CMS lines. The polymorphism identified with T86 primer was further confirmed by amplification products of the F_2 individuals, which showed the same DNA band (400 bp) present in some F2 lines, whereas it was absent in other F2 lines (Fig 1 and 2).

DISCUSSION

It is worth noting that, selection for floral traits that increased cross-pollination such as stigma area and SER improve hybrid rice seed production (Taillebois and Guimaraes, 1988). It is possible to improve

outcrossing rate in rice through breeding by using parents possessing a high stigma exsertion rate (Zhang and Lu, 1995). The present study showed that the stigma exsertion rate (SER) plays a key role in the outcrossing process. SER was very significantly related to outcross rate and stigma activity. The exserted stigma can be easily damaged by environmental conditions, such as wind, water stress, and physical interruption during the flowering period. Observation of stigma exsertion on the flowering day for those spikelets flowering on that day only should avoid such damages and improve data accuracy. Therefore, data generated in the present study using this observation technique for 15 panicles should accurately describe a genotype for stigma exsertion. In this study the SER in the two CMS lines (G46A and IR69625A) was higher than Giza 178R, while it was intermediate in the F1 hybrids. Whereas SER was varied in F2 selected lines (Table 2 and 3). Therefore, any CMS line, which can inherit this character, can greatly improve the outcrossing rate of rice.

Molecular marker analysis indicated that there was one DNA marker (T86) which showed amplification band with CMS lines and some of F2 selected lines, which suggested that this marker is associated with the SER trait in these lines (Fig 1 and 2). In addition, the lines that showed amplification products are the same lines which showed highest SER comparing with other lines. These results suggested that T86 primer is specific marker related to SER trait. Several other molecular markers are also associated with stigma characters on different chromosomes have Table (4) (Wen Gui Yan *et al.*, 2009). It was reported that a highly significant QTL (qES3) was confirmed at the centromeric region on chromosome 3 and increased the frequency of the exserted stigmas in the IR24 variety (Miyata *et al.*, 2007). Xu and Shen (1988) reported that there are minor genes, which together with some major genes control this character. Research findings showed that double stigma exsertion favor outcrossing more than single stigma exsertion (Xu *et al.*, 1988). Therefore, much effort is needed to be geared towards breeding lines with double stigma exsertion. Exsertion of stigmas and stigma area increase the probability of out-crossing for production of hybrid seed in comparison with the exsertion of stigma with small stigma area (Wen Gui Yan *et al.*, 2009). These results indicate that improvement in floral traits of female and male parents has to be done separately to increase out crossing potential in rice in relation to hybrid rice breeding and seed production programs. When a molecular marker is associated with a phenotypic trait, it should associate with others that highly

correlate with this trait in theory (Wen Gui *et al.*, 2009). The present study identified a positive association among stigma exsertion rate, stigma area, opining angel of spikelets, seed set rate, and fertility percentage of spikelets. One molecular maker (T86) was associated with stigma exsertion. Furthermore, the data suggest the same marker was associated with the same traits positively correlated with stigma exsertion. These correspondences between trait–trait correlation and trait–marker association further validated the resulting association mapping of stigma and spikelet characteristics (Wen Gui Yan *et al.*, 2009). Our results demonstrated the usefulness of DNA markers for the practical molecular breeding of floral traits in rice.

Table (2): Floral characteristics of G46 CMS line, Giza 178R and their hybrid combinations (average of two years).

Character		Floral Characteristics								SSR (%)	SFP (%)
			Spikelet Characters			Stigma Characters					
Genotype		HD (days)	Sp. L (mm)	DOST (mm)	OAS (°)	St. L (mm)	St. W (mm)	St. A (%)	SER (%)		
G46A		97	0.780	0.40	29.5	1.19	0.46	0.55	52.3	00.0	00.0
G46B		98	0.780	0.40	29.5	1.10	0.43	0.48	49.5	90.2	98.3
Giza 178R		111	0.720	0.34	23.3	0.89	0.33	0.30	36.6	86.1	95.5
F1 Population		113	0.780	0.38	26.7	0.88	0.46	0.41	43.6	82.2	96.6
F2 Lines	L1	84	0.750	0.42	29.6	0.96	0.53	0.51	49.7	90.2	96.5
	L2	88	0.740	0.41	29.6	1.02	0.45	0.46	51.2	89.7	97.4
	L3	89	0.800	0.45	26.7	0.88	0.39	0.34	32.4	84.5	94.9
	L4	81	0.890	0.42	23.6	0.73	0.33	0.24	35.2	85.7	92.0
	L5	91	0.732	0.36	29.6	1.11	0.43	0.48	45.9	90.1	95.0
	L6	88	0.745	0.38	26.7	0.98	0.34	0.33	34.2	87.1	90.1
	L7	91	0.889	0.43	23.6	0.91	0.38	0.35	34.7	82.5	92.1
	L8	85	0.810	0.39	26.7	0.81	0.34	0.28	32.1	84.1	93.2
	L9	90	0.780	0.38	26.7	0.89	0.33	0.30	37.1	86.2	90.0
	L10	87	0.810	0.36	26.7	0.90	0.40	0.36	33.4	81.8	91.1

HD: Heading Date; Sp. L.: Spikelet Length; DOST: Distance between Opening Spikelet Tips; OAS: Opening Angle of Spikelet; St. L.: Stigma Length; St. W.: Stigma Width; St. A.: Stigma Area; SER: Stigma Exsertion Rate; SSR: Seed Set Rate; SFP: Spikelet Fertility Percentage.

Table (3): Floral characteristics of IR69625 CMS line, Giza 178R and their hybrid combinations (average of two years).

Character / Genotype		Floral Characteristics								SSR (%)	SFP (%)
		HD (days)	Spikelet Characters			Stigma Characters					
			Sp. L (mm)	DOST (mm)	OAS (°)	St. L (mm)	St. W (mm)	St. A (%)	SER (%)		
IR69625A		112	0.825	0.40	26.7	1.08	0.51	0.56	54.50	00.0	00.0
IR69625B		115	0.824	0.40	26.7	1.03	0.48	0.50	53.25	88.2	98.7
Giza 178R		112	0.720	0.34	23.3	0.89	0.33	0.30	37.30	86.4	95.7
F1 Population		91.0	0.790	0.39	26.7	0.96	0.44	0.42	45.50	84.2	97.5
F2 Lines	L1	96.0	0.800	0.41	26.7	1.03	0.48	0.50	55.10	85.8	93.6
	L2	96.0	0.820	0.40	26.7	1.05	0.48	0.50	46.80	83.6	94.1
	L3	90.0	0.790	0.39	26.7	0.99	0.47	0.47	42.20	86.7	90.6
	L4	90.0	0.780	0.38	26.7	1.02	0.44	0.45	46.80	83.7	97.2
	L5	96.0	0.831	0.37	20.7	0.89	0.38	0.34	37.80	80.6	85.1
	L6	95.0	0.800	0.32	20.7	0.96	0.44	0.42	39.30	82.5	89.8
	L7	93.0	0.811	0.35	20.7	0.94	0.41	0.39	38.50	78.0	83.2
	L8	94.0	0.720	0.34	23.3	0.94	0.45	0.42	36.70	78.0	85.6
	L9	97.0	0.821	0.32	20.7	0.96	0.44	0.42	38.21	79.1	88.1
	L10	97.0	0.843	0.31	20.7	0.89	0.39	0.35	37.22	83.4	79.6

HD: Heading Date; Sp. L.: Spikelet Length; DOST: Distance between Opening Spikelet Tips; OAS: Opening Angle of Spikelet; St. L.: Stigma Length; St. W.: Stigma Width; St. A.: Stigma Area; SER: Stigma Exsertion Rate; SSR: Seed Set Rate; SFP: Spikelet Fertility Percentage.

Table (4): Identification of molecular markers in different chromosomes associated with stigma characters in rice (Wen Gui Yan *et al.*, 2009)

Stigma Character	Chr. No.	Position	Markers
Stigma length	3	83.5	RM1334
	10	0.8	RM474
Stigma L/w ratio	5	3.0	RM507
	7	96.9	RM118
	10	71.4	RM484
Single Stigma Exsertion	1	98.5	RM5
	6	0.5	RM133
	9	7.8	RM105
	10	55.2	RM25669
Double Stigma Exsertion	1	98.5	RM5
	1	102.3	RM3642
	5	104.4	RM178
	7	78.9	RM455
	8	46.9	RM44
	11	8.7	RM7203
Total Stigma Exsertion	5	104.4	RM178
	7	78.9	RM455
	8	78.5	RM284
	9	7.8	RM105
	10	55.2	RM25669

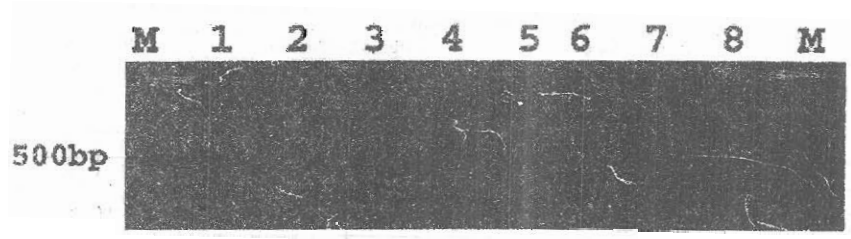

Fig. (1). The amplification of T86 primer fro (G46A/Giza 178R)
M: DNA ladder, 1: G46A; 2-7: selected lines from F2 population; 8: Giza 178R

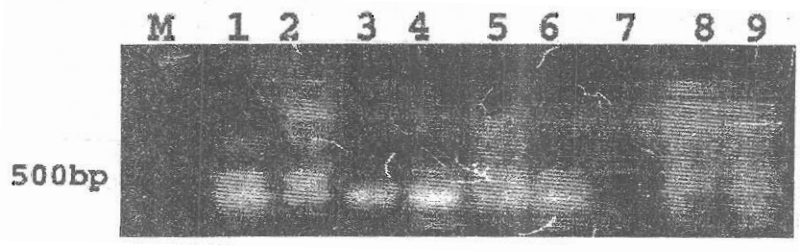

Fig.(2). The amplification of T86 primer for (IR69625A/Giza 178R)
M: DNA ladder, 1: IR69625A; 2-9: selected lines from F2 population

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الملخص العربي

التقييم والتحليل الجزئيى لصفات الزهرة فى الارز

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تم تقييم الصفات الزهرية لسلالتين عقيمتين CMS من مصدرى عقم مختلفين في مركز بحوث الأرز بسخا RRTC أثناء موسمى 2006 2007 . وقد وجدت تباينات بين هاتين السلالاتين في كل هذه الصفات. وقد لوحظت صفات ال stigma للزهرة فى كلا السلالتين و F_1 وF_2 . وقد تم التحليل الجزيئي لتحديد DNA markerالمتخصص والمرتبط بصفات ال stigma.وقد أوضحت النتائج أن primer T_{86} مرتبط ببعض صفات ال stigma.