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Full Length Research Paper

Identification of rice hybrids and their parental lines using total soluble protein and fertility restorer gene markers

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For identification of rice hybrids and their parental lines, thirty populations produced from hybridization between a cytoplasmic male sterility (CMS) line (IR69625A), and each of the three restorer lines (Giza178R, Giza181R and Giza182R) during 2009, 2010 and 2011 seasons were used. Electrophoresis of proteins showed promising results in genetic purity determination of hybrids and parental lines. The presence or absence of a specific band was much useful for differentiating the highest from the lowest parents in restoring ability. Five markers linked to fertility restorer genes: M2, RM3425, RM258, RM1108 and RM5373 were screened on DNA templates from the CMS line and its three restorer lines. Only RM1108 marker was able to show polymorphism between the highest and lowest Giza181R parents. This marker could be assessed for identification and testing of seed genetic purity of restorer lines.

Key words: Hybrid rice, genetic purity, SDS-PAGE, fertility restorer genes.

INTRODUCTION

Rice (*Oryza sativa* L.) is the major staple cereal food crop fulfilling about 60% dietary requirement, 20% calorie and 14% protein requirement of the world's population. In the present decade, the rate of increase in rice production is lower (1.5% per year) than the increase in population (1.8% per year). The present world population of 6.3 billion is likely to reach 8.5 billion by 2030. Out of this, 5 billion people will be rice consumers and there is a need of 38% more rice by 2030. To meet this challenge, there is a need to develop rice varieties with higher yield potential and greater stability (Khush, 2006). Hybrid rice technology is one of the strategies to meet this immense challenge imposed by ever growing populations. Hybrid rice varieties have clearly shown a yield advantage of 1.0 to 1.5 tonnes per hectare (20 to 30%) over conventionally bred modern varieties (Virmani et al., 2003).

In the three-way method, hybrid seeds are produced by crossing a CMS line with a restorer line, and the CMS line is multiplied by crossing it with a maintainer line (Virmani et al., 1997). This compound system makes it difficult to produce pure hybrid seeds constantly (Mao, 2001). It was reported that 1% impurity in the hybrid rice seeds caused the yield reduction of 100 kg per hectare (Mao et al., 1996). Therefore, it is very important to manage the seed purity of hybrid varieties and their parental lines.

Production of rice hybrids using a CMS system is based on cytoplasmic male sterility and fertility restoration systems. Cytoplasmic male sterility is caused

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by lesion or rearrangement of mitochondrial genome resulting in its inability to produce functional pollen. But CMS can be restored by nuclear genes (*Rf* genes) governing fertility restoration (Nematzadeh and Kiani, 2010). Fertility-restorer (*Rf*) genes exist widely in *Oryza* species with the AA-genome, and fertility of a given CMS type is controlled by several *Rf* alleles in various wild restorer accessions (Li et al., 2005).

In order to increase hybrid rice breeding efficiency and improve the restoring ability for the restorer lines: Giza178R, Giza181R and Giza182R, the present study was undertaken for identification of rice hybrids and their parental lines using total soluble protein and fertility restorer gene markers.

MATERIALS AND METHODS

Plant material

One wild-abortive CMS line; IR69625A with three restorer lines; Giza178R, Giza181R and Giza182R were used in this study as parental lines for production of F_1 hybrids. Seeds of the WA-CMS line as well as the restorer lines were kindly obtained from Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt.

Field experiment

Three periodical sowing dates were applied with 15 days intervals to overcome the differences of heading date among the parental lines during May 2009 season. Each line was planted in four rows, five meters length and 20 cm apart between plants and rows under isolated plots. The hand-crossing technique was used depending on the sterility of CMS line (female parent). A total of 30 single crosses were made and harvested separately for each restorer line under investigation. The seeds of each single cross (F_1) were kept to be grown with some seeds of its restorer line for identification experiment, while the other remaining seeds of the restorer lines were kept to be grown for multiplication experiment.

In 2010 season, the 30 populations for each restorer line were sown in the nursery with their crosses during the first week of May for identification experiment and after 21 days, the other remaining seeds of each restorer line population were sown for multiplication experiment. Seedlings were carefully pulled from the nursery after 30 days from seeding and transferred to the permanent field at the rate of one seedling/hill. Five replications were grown in randomized complete block design, each consisting of one row for the restorer line and another row for its F_1 crosses (for all of the thirty populations). Each row was five meters long and contained 25 individual plants. The hand-crossing technique was also applied in 2010 season. All the previous procedures were repeated in 2011 season. Thestandard agronomic practices and Plant protection measures were followed in raising the crop as recommended by RRTC (2008).

Agronomic traits

Traits of seed set (%) and grain yield/plant (g) were evaluated using five replicates for the thirty selected populations of F_1 crosses and their restorer lines. Seed set % was calculated as indicator for the restoring ability

$$\text{seed set \%} = \frac{\text{No. of filled grains}}{\text{No. of total grains}} \times 100$$

, while grain yield/plant was measured as the weight of grain yield of each individual plant at 14% moister content.

SDS-Polyacrylamide gel electrophoresis

Total soluble proteins were extracted from seedling leaves (21 days old) of the highest and lowest six parents in restoring ability (based on seed set %) as well as CMS line in addition to their F_1 crosses. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of total soluble protein was carried out by using 12.5% polyacrylamide gel according to Laemmli (1970). Bands with different molecular weights (MW) were determined against pre-stained high molecular weight standard marker (PINK Prestained Protein Marker, Cat. No. MWP02), with molecular weights ranging from 15 to 175 kDa.

Genomic DNA isolation and PCR analyses

Total genomic DNA was isolated from young, healthy leaves of the highest and lowest parents in restoring ability, for each restorer line, as well as CMS line by using the DNeasy Plant Mini Kit (QIAGEN GmbH, Cat. No. 69104).

A total of five primers reported to be linked to *Rf* genes were screened on DNA templates. The details of primers sequences along with their references are given in Table 1. All primers were introduced from Ferments Company, Germany. Sequences of primers were directly down-loaded from gramene website (www.gramene.org).

The PCR amplification was performed according to Williams et al. (1990). PCR amplified products were run on 1.5% agarose gel against a known DNA Ladder (50 bpDNA ladder, INtRON Biotechnology and Jena Bioscience).

Statistical analysis

Data of seed set % and grain yield/plant traits were subjected to analysis of variance for randomized complete block design as suggested by Panse and

Table 1. Details of the used primers and their nucleotide sequences.

Primer	F/R Primer 5´→3´	CL.	Linked *Rf* gene	Repeat motif	Annealing temperature	References
M2	F-TTGGGAGATAAAATGAGGATGTGG R-TATAACTCTGGACGACAACGACGG	10	*Rf-1*	-	65	Komori and Nitta (2004)
RM3425	F-AGCAGCAGCAAGAACCCTAG R-TTGGTGATCGGTGATGGTC	1	*Rf-3*	(CT) 18	55	Attia et al. (2009)
RM258	F-TGCTGTATGTAGCTCGCACC R-TGGCCTTTAAAGCTGTCGC	10	*Rf-4, Rf-5, Rf-(u1) Rf-6(t)*	(GA) 21 (GGA) 3	55	McCouch et al. (2002), Mishra et al. (2003)
RM1108	F-GCTCGCGAATCAATCCAC R-CTGGATCCTGGACAGACGAG	10	*Rf-1, Rf-4, Rf-5*	(AG) 12	55	Sattari (2004)
RM5373	F-GGAGATGCTATAGCAGCAGTG R-ATTGCTCCTTACCACCTTGC	10	*Rf-5, Rf-6(t)*	(TC) 13	50	Liu et al. (2004)

F/R Primer: forward/reverse primer; CL: chromosomal location.

Sukhatme (1954). Analysis of variance was used to estimate the genotypic (σ^2_g) and phenotypic variances (σ^2_{ph}) according to the formula suggested by Burton (1952). Heritability in broad sense (h^2_b) was estimated as the percentage of genotypic to phenotypic variance (Hansen et al., 1956).

RESULTS AND DISCUSSION

Mean performance and heritability of agronomic traits

Mean performance for the three restorer lines as well as their hybrids for seed set % and grain yield/plant traits in 2010 and 2011 seasons were shown in Table 2. Results revealed a wide range of differences among the populations (No. 1 to 30) for all the studied parental lines as well as their hybrids.

For restorer line Giza178R, the desirable mean values were obtained by populations No. 2, 14 and 18 in 2010 season and in 2011 season by populations No. 5, 6 and 12 for seed set % trait. The obtained values were better for selection in 2011 season, whereas the mean value of the parental populations (No. 1 to 30) was 79.89% in 2010 season and 93.13% in 2011 season. Moreover, the produced hybrid populations (No. 1 to 30) revealed seed set percentages of 88.70 and 95.19% in the two seasons (2010 and 2011) respectively. For grain yield/plant trait, populations No. 4, 8 and 21 in 2010 season and populations No. 7, 10 and 24 in 2011 season showed the desirable mean values. The mean values of parental populations (No. 1 to 30) were 35.45 and 51.51 g in 2010 and 2011 seasons, respectively. Also, the general mean values for grain yield/plant trait of hybrid populations (No. 1 to 30) were 62.75 and 83.03 g in the two seasons (2010 and 2011), respectively.

Regarding the restorer line Giza181R, results showed that the desirable mean values for seed set % trait were obtained from populations No.12, 16 and 28 in 2010 season and in 2011 season from populations No. 2, 10 and 20. The mean values of parental populations (No. 1 to 30) were 77.48 and 89.06% in 2010 and 2011 seasons, respectively. Also, general mean values of hybrid populations (No. 1 to 30) were 87.09 and 93.98% in 2010 and 2011 seasons, respectively. Concerning grain yield/plant trait, the desirable mean values were obtained by Giza181R populations No. 6, 16 and 21 in 2010 season and by populations No. 8, 12 and 29 in 2011 season. The general mean values of parental populations (No. 1 to 30) were 31.17 and 63.06 g in 2010 and 2011 seasons, respectively. Moreover, the general mean values of hybrid populations (No. 1 to 30) were 73.35 and 118.50 g in 2010 and 2011 seasons, respectively.

The desirable mean values for seed set % trait were found for Giza182R populations No. 13, 25 and 28 in 2010 season and with populations No. 8, 24 and 27 in 2011 season. The general mean values of the parental populations (No. 1 to 30) were 90.08 and 91.82% in 2010 and 2011 seasons, respectively. For hybrid populations (No. 1 to 30), the general mean values were 88.57 and

Table 2. Mean performance values of the three restorer lines: Giza178, Giza181 and Giza182, and their crosses for seed set and grain y ield/plant traits.

Season		Seed set (%)									Grain yield/plant (g)								
		Giza178			Giza181			Giza182			Giza178			Giza181			Giza182		
		Population	*Parent*	*Hybrid*	*Population*	*Parent*	*Hybrid*	*Population*	*Parent*	*Hybrid*	*Population*	*Parent*	*Hybrid*	*Population*	*Parent*	*Hybrid*	*Population*	*Parent*	*Hybrid*
	H	28	93.33	97.49	2	87.39	94.52	4	93.58	95.50	19	50.57	85.59	13	55.51	114.48	17	106.31	158.20
	L	22	66.45	75.23	5	68.31	71.29	8	82.42	81.33	1	22.30	34.48	5	19.41	43.13	6	35.37	23.07
2010	S	2	79.45	87.35	12	77.33	87.42	13	90.27	88.43	4	33.64	62.62	6	31.73	71.51	23	67.90	65.11
	S	14	80.46	89.32	16	77.47	86.76	25	89.48	89.23	8	37.58	62.91	16	30.60	76.07	25	71.91	87.24
	S	18	79.35	89.53	28	78.51	87.33	28	90.18	88.31	21	34.62	63.12	21	29.92	70.53	27	67.82	65.40
$\bar{X}$		-	79.89	88.70	-	77.48	87.09	-	90.08	88.57	-	35.45	62.75	-	31.17	73.35	-	70.00	79.70
	H	17	97.61	97.48	27	94.42	96.39	30	95.47	98.42	28	79.41	103.31	9	99.08	203.63	19	93.76	182.49
	L	13	85.46	91.47	1	72.50	90.66	3	82.27	79.48	25	25.58	60.12	21	30.48	44.66	3	65.27	34.46
2011	S	5	92.47	95.37	2	88.41	93.68	8	91.37	93.52	7	51.46	81.87	8	64.48	119.77	6	73.38	99.26
	S	6	93.59	94.58	10	89.40	94.21	24	91.54	93.61	10	51.78	86.27	12	60.99	115.23	18	78.69	99.74
	S	12	93.45	95.35	20	89.40	94.18	27	92.37	95.29	24	50.43	82.79	29	65.71	121.50	21	78.57	121.37
$\bar{X}$		-	93.13	95.19	-	89.06	93.98	-	91.82	93.92	-	51.51	83.03	-	63.06	118.50	-	75.95	103.35

H: highest value; L: lowest value; S: selected values.

93.92% in 2010 and 2011 seasons, respectively. Concerning the performance of grain yield/plant trait, the desirable mean values were for Giza182R populations No. 23, 25 and 27 in 2010 season and in 2011 season with populations No. 6, 18 and 21. The general mean values of parental populations (No. 1 to 30) were 70.00 g in 2010 season and 75.95 g in 2011 season. In case of grain yield/plant trait for the hybrid populations (No. 1 to 30), the mean values were 79.70 and 103.35 g in 2010 and 2011 seasons, respectively.

Results for all the studied restorer lines: Giza178R, Giza181R and Giza182R, indicated that the selection was in the direction of the desirable values of both traits in 2011 season for the parental lines and their hybrids. Consequently, the produced seeds from the selected populations could be used as nucleus seeds which have special desirable mean value for both seed set % and grain yield/plant traits.

Mean square values of Giza178R, Giza181R and Giz182R populations (No. 1 to 30) as well as their hybrids for seed set % and grain yield/plant are presented in Table 3. The thirty populations showed highly significant differences for the parental lines and their hybrids in 2010 and 2011 seasons indicating that the average of improvement was significant in all populations for all restorer lines.

These results were in agreement with those obtained in rice by Bagheri and Jelodar (2010) who found that analysis of variance revealed significant differences among genotypes, crosses, lines, testers and line×tester interactions for spikelet fertility and grain yield traits. On the other hand, Agbo and Obi (2005) reported that selecting upland rice genotypes with stable and high number of filled grains would sustain high yields in such genotypes especially as the yield

Table 3. Mean square values of the restorer lines: Giza178, Giza181 and Giza182, as well as their hybrids for seed set and grain yield/plant traits.

Season	S.O.V	d.f	Seed set %						Grain yield/plant					
			Giza178		Giza181		Giza182		Giza178		Giza181		Giza182	
			Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid
2010	Reps.	4	1.82	0.14	1.52	0.40	0.37	1.24	2.64	1.33	0.60	2.08	0.10	108.539
	Population	29	208.40**	8.68**	142.68**	85.55**	29.32**	95.03**	531.14**	725.19**	277.83**	1512.79**	305.53**	7696.520**
	Error	116	0.84	0.08	0.63	0.95	0.99	0.86	2.24	1.74	2.12	1.98	1.37	117.414
2011	Reps.	4	0.09	0.44	0.13	0.07	0.13	0.06	1.62	0.68	0.80	1.88	0.68	0.928
	Population	29	62.51**	165.85**	137.78**	9.57**	33.37**	75.85**	371.69**	800.50**	1828.81**	7600.16**	1633.94**	10017.600**
	Error	116	0.11	0.90	0.07	0.06	0.07	0.06	1.27	1.42	1.11	1.36	0.99	1.216

**: Significant at 0.01 level.

Table 4. Heritability in broad sense for seed set (%) and grain yield/plant traits in 2010 and 2011 seasons for the parental restorer lines and their hybrids.

Season	Seed set %						Grain yield/plant					
	Giza178		Giza181		Giza182		Giza178		Giza181		Giza182	
	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid	Parent	Hybrid
2010	98.74	97.57	98.52	98.45	95.56	97.84	99.09	99.58	99.03	99.73	99.52	97.15
2011	99.68	99.20	99.85	98.64	99.41	99.84	99.23	99.74	99.90	99.96	99.90	99.98

components are complementary in action.

Regarding the heritability of seed set % and grain yield/plant traits, Table 4 presented the heritability values in broad sense in 2010 and 2011 seasons. Both traits gave high values of heritability for the three restorer lines and their hybrids in 2010 season. Similar trend was found for the results obtained in 2011 season, whereas the heritability in broad sense was higher than the recorded values in 2010 season. This indicates that the environmental effect was very low and these traits were controlled by additive and non-additive genetic variances and could be selected in early generations. These findings were in agreement with the obtained results by Augustina et al. (2013) who reported that the evaluated agronomic traits (15 traits) showed high heritability estimates ranging from 93.00 to 99.88. Similar results were obtained by Akinwale et al. (2011) who found that high to medium heritability and genetic advance, in twenty rice genotypes (*Oryza sativa* L.), were recorded for the number of grains/panicle, grain yield, panicle weight and the number of panicles/plant. This suggested that these traits were primarily under genetic control and selection for them could be achieved through their phenotypic performance. Bisne et al. (2009) reported that heritability with genetic advance is more helpful in predicting the gain under effective selection.

Identification of total soluble proteins of the parental lines and their hybrids

Total soluble proteins, of the highest and lowest six parents in restoring ability (based on seed set %), as well as their F_1 crosses, were presented in Figure 1 and Tables 5 to 7. For description of total protein patterns, bands number and intensity were taken as the criteria.

For the restorer line Giza178R and its crosses, total protein profiling showed a total number of 30

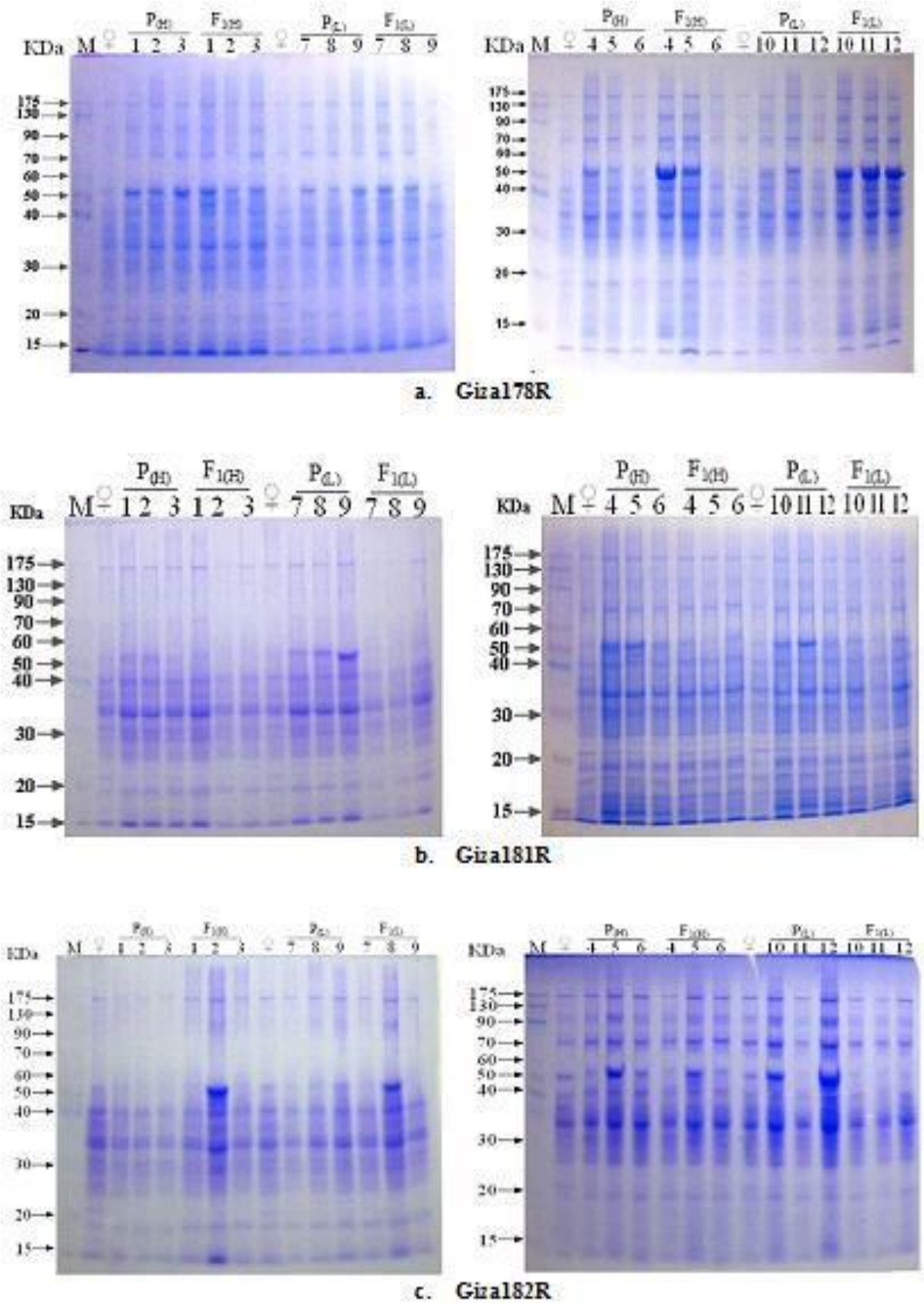

Figure 1. Protein banding pattern for the highest and lowest six parents in restoring ability as well as their crosses, for the three restorer lines: (a) Giza178R, (b) Giza181R and (c) Giza182R.

Table 5. Description of total soluble protein of the six highest and lowest yield populations of IR69625A/Giza178R as well as their parents.

Bands No.	The highest													The lowest											
	♀	Parent						F_1						♀	Parent						F_1				
		1	2	3	4	5	6	1	2	3	4	5	6		7	8	9	10	11	12	7	8	9	10	11	12
1	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	++	+
3	+	+	++	++	+++	++	+	++	+	++	+++	+++	+	+	+	++	++	++	+++	+	++	++	+	+++	+++	++
4	+	-	+	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
5	+	++	++	++	++	+	-	++	+	++	+++	++	-	+	+	+	+	-	+	-	++	++	+	++	+++	+++
6	+	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	+	+	+
7	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	+	+	+	+	-	-	+	++	++	++
8	++	++	++	-	+++	+++	++	-	-	-	+++	+++	++	++	-	-	-	++	+++	++	+++	+++	++	+++	+++	+++
9	+	++	++	+++	+	+	+	+++	++	+++	-	+	-	+	+++	+++	++	-	+	-	+++	+++	+	+	+	+
10	+	-	-	-	+	+	+	+	-	+	++	++	-	+	-	-	-	-	+	-	+	-	-	+	++	++
11	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
12	+	+++	++	+++	+++	++	-	+++	++	+++	+++	+++	+	+	+++	++	+++	++	+++	+	+++	+++	++	+++	+++	+++
13	-	+	++	-	+	-	-	+++	++	+	+	+	+	-	-	-	+	+	+	-	++	++	-	++	+	+
14	+	++	++	++	++	++	+	++	+	++	+++	+++	+	+	++	+	++	++	++	+	++	++	+	+++	+++	+++
15	-	++	++	++	-	-	-	++	+	++	-	-	-	-	+	+	+	-	-	-	++	+	+	-	-	-
16	+	+	+	+	++	++	+	+	-	+	-	-	-	+	+	+	+	-	++	-	+	+	+	+++	+++	+++
17	++	++	+++	+++	+++	++	++	+++	++	+++	+++	+++	++	++	++	++	++	++	+++	++	+++	++	++	+++	+++	+++
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-
19	+	-	-	-	+	+	-	+	+	++	+	++	-	+	-	+	+	+	+	-	-	-	-	-	++	++
20	+	++	++	++	++	++	+	+++	+	+++	++	+++	+	+	+	+	+	++	++	+	++	+	+	+++	+++	+++
21	+	-	-	-	-	-	-	-	-	-	-	++	+	+	-	-	-	+	-	-	+	-	-	+	-	+
22	+	+	+	++	++	++	+	++	+	++	++	+++	+	+	+	+	+	++	++	-	++	+	+	++	++	++
23	++	+	+	+	-	-	+	+	-	+	+	++	+	++	-	-	-	-	-	-	-	-	+	-	++	++
24	+	+	+	-	-	-	-	+	-	+	+	++	-	+	+	+	+	-	-	-	++	+	+	+	++	++
25	+	++	++	++	+++	+++	+	++	+	++	++	+++	+	+	++	++	+	++	+++	+	++	++	+	+++	+	+
26	+	-	-	-	-	-	-	-	-	-	+	++	-	+	-	-	-	+	+	-	-	-	-	+	+	+
27	++	+	++	++	+++	+++	++	++	+	++	+++	+++	++	++	++	++	+	++	++	+	++	++	+	+++	+++	+++
28	+	-	-	-	++	++	+	-	-	-	++	+++	++	+	-	-	-	++	++	+	-	-	-	++	++	++
29	+	-	-	-	-	++	-	-	-	-	+++	+	-	+	-	-	-	+	-	-	-	-	-	++	++	++
30	+	+	++	++	+++	++	+	++	+	++	+++	+++	+	+	+	+	+	++	++	+	++	++	+	+++	+++	+++
Total	24	17	18	14	19	19	14	21	14	20	22	22	15	24	14	16	19	17	19	11	20	17	18	23	24	25
Mean	24			16.8							19			24			16							21.2		

- : absent; + : light intensity; ++ : medium intensity; +++ : dark intensity.

Table 6. Description of total soluble protein of the six highest and lowest yield populations of IR69625A/Giza181R as well as their parents.

Bands No.	The highest													The lowest											
	♀	Parent						F_1						♀	Parent						F_1				
		1	2	3	4	5	6	1	2	3	4	5	6		7	8	9	10	11	12	7	8	9	10	11	12
1	-	-	-	+	+	+	-	+	-	-	+	-	+	-	-	+	+	+	+	-	-	-	-	-	+	+
2	+	++	+	++	++	++	+	++	+	+	++	+	++	+	++	+	++	++	++	+	+	+	+	++	+	++
3	+	++	+	+	+	+	-	++	+	+	+	-	+	+	+	+	++	++	+	+	+	+	++	+	-	++
4	+	++	+	+	+++	++	+	++	+	+	++	+	++	+	++	+	++	++	++	+	-	-	+	++	-	+++
5	+	++	++	+	+++	++	+	++	+	+	++	+	++	+	++	+	+	++	++	++	-	-	+	++	-	+++
6	+	-	++	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-
7	++	++	-	+	+++	++	-	++	-	-	-	-	+	++	-	-	++	-	+	+	-	-	-	-	-	++
8	+	++	+	+	+++	++	++	++	+	+	++	++	++	+	++	+	++	++	++	++	+	-	+	++	+	++
9	+	+	-	+	++	++	+	+	-	-	+	+	++	+	+	+	-	++	++	+	-	-	+	++	+	+
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
11	-	++	++	-	-	+	-	++	+	+	+	+	-	-	+++	+++	-	-	-	-	+	+	++	-	-	+
12	+	++	++	+	+++	+++	++	+	-	-	++	+	++	+	+	+	+++	+++	+++	+	-	-	+	++	+	++
13	-	+	+	+	++	++	+	+	-	-	+	+	+	-	+	++	++	+	+	+	-	-	+	+	-	+
14	+	++	+	+	+++	++	++	++	+	+	++	+	++	+	+	+	++	++	++	++	+	+	++	++	+	++
15	-	++	+	+	-	-	-	-	+	-	-	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-
16	+	++	+	+	++	+	+	++	+	+	+	+	+	+	++	+	++	++	++	+	-	-	++	+	-	++
17	-	++	+	+	-	-	-	++	+	+	-	-	-	-	++	+	++	-	-	-	+	+	++	-	-	-
18	++	+++	+++	++	+++	+++	++	+++	+	++	++	++	++	++	+++	+++	+++	++	+++	++	+	+	+++	++	+	+++
19	-	-	-	+	++	++	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+	++	-	++
20	+	++	++	+	+++	+	++	++	+	+	++	+	+	+	+	+	++	++	++	++	+	+	++	++	+	++
21	+	++	++	+	+++	++	+	+++	+	+	++	++	++	+	++	++	+++	++	++	++	+	+	++	++	+	++
22	+	-	-	-	+	+	-	+	-	-	+	-	+	+	+	++	++	+	-	-	-	-	+	+	-	+
23	+	++	++	+	+++	++	++	++	+	+	++	++	++	+	++	+	++	+++	++	++	+	+	++	++	+	+++
24	-	+	+	+	++	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	++
25	++	+	-	++	++	++	+	++	-	-	++	++	++	++	+	+	++	++	++	+	-	-	++	++	+	++
26	+	++	++	++	++	+	-	+++	++	++	++	+	+	+	++	+	+++	++	++	+	+	-	+	++	+	++
27	+	++	++	++	+++	++	++	+++	++	++	++	++	++	+	+++	++	++	+++	++	++	+	+	+++	++	+	+++
28	+	++	++	++	++	+	+	+++	-	++	++	+	+	+	+++	-	+++	++	++	+	++	-	-	+	+	++
29	++	++	+	++	++	++	-	++	+	+	-	-	-	++	++	+	++	-	-	+	+	+	++	+	+	+
30	+	++	++	++	+++	++	++	++	+	+	++	++	++	+	++	++	++	+++	++	++	+	+	++	++	+	+++
31	+	++	++	++	+++	++	+	++	+	++	+	+	+	+	++	++	++	++	++	++	+	+	++	++	+	++
32	+	+++	+++	++	+++	+++	++	+++	+	+	++	+	+	+	+++	++	+++	+++	+++	+++	+	+	++	++	+	+++
Total	24	27	25	28	27	28	20	29	20	21	26	22	26	24	27	27	27	24	24	24	17	14	27	25	19	28
Mean	24				25.8					24				24				25.5							21.7	

- : absent; + : light intensity; ++ : medium intensity; +++ : dark intensity.

Table 7. Description of total soluble protein of the six highest and lowest yield populations of IR69625A/Giza182R as well as their parents.

Bands No.	The highest													The lowest											
	♀	Parent						F_1						♀	Parent					F_1					
		1	2	3	4	5	6	1	2	3	4	5	6		7	8	9	10	11	12	7	8	9	10	11	12
1	-	-	-	-	+	++	+	-	-	-	-	-	+	-	-	-	-	++	-	++	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	++	-	-	-	-	-	-
3	+	+	+	+	+	++	++	++	+++	++	+	++	++	+	+	++	++	++	+	+++	+	+++	+	+	+	++
4	+	+	-	-	+	++	++	+	-	-	+	++	++	+	+	-	-	++	+	++	+	-	-	+	+	+
5	+	+	+	+	++	+++	++	+	+++	+	+	++	++	+	+	++	++	+++	+	+++	+	+++	-	+	+	+
6	+	+	-	-	+	++	++	+	++	+	+	+++	+++	+	+	++	++	+++	++	+++	+	++	-	++	+	++
7	+	-	-	-	+	++	++	+	++	+	++	++	++	+	-	+	+	++	+	++	-	++	-	+	+	++
8	++	-	-	-	++	+++	++	+	++	-	++	+++	+++	++	-	-	++	+++	++	+++	-	++	+	++	++	+++
9	+	+	+	-	+	++	+	+	+	+	+	++	+	+	+	-	+	++	+	++	+	+	-	+	+	+
10	+	+	+	+	-	+	+	+	++	+	-	++	+	+	+	++	++	+++	+	+++	+	++	+	+	+	++
11	-	+	+	+	-	-	-	++	++	+	-	-	-	-	+	++	++	-	-	-	+	++	+	-	-	-
12	-	++	+	+	++	-	-	++	-	++	+	-	++	-	+	++	++	-	+	-	+	-	+	+	+	-
13	+	-	-	-	-	+++	++	-	+++	-	-	+++	-	+	-	-	-	+++	-	+++	-	+++	-	-	-	+
14	-	-	-	-	-	+	+	+	+++	++	-	++	+	-	+	++	++	+	-	+++	++	+++	++	-	-	+
15	-	+	+	+	-	-	-	+	++	+	-	-	-	-	+	++	-	-	-	-	+	-	+	-	-	-
16	+	++	++	+	++	+++	+++	++	+++	++	++	+++	++	+	++	++	++	+++	++	+++	++	+++	++	++	++	+++
17	-	+	+	-	-	-	-	++	+++	+	-	-	-	-	-	-	++	-	-	-	+	+++	++	-	-	-
18	+	+	+	+	-	+++	++	++	+++	+	+	+++	++	+	+	++	++	+++	-	+++	+	+++	++	++	+	+++
19	-	+	+	+	-	-	+	++	+++	++	+	-	-	-	+	++	+++	-	-	-	++	+++	++	+	+	++
20	++	+	+	+	++	+++	+++	++	+++	++	++	+++	+++	++	+	++	+++	+++	++	+++	++	+++	++	++	++	+++
21	+	+	+	+	+	++	++	+	++	+	+	++	+	+	+	+	++	++	+	++	+	++	+	+	+	++
22	+	+	+	+	+	++	++	++	+++	+	+	++	+	+	+	++	++	++	+	++	+	+++	+	+	+	++
23	+	+	-	+	+	++	++	++	+++	++	+	++	+	+	+	++	++	++	+	+++	+	+++	+	++	+	++
24	+	+	+	+	++	+++	++	++	+++	++	+	++	++	+	+	+	++	+++	+	+++	+	+++	+	++	+	++
25	-	-	-	-	-	-	-	+	++	+	-	-	-	-	-	+	++	+	-	+	+	++	+	-	-	-
26	++	+	-	-	+	++	++	+	++	-	+	+	+	++	-	-	+	++	+	+++	-	++	-	+	+	+
27	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	+	-	+	-	-	-
28	+	++	+	+	++	+++	+++	++	+++	++	++	+++	++	+	++	++	++	+++	++	+++	++	+++	++	++	+	++
29	+	+	-	-	+	++	++	+	++	-	+	++	+	+	-	+	+	++	+	++	-	++	+	+	+	++
30	++	+	-	-	+	++	++	+	++	-	+	++	+	++	-	+	+	++	+	++	-	++	+	+	+	++
31	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
32	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
33	+	++	-	-	+	++	++	++	+++	+	+	++	+	+	-	+	++	++	-	+++	+	+++	+	+	-	++
Total	24	23	16	15	20	23	24	27	26	23	21	22	23	24	19	23	26	25	19	25	23	25	22	22	21	23
Mean	24				20.2						23.7			24				22.8					22.7			

- : absent; + : light intensity; ++ : medium intensity; +++ : dark intensity.

polypeptide bands with the presence or absence of particular bands (Figure 1a and Table 5). The results indicated that only seven common monomorphic bands (No. 3, 14, 17, 20, 25, 27 and 30) appeared in the highest and lowest groups of the parent Giza178R, as well as their F_1 crosses, in addition to another band (No. 11) which was parental monomorphic; though it did not appear in the highest and lowest Giza178R parents, it appeared in only one individual of the highest and lowest F_1 crosses. Among the polymorphic bands (22 bands), it was obvious that band No. 7, with MW of ~88 kDa, was observed in four individuals of the lowest parental group and was inherited to their crosses. In spite of the absence of this band in all individuals of the highest parental group, it appeared in three of their F_1 crosses. On the other hand, band No. 23 with MW of ~24 kDa appeared in most individualsof the highest parental group and CMS line, but disappeared in all individuals of the lowest parental group. This band was inherited to a large number of the highest and lowest F_1 crosses. The presence or absence of specific band was much useful for differentiating the highest from the lowest parents in restoring ability. The mean number of bands was increased in both the highest and lowest F_1 crosses groups (19 and 21.2 bands, respectively) compared to their parental groups (16.8 and 16 bands, respectively). This increase in bands number may be as a result of the expression of gene(s) that play an important genetic role in the restoring process of CMS line.

With respect to the restorer line Giza181R and its crosses, variations in number and intensity of bands were shown in Figure 1b as well as Table 6. The protein profiling showed a total number of 32 polypeptide bands with diverse molecular weights. Ten common monomorphic bands (No. 2, 14, 18, 20, 21, 23, 27, 30, 31 and 32) were detected between the highest and the lowest parental groups of Giza181R and their F_1 crosses, but bands No. 4, 5, 8, 12, 13, 16 and 24 were parental monomorphic, and they appeared in all the highest and lowest yield parents, while the other bands were polymorphic. Band No. 19 with MW of ~33 kDa was completely absent in the lowest parents, but appeared in some individuals of the highest parental group as well as some of the highest and lowest crosses' groups. Concerning the mean number of bands, it was nearly equal in the highest and lowest groups of the restorer parent Giza181R (25.8 and 25.5 bands, respectively), while it was increased in the highest crosses group (24 bands) than the lowest one (21.7 bands). This result revealed that the mean number of bands decreased in the F_1 crosses groups compared to that of the parental groups, indicating that the presence of restorer genes reduced the amount of extracted protein. This was in line with the previous reports which suggested that restorer (*Rf*) genes act to reduce the accumulation of CMS-associated RNAs and/or proteins in F_1 hybrids. Wang et al. (2006) reported that RF1A cleaves the atp6-orf79 transcript, and RF1B promotes its degradation, suggesting two distinct pathways for restoration. Also, Kazama et al. (2008) suggested that RF1 can bind directly to the CMS-RNA atp6-orf79.

The protein banding patterns for the restorer line Giza182R and its crosses were illustrated in Figure 1c and Table 7. The obtained data revealed a total number of 33 bands. Certain common monomorphic bands such as No. 3, 16, 20, 21, 22, 24 and 28 were observed in the highest and lowest parental groups and their crosses, but band No. 5 was found in all the highest and lowest parental groups and their crosses, except only one individual in the lowest crosses. This might be due to prominence of proteins. It was obvious that band No. 25 (~23 kDa), which was absent in the highest parental group, appeared in four individuals of the lowest parental group as well as some of the highest and lowest F_1 crosses. The protein profile of hybrids revealed that the male line exhibited some bands which supposed to appear in the hybrids but were absent. This might be due to the dominant nature of protein and was not expressed in heterozygous condition of the hybrid. For the mean number of bands, the highest number of protein bands (23.7 bands) was recorded in protein profile of the highest crosses group which was higher than their male parents, indicating the contribution of that protein from female parent to hybrid. The results of the lowest group of parents and their crosses revealed that changes in the mean number of bands were limited (22.8 and 22.7 bands, respectively). Thus, the bands of the parental line and its F_1 were almost the same but there were obvious differences in intensity.

The importance of varietal characterization through electrophoretic banding pattern was earlier reported in rice genotypes by Nethra et al. (2007), Patra and Chawla (2010) and Galani et al. (2011). Electrophoresis of proteins had showed promising results in genetic purity determination of hybrids and parental lines (Aksyonov, 2005).

Molecular characterization of fertility restoration

Polymerase chain reaction analysis for M2, RM3425, RM258, RM1108 and RM5373 markers, which were reported to be linked to *Rf* genes, was conducted with purified DNA samples of the highest and lowest parents in restoring ability (based on seed set %) of the three tested restorer lines; Giza178R, Giza181R and Giza182R, as well as the CMS line. For the four markers: M2, RM3425, RM258 and RM5373, only one DNA band with different sizes for each marker was observed in all the highest and lowest parents for the three varieties (Figure 2a, b, c and e), whereas only RM1108 marker showed polymorphism between the highest and lowest Giza181R parent (Figure 2d).

Based on the previous studies, it was clear in this study that M2 marker showed one band with an expected size of 520 bp in all the highest and lowest restorer lines, but not in the CMS line (Figure 2a). M2 is a dominant

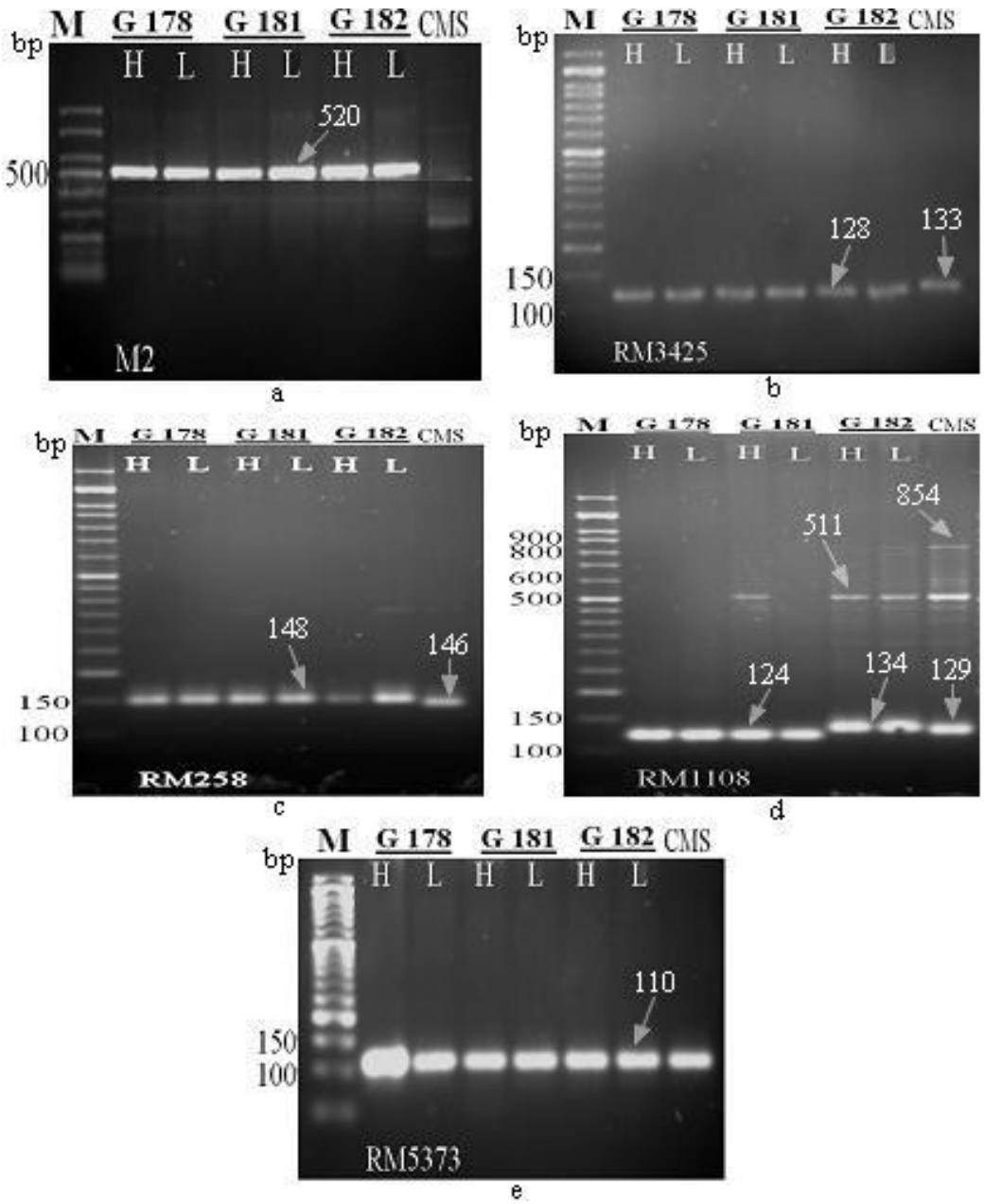

Figure 2. Profiles of DNA amplification products generated from M2, RM3425, RM258, RM1108 and RM5373 markers for the highest (H) and lowest (L) parents of the three restorer lines (Giza178, Giza181 and Giza182), as well as the CMS line (IR69625A). M = 50 bp DNA size marker.

PCR-based marker that detected the genomic region tightly linked to the *Rf-1* allele, from which many restorer lines have been developed (Li and Yuan, 1986) and it is expected that many restorer lines are recognized by M2. The genotype at the *Rf-1* locus is *Rf-1*/*Rf-1* in restorer lines. Thus, restorer seeds contaminated with seeds of *Rf-1*/*rf-1* or *rf-1*/*rf-1* should be discarded before the seed production of hybrids. This treatment decreases the possibility that sterile plants would be generated by fertilizing the CMS plants by *rf-1* pollens in hybrid seed production. This result supported the fact that M2 could be useful to test the seed purity of F_1 hybrids and their parents efficiently.

With respect to the RM3425 marker, our results revealed that a certain band with an expected size of 128 bp appeared in the three highest and the three lowest restorer lines, while another band was observed as a different allele with a molecular size of 133 bp in the CMS line (Figure 2b). Therefore, it is believed that all restorer lines carry the same allele which may be capable of

fertility restoration for the CMS line. It is well known that RM3425 is a co-dominant SSR marker that linked to *Rf-3* locus (Attia et al., 2009), which is located on the short arm of chromosome 1 of Indica rice (Alavi et al., 2009). However, *Rf-3* is a major restorer gene to cytoplasmic male sterile type wild abortive (WA) (Garg et al., 2006).

Concerning the RM258 marker, the results showed that one band with expected size of 148 bp was observed in the three highest and the three lowest restorer lines. In addition, another band with a molecular size of 146 bp was revealed only in the CMS line. So, it was clear that all restorer lines carry the same band which may link to one or more of *Rf* loci: *Rf-4*, *Rf-5*, *Rf-(u1)* or *Rf-6(t)* (Figure 2c). This finding was in agreement with that of Hashemi et al. (2009), who reported that the RM258 loci were monomorphic. Based on the previous studies, at least three major genes [*Rf-3*, *Rf-4* and *Rf-*(*u1*)] along with many QTLs control the trait of fertility restoration for WA cytoplasm in different restorer lines and each restorer line may have a different combination of alleles of these genes and QTLs which ultimately decide the degree of fertility restoration ability of that particular line. Huang et al. (1999) identified a microsatellite marker RM258 linked with fertility restorer gene *Rf-5* for Hl-type CMS at a genetic distance of 7.8 cM on chromosome 10. On the other hand, Mishra et al. (2003) reported that RM258 marker located on chromosome 10 was found to be linked to the restorer gene *Rf-(u1)* in a basmati quality restorer line PRR78 R and it was mapped at a distance of 9.5 cM from the restorer locus. Linkage analysis on F_2 recessive class showed that RM258 is flanked to restorer gene *Rf4* at a distance of 3.1 cM (Nematzadeh and Kiani, 2010). *Rf-6(t)* gene which confers partial fertility in plants with WA-type CMS (Tan et al., 1998; Jing et al., 2001), was also identified on chromosome 10 in the region between the SSR markers RM311 and RM258 (McCouch et al., 2002).

With respect to RM5373 marker, the results showed the presence of a DNA band with an expected size of 110 bp. This band was identified by all the studied restorer lines (the highest and the lowest), as well as the CMS line (Figure 2e). No evidence for the presence of polymeric bands appeared. Therefore, the RM5373 marker could not distinguish between any of the studied lines which carry the same allele. RM5373 is a SSR marker which has been reported to link to *Rf-5* and *Rf-6(t)* loci that are located on chromosome 10. Liu et al. (2004) mapped the nuclear fertility-restorer genes *Rf-5* and *Rf-6(t)* of HL-type CMS on chromosome 10. The first *Rf* locus was *Rf-5*, co-segregated with the SSR marker RM3150, and was flanked by RM1108 and RM5373, which were 0.9 cM and 1.3 cM away, respectively. Another *Rf* locus, designated as *Rf-6(t)*, co-segregated with RM5373, was flanked by RM6737 and RAPD marker SBD07 at genetic distances of 0.4 cM.

In the case of RM1108 marker, it was observed that more than one band were amplified. The first band with an expected size of 124 bp was revealed in the highest and lowest Giza178R and Giza181R parents. The second band with a molecular size of 134 bp appeared in the highest and lowest Giza182R parents. Another band with a molecular size of 129 bp appeared only in the CMS line. Therefore, the two different alleles which were observed in the three restorer lines (the highest and the lowest) with molecular sizes of 124 and 134 bp may link to one of *Rf* loci; *Rf-4*, *Rf-5* or *Rf-1*. But Giza182R parent may carry *Rf* loci different from those in the other two restorer parents (Giza178R and Giza181R) as shown in Figure 2d. Moreover, two extra bands with different sizes appeared as shown in Figure 2d. The DNA band with the molecular size of 511 bp was of specific interest, while it appeared in the highest parent of the restorer line Giza181R and was absent in the lowest parent of the same line. It was expected that this band should appear in the highest parents of the other two restorer lines (Giza178R and Giza182R) and disappear in the lowest parents of both restorer lines. It was surprising that this band (511 bp) was absent in the highest and lowest parents of the restorer line Giza178R and present in the highest and lowest parents of Giza182R in addition to the CMS line. The second band with a molecular size of 854 bp was observed only in the CMS line. Sattari (2004) demonstrated that *Rf-4* for the WA-CMS system is linked to RM1108 (1.6 cM) in a chromosomal region that is also known to be associated with the *Rf-1* and *Rf-5* loci. A previous report of Jing et al. (2001) reported that the three rice nuclear fertility restorer genes [*Rf-1*, *Rf-4* and *Rf-5(t)*] for the three types of CMS (BT, WA and HL) could be allelic for different alleles or haplotypes of a single nuclear locus that can restore the fertility of different types of CMS.

Yashitola et al. (2002) indicated that confirmation of genotypes by an unlinked marker is a reliable approach for assessing hybrid seed purity. A single polymorphic marker should suffice to ascertain hybrid seed purity in rice. Also, Nandakumar et al. (2004) successfully employed a single restorer gene linked marker assessment for testing genetic purity of hybrid seeds that substantially reduced the time, space and labor.

Conclusion

Although the seed purity of hybrids and their parents can be estimated by growing the plants in the field and checking fertility and other traits, it requires a significant amount of time and resources. Protein profile and DNA marker technology is expected to solve this problem. Proteins are not sensitive to environmental fluctuations and its banding pattern is very stable which advocate for cultivar identification purpose in crop. It may now be possible to test seed purity by genotyping plants with PCR-based markers. The use of molecular markers linked to *Rf* genes can enhance the selection efficiency, save time and avoid the complications associated with

phenotype-based screening.

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