Establishment of a salt tolerant somatic hybrid through protoplast fusion between rice and ditch reed

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ABSTRACT

Inter-generic somatic hybridization of rice and ditch reed protoplasts, followed by selection for salt tolerance, yielded 12 salt-tolerant lines. One line was field tested for four seasons in hypersaline soils and the yield per feddan ranged between 3.2 and 4.5 tons. Compared to rice, grains of this somatic hybrid had slightly higher contents of protein and carbohydrates. Among amino acids, proline content of the hybrid was almost 30 times that of rice, cysteine was almost 20 times and glysine more than 10 times. Grains had astonishingly higher content of iron, manganese and zinc. The RAPD technique was used on the genomic DNA of parents and the somatic hybrid. Eleven decamer primers were used and 221 amplification products were observed. Twenty one bands were amplified from the somatic hybrid that were not amplified from either parental DNA. Ten RAPD markers derived from the ditch reed parent were identified in the salt-tolerant somatic hybrid. With respect to the anatomical analysis, the blade leaf, midrib, mesophyll and protoxylum vessels were similar in thickness to those of ditch reed. In general, the stem anatomical parameters of the somatic hybrid were intermediate between those of rice and ditch reed.

Keywords: *Inter-generic somatic hybridization, Salt tolerance, Protoplast Fusion, RAPD, PCR.*
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INTRODUCTION

Salinity remains one of the oldest and most serious problems in agriculture. The total area affected by mineral toxicity is about 25% of the world's potentially arable land (Reghave and Nabors, 1985). Nearly one third of the 230 million hectares of lands irrigated worldwide has become saline (McWilliams, 1986). Interestingly, a large number of photo-synthetically efficient (C4) plant species are productive under a wide variety of abiotic stresses. Halophytic species grow throughout their life cycle in salt marshes, and estuaries or saline deserts. These tolerant species are potential sources for salt-tolerance genes, as well as other stress-tolerance genes.

Phragmites communis, known as ditch reed, is a bamboo-like grass that is tolerant to brackish water and flooding conditions. Somatic cell hybridization of rice (*Oryza sativa*) and ditch reed *via* protoplast fusion was considered to be a useful approach for overcoming sexual incompatibility barriers and for the introduction of salt-tolerance trait

into rice. Cell fusion was reported previously between rice and other plants such as soybean (Niizeki *et al.*, 1985), weedy barnyard grass (Terada *et al.*, 1987), and alfalfa (Niizeki *et al.*, 1992). This is the first report on a somatic hybrid between rice and ditch reed. Selected rice X ditch reed somatic hybrid would combine the economical traits from rice with the abiotic stress tolerance from ditch reed. These hybrids could then serve as a source from which salt-tolerance genes can be identified, isolated, and transferred to other plants via recombinant DNA techniques.

In this study, a salt-tolerant somatic hybrid between rice and ditch reed was produced by fusion of protoplasts from rice and ditch reed. The salt-tolerant somatic hybrid produced was characterized,when field tested, with respect to yield and grain quality. RAPD-PCR analysis (Williams *et al.*, 1990; Dioh *et al.*, 1997) was also performed on the genomic DNA of the hybrid and the two parents as a starting step towards the molecular cloning of salt tolerance gene(s).

MATERIALS AND METHODS

This work started 1991 in the Plant Biotechnology Centre, Faculty of Agriculture, Cairo University.

Initiation of callus from rice and ditch reed: Calli were initiated from mature rice embryos (var. Giza-176) and from ditch reed leaf blades. Mature rice seeds or ditch reed leaf blades were surface sterilized in 1.5% sodium hypochlorite for 45 min., washed three times in sterile distilled water, and placed on MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 3% sucrose. In addition, rice embryo medium contained 1 mg/l 6-Benzyl-aminopurine (BAP), and 100 mg/l glutamine. Rice embryos or ditch reed leaf blades were cultured in the dark for one week then transferred to grow under low light intensity (10 $\mu E/m^2/s$) (Micro Einsteins/ meter square/ second) at a temperature of 30ºC. Callus developed from the scutellum of the embryo within three weeks was sub-cultured for 2 more weeks onto fresh culture medium with the same compounds.

Protoplast isolation: Protoplasts were isolated from callus derived from rice mature embryo. In brief, the callus (3g) was placed in petri dishes containing 30 ml of an enzyme mixture consisting of 3% cellulase (Onozuka Sigma Chemical Co.), 1% pectinase (Sigma Chemical Co.) and 0.4 M mannitol, at pH 4.5, with 400 mg/l ampicillin (Flow Lab), 10 mg/l gentamycin (Flow lab), 10 mg/l tetracycline (Sigma Co.) and CPW salts (El-Shihy 1986). The mixture of protoplasts and enzymes was maintained statically in the dark for 6 hours at 30ºC. Ditch reed protoplasts were isolated by the same protocol used for rice protoplasts. The isolation and purification procedures established by El-Shihy (1990) were applied. Cell viability of the protoplasts was tested by staining with fluorescein diacetate (FDA) according to Widholm (1972). The yield per gram fresh weight was 5×10^5 rice protoplasts (viability of 60%), and 2.5×10^5 ditch reed protoplasts (viability of 55%). Cell wall regeneration was tested by staining with calcofluor white (Nagata and Takebe, 1970). Cell division was tested using hematocytometer slide and photomicroscope and data were calculated as percentage of the total cells.

Cell fusion: The electrofusion was established by using BIOJET (Bio-med, Germany). The stainless steel electrodes were removed from the polymethylmethacylate ring chamber and autoclaved. The polymethylmethacylate ring chamber was sterilized with 70% ethanol. The sterilized components were assembled in a laminar flow hood. A mixture of protoplasts (2.5×10^7/ml) was suspended in 0.4 M

mannitol, 0.1% MES (2-(N-Morpholino) ethane sulfonic acid (Sigma Chemicals), pH 5.8 and 200 ul aliquots of the suspension was sucked into a sterile syringe and injected into the chamber. Then the whole set-up was transferred to a refrigerator for precooling at 4◦ C for 15 min. An AC electric pulse (3000KHZ, 250 V/cm, 10 sec x3) was followed by DC pulses (3KV/cm, 10μsec x3) at room temperature and protoplasts were cultured immediately at a density of $5x10^7$/ml in MS culture medium with 2 mg/l 2,4-D, 3% sucrose at pH 5.8. Five grams sea salt (Sigma Chemical Co.) were added to one litre of the culture medium and the protoplasts were incubated in the dark at 30◦C.

Plant regeneration: Hybrid calli were transferred onto a regeneration medium containing MS medium, 3% sucrose, 2 mg/l BAP, and 0.5 mg/l 2,4-D, and maintained at 30 ◦C under light intensity of 30 $\mu E/m^2/s$ and a photoperiod of 8 hours dark. Within 3- 4 weeks shoots were transferred onto MS medium with 2 mg/l NAA, and 3% sucrose for two weeks for rooting. They were then transferred to pots in the greenhouse and grown to maturity and seed set. For five seasons (1994-1998) larger sized seeds were selected and grown under salinity stress using sea water for irrigation, both in the greenhouse and in a small area of land in the college farm in Giza.

Field test: The field test started 1999 and continued for four successive seasons in hypersaline soils in two governorates (Beni Suef and Fayoum). The salinity of water used for irrigation ranged between 1000 and 2000 ppm.

Chemical analysis of grains: Total nitrogen was determined in rice and new line of rice using automated Kjeldahl procedure according to (A.O.A.C., 1998). Total hydrolysable carbohydrates were colorimetrically determined as outlined by Dubois *et al.* (1956). Amino acid determination was performed according to the method of Widner and Eggum (1966). The system used for the analysis was high performance amino acid analyzer (Beckman 7300). Thiamine (vitamin B1) and riboflavine (vitamine B2) were determined according to Bongar (1992), using Beckman HPLC system.

Crude ash was measured by official methods of analysis (A.O.A.C., 1998). For the determination of total phosphorus, potassium, calcium, magnesium, manganese, iron, zinc, cupper, molybdenum, boron, lead, titanium and sodium, the wet digestion method using sulfuric and perchloric acids was used. Phosphorus was estimated colorimetrically according to King (1951). The other major and trace elements were determined using atomic absorption spectrophotometer (GBS 932 AA).

Anatomical analysis: Paraffin blocks containing leaves and stem (as described by Johanson, 1940) were cross-sectioned (17 μ thick) with rotary microtome. The prepared slides were then paraffinied and stained with safranin and malachite green. The sections were examined and photographed to study the difference and similarity between the rice, hybrid and ditch reed plants.

RAPD-PCR screening: Genomic DNA extractions and RAPD amplification were performed as described by Arnau *et al.* (1994) and Lin *et al.* (2000). PCR analysis was performed with a Perkin Elmer Cetus 9700 GeneAmp apparatus under the following conditions: one cycle of 3 min at 95°C and 45 cycles of 1 min at 95°C, 2 min at 35°C, 2 min at 72°C followed by one cycle of 15 min at 72°C. Eleven decamer primers were used (Table 4) at 1 μM with 50 ng of DNA, 2.5 μl of 10X buffer ($MgCl_2$ at 1.5 mM final), 0.2 mM dNTP, 1 U *Taq* polymerase and

autoclaved water up to 25 µl. Amplification products were separated by gel electrophoresis on 1.5% agarose gel in TAE at 5 V/cm for 3 hr. Analysis was repeated twice from extraction to electrophoresis of amplification products.

RESULTS AND DISCUSSION

Although the ditch reed derived callus grew well on culture media with or without relatively high salinity level, the protoplasts obtained from this callus exhibited cell wall regeneration after 48 hr, but no further growth was observed. On the other hand, rice callus-derived protoplasts grew till plantlet formation and maturity on a culture medium without salt. With salinity of 5 g/l sea water, cell wall regeneration was evident after 48 h but no cell division occurred. Under relatively high salinity (5 g/l sea water) the hybrid protoplasts were the only protoplasts exhibiting cell division (5.2% after 4 days), colony formation(Figure 1.A) and callus formation till whole plants.

After two weeks, small colonies were obtained without any addition of fresh medium. When 0.5 ml of fresh medium lacking mannitol and 2,4-D was used to dilute 5 ml of the culture medium every two days, the small colonies continued their growth till callus formation. The growth of callus continued on solidified agar (0.8%) MS medium with 2 mg/l 2,4-D at a relatively low light intensity (10µE/m^2/s). These calli continued their growth till the production of whole plantlets (Figure 1.B). Hundreds of hybrid calli were obtained and hundreds of different hybrid plantlets were produced as well. Some of the plantlets had phenotypes similar to ditch reed plants and some to rice plants and some were intermediate between both. The salt-tolerant hybrid plants which were quite similar to the rice plants were selected.

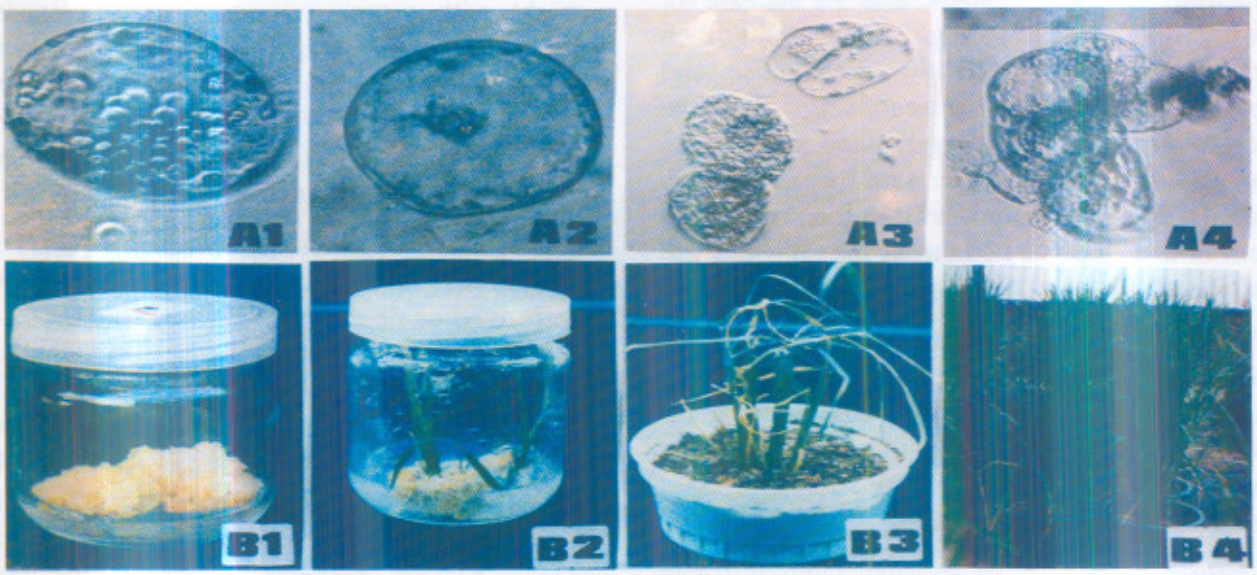

Fig. (1): A) Rice protoplast (A1) and ditch reed protoplast (A2) followed by cell fusion (A3) and small colony formation (A4), B) Callus derived from hybrid protoplasts (B1) followed by plant regeneration and whole plant formation (B2) then transferred into pots (B3) to continue its growth in the greenhouse (B4).

In all, 12 lines were selected, including one that was very tolerant to high salinity. In 1993, rooted plantlets of this line were transferred to pots in the greenhouse and grown to maturity and seed set. For five seasons (1994-1998) larger sized seeds were selected and grown under salinity stress using sea water for irrigation, both in the greenhouse and in a small area of land in the college farm (in Giza). The first field test started in 1999 for four successive seasons. (Table 1) shows the yield of this somatic hybrid in hypersaline soils (Figure 2). All the Giza-176 rice plants grown for comparison in the same fields died off. The average grain production per feddan ranged between 3.2 and 4.5 tons.

Table (1): Yields of grains and protein of the somatic hybrid in two governorates.

Season	Governorate	Area (Feddan)*	Salt in Soil (ppm)	Yield / Feddan (Ton)	Weight of 100 grains (gm)	Crude Protein (%)**	Protein Yield (kg) / feddan
1999	Beni Suef	0.50	32000	3.20	3.20	9.65	308.8
2000	Beni Suef	5.00	32000	3.60	3.15	9.60	345.6
2001	Fayoum	6.00	32000	4.32	3.21	9.62	315.6
2001	Beni Suef	10.00	33000	4.18	3.20	9.65	403.4
2002	Beni Suef	5.00	32000	4.50	3.20	9.65	434.3
2002	Fayoum	5.00	32000	4.50	3.21	9.64	433.8

(*) Feddan =4200 m^2

(**) Percentage of crude protein = nitrogen content x 6.25.

Fig. (2): Somatic hybrid grown in hypersaline soils.

Compared to Giza 176, grown in Giza, grains of this somatic hybrid had slightly higher content of crude protein and carbohydrates (Table 2). Among the amino acids, proline content of the somatic hybrid was almost 30 times that of the Giza rice, cysteine was almost 20 times, and glycine more than 10 times. The ash content was more than double that of the Giza-176 rice. Moreover, it had astonishingly higher contents of iron, manganese and zinc. The blade leaf, midrib, mesophyll and protoxylum vessels were similar in thickness to those of ditch reed. In general, the stem anatomical parameters of the somatic hybrid were intermediate between those of rice and ditch reed (Table 3 and Figure 3).

Table (2): Nutrients, amino acids contents and major and trace elements of rice (R) and somatic hybrid (SH).

Component	R	SH	SH/R
Crude Protein (%)*	8.40	9.65	1.15
Ash (%)	1.40	3.38	2.41
Total carbohydrates (%)	63.40	70.55	1.11
Thiamin (mg/100g fwt)	0.04	0.04	1.00
Riboflavin (mg/100g fwt)	0.35	0.36	1.03
Elements (mg/g dry wt):			
Potassium (K)	4.85	12.39	2.55
Phosphorus (P)	15.12	32.48	2.15
Calcium (Ca)	17.35	38.25	2.20
Magnesium (Mg)	1.45	5.65	3.90
Manganese (Mn)	0.05	0.89	17.80
Iron (Fe)	0.11	3.89	35.36
Zinc (Zn)	0.80	4.92	6.15
Copper (Cu)	0.04	0.12	3.00
Sodium (Na)	1.00	2.85	2.85
Molybdenum (Mo)	1.25	2.85	2.28
Boron (B)	1.71	3.84	2.25
Lead (Pb)	0.55	0.72	1.31
Titanium (Ti)	1.90	2.28	1.20
Amino Acids (%):			
Asparatic acid	0.54	2.92	5.41
Threonine	0.21	0.21	1.00
Serine	0.31	0.32	1.03
Glutamic acid	1.10	4.55	4.14
Proline	0.20	5.85	29.25
Glycine	0.30	3.20	10.66
Alanine	0.31	0.55	1.77
Cysteine	0.05	0.98	19.60
Valine	0.21	0.75	3.57
Metheonine	0.05	1.24	2.48
Isoleucine	0.15	0.15	1.00
Leucine	0.42	0.43	1.02
Tyrosine	0.21	0.22	1.05
Phynylalanine	0.30	0.30	1.00
Histidine	0.12	0.19	1.58
Lysine	0.22	1.94	8.82
Arginine	0.42	0.78	1.86

(*) Percentage of crude protein = nitrogen content x 6.25.

Table (3): Leaf and stem anatomical parameters (in μm) in rice (R), somatic hybrid(SH), and ditch reed (D).

Organ	Parameters	R	SH	D
Leaf	Blade Leaf Thickness	140	220	205
	Midrib Thickness	1200	820	890
	Mesophyll Thickness	90	170	150
	Protoxylem Vessel Thickness	25	35	40
	Metaxylem Vessel Thickness	45	30	60
	Phloem Thickness	80	50	50
	Vascular bundles Thickness	140	130	160
	Cuticle Thickness (Upper Epidermis)	20	18	10
	Air Spaces (average)	2600	860	Not Present
Stem	Diameter	720	815	1055
	Layers of The Ground Tissue(*)	(18)	(19)	(20)
	Thickness of Ground Tissue	680	770	1015
	Thickness of Vascular Bundles	85	98	195
	Air spaces (average)	1090	2040	2135

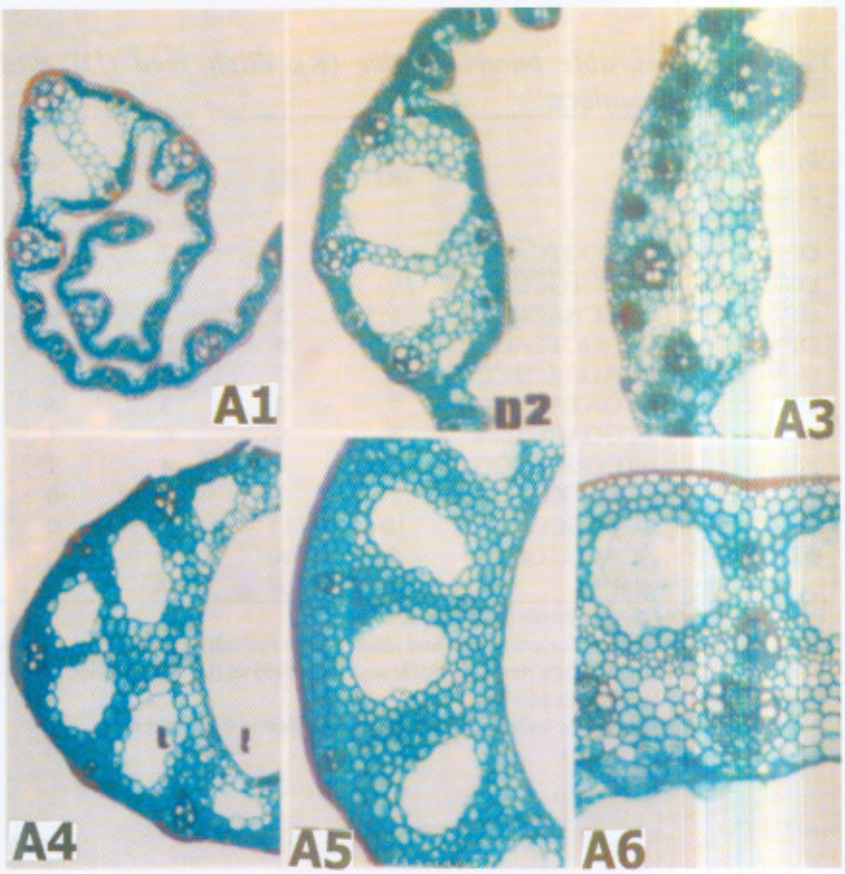

Fig. (3): Leaf anatomical parameters (100x) in rice (A1), somatic hybrid (A2) and ditch reed (A3). Stem anatomical parameters (100x) in rice (A4), somatic hybrid (A5) and ditch reed (A6).

Decamers were used to amplify RAPD bands from genomic DNA of the selected salt-tolerant somatic hybrid and the two parents. RAPD marker amplification from parental and hybrid lines was highly reproducible in all the assays performed. Out of 25 primers examined, only 11 decamers produced scorable bands. The remaining decamers either did not produce any bands or the bands were not clear enough to be evaluated. In all, 221 amplification products were observed. In the evaluation of band number, only those bands with enough intensity and difference in size from neighboring fragments were used. The size of the RAPD bands produced was between 230 bp to about 2100 bp (Figure 4 and Table 4 and 5). Several bands were amplified from the somatic hybrid that were not amplified from either parental DNA. Twenty-one new nonparental RAPD bands were identified in the hybrid (Table 4) and might represent stable genomic modifications. The highest number of new nonparental RAPD bands observed for the somatic hybrid was 6 bands for primer OPK-10 or primer OPP1 (Table 4). Quantitative DNA changes were also found in the somatic hybrid as compared to one or both of the parental lines. Ayliffe *et al.* (1994) reported that nonparental bands may arise in RAPD due to the formation of heteroduplex molecules between two allelic sequences of different size. The high genetic variability observed in the somatic hybrid may be due to genetic recombination in protoplasts or during regeneration after protoplast fusion.

Table (4): Scorable bands in rice (R), ditch reed (D), and somatic hybrid (SH) for the 11 decamers.

Primer & Sequence		(R)	(D)	Shared Bands in (R) and (D)	(SH)[1] (R)[2]	(D)[3]	(R&D)[4]	New[5]	Total
OPK-10	GGTGGTCAAG	3	2	2	1	0	1	6	8
OPK-03	CTCCTGCCAA	9	4	1	4	1	0	0	5
OPP-01	GTGCAACGTG	5	5	5	0	0	3	6	9
OPM-13	CCAGCTTAGG	3	5	0	2	2	0	2	6
OPK-15	GTAGCACTCC	6	7	2	1	4	0	3	8
OPP-03	CTGATACGCC	7	5	3	3	1	1	1	6
FS15	TCGGACGTGA	7	9	6	1	1	6	1	9
FS26	ATCGGCTGGG	7	4	4	2	0	3	1	6
FS27	AGCCGGCCTT	7	7	7	0	0	5	0	5
FR27	ACGCGCGGGA	10	11	10	0	1	9	1	11
BP22	ACAGGTGGTT	9	10	6	2	0	4	0	6
	Total	73	69	46	16	10	32	21	79

[1]Parental origin of RAPD bands in the somatic hybrid.
[2]Bands derived from the rice parent(R) and identified in the salt-tolerant somatic hybrid(SH).
[3]Bands derived from the ditch reed parent(D) and identified in the salt-tolerant somatic hybrid(SH).
[4]Bands shared between both parents.
[5]Bands identified in somatic hybrid (SH)and not identified in both parents.

Table (5): Polymorphic bands and polymorphism (%) for each decamer used.

Decamer	M. Wt. (bp)	Polymor. R	Polymor. RD	Polymor. D	% Polymor.	Decamer	M. Wt. (bp)	Polymor. R	Polymor. RD	Polymor. D	% Polymor.
OPK.10	1600	-	+	-	88.8	OPM.13	1400	-	+	-	100
	1550	-	+	-			1200	+	+	-	
	1120	-	+	-			1000*	-	+	+	
	750	+	-	+			800	-	-	+	
	560	+	+	-			750	+	+	-	
	500	-	+	-			610	-	-	+	
	410	-	+	-			500	+	-	-	
	340	-	+	-			480*	-	+	+	
OPK.03	800	+	-	-	100		380	-	-	+	
	750*	-	+	+			300	-	+	-	
	650	+	+	-		OPK.15	1200	+	-	+	100
	520	+	-	-			1000*	-	+	+	
	450	+	-	+			850	+	+	-	
	400	+	+	-			650	+	-	-	
	350	+	+	-			610*	-	+	+	
	300	+	+	-			580	+	-	-	
	270	+	-	-			550	-	+	-	
	260	-	-	+			500*	-	+	+	
	250	+	-	-			460*	-	+	+	
	230	-	-	+			400	+	-	+	
OPP.01	1600	-	+	-	72.2		370	-	-	+	
	1450	-	+	-			350	-	+	-	
	1300	-	+	-			320	+	-	-	
	940	-	+	-			270	-	+	-	
	850	+	-	+		FS15	1919	+	+	-	40
	770	-	+	-			1554*	-	+	+	
	700	+	-	+			1128	-	+	-	
	600	-	+	-			472	-	-	+	
OPP.03	1600	-	-	+	90	BP22	1412	+	-	+	63.6
	1500	+	+	-			961	+	+	-	
	1300	+	-	-			910	+	-	+	
	940	+	+	-			506	+	+	-	
	770	+	-	+			498	-	-	+	
	560	-	+	-			484	-	-	+	
	530	+	-	+			472	+	-	-	
	480	+	+	-		FS27	1412	+	-	+	28.6
	470*	-	+	+			837	+	-	+	
FS26	910	+	+	-	62.5	FR27	1636*	-	+	+	25
	865	+	+	-			1412	+	-	+	
	837	-	+	-			1081	-	+	-	
	544	+	-	-							
	484	+	-	+							

*Markers derived from the ditch reed parent and identified in the salt-tolerant somatic hybrid.

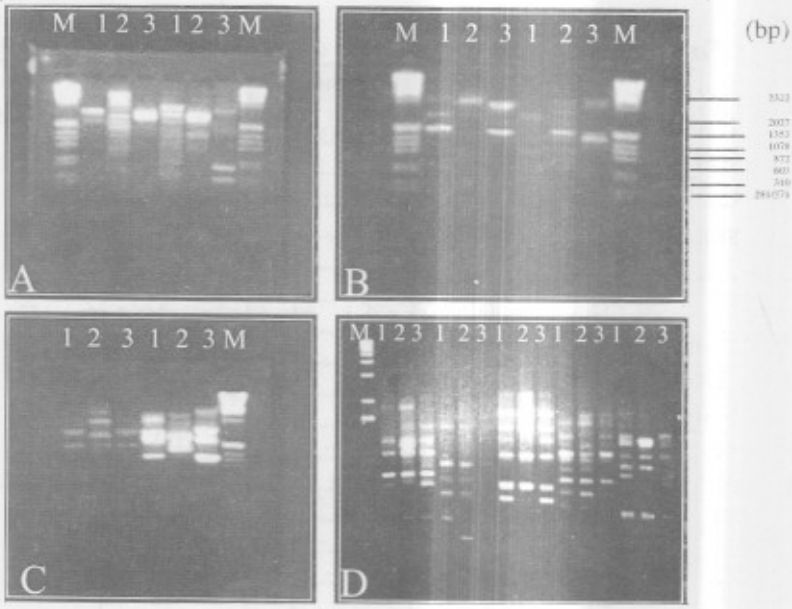

Fig. (4): RAPD-based amplification of genomic DNA using 11 decamers. RAPD profiles were amplified from DNA extracted from rice (1), rice X ditch reed (2), and ditch reed (3). Amplified products were separated by agarose gel electrophoresis. (A) decamers OPK10 and OPK03; (B) decamers OPM13 and OPK15; (C) decamers OPP01 and OPP03; and (D) decamers FS15, FS26, FS27, FR27, and BP22. M indicate molecular weight marker.

RAPD markers characteristic of one or the other parent investigated were identified. Primer FR27 showed the highest numbers of RAPD bands observed for rice (10), ditch reed (11), and the somatic hybrid (11). In all, RAPD bands identified for rice, ditch reed, and the somatic hybrid were 73, 69, and 79, respectively. In the salt-tolerant somatic hybrid, 10 RAPD bands were derived from the ditch reed parent. These RAPD bands will be converted to SCARs (Paran *et al.* 1991; D'Esposito *et al.* 1994) or RFLP markers and used to localize the respective markers on restriction fragments from the ditch reed and the hybrid line. It is expected that the RAPD markers identified in this study will aid the positional cloning (Bent *et al.* 1994) of salt tolerance genes as well as the screening procedure for salt-tolerance.

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الملخص العربي

تكوين هجين خضري مقاوم للملوحة عن طريق دمج الخلايا العارية (البروتوبلاستات) لنبات الأرز ونبات الغاب

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اجري تهجين خضري بين جنسي الأرز (الصنف جيزة ١٧٦) والغاب، بدمج الخلايا العارية لهذين الجنسين، ثم الانتخاب لمقاومة الملوحة، نتج عنه اثنى عشر خطا، أختبر أحد الخطوط في الحقل في تربة عالية الملوحة فتراوح إنتاج الفدان ما بين ٣,٢ طناً و ٤,٥ طناً. اتضح أن حبوب الهجين الخضري تحتوي على نسبة بروتين ونسبة كربوهيدرات أعلى قليلا من الأرز جيزة ١٧٦ ، كما ظهر من التحليل الكيماوي أن الهجين الخضري يحمل من حامض البرولين ما يقرب من ٣٠ ضعف ما يحمله الأرز جيزة ١٧٦ ، ونحو ٢٠ ضعفاً من حامض السيستين وأكثر من عشرة أضعاف من حامض الجلايسين. كانت حبوب الهجين الخضري تحوي قدراً كبيراً للغاية من الحديد والمنجنيز والزنك. استعملت تقنية الرابيد على دنا الأبوين والهجين الخضري، كما استخدمت بادئات عشرية، وظهر أن هناك ٢١ شريطاً في الهجين لا يوجد لها مثيل في دنا الأبوين، كما أمكن كشف عشرة واسمات رابيد في الأرز موجودة في الهجين الخضري. كانت المقاييس التشريحية للهجين الخضري على وجه العموم تقع ما بين المقاييس المناظرة للأرز والغاب.