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Intergeneric somatic hybrids of rice [*Oryza sativa* L. (+) *Porteresia coarctata* (Roxb.) Tateoka]

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Abstract Somatic hybrid plants were obtained following the electrofusion of rice (*Oryza sativa* L. cv 'Taipei 309', 2n = 2x = 24) cell suspension–derived protoplasts with non-dividing leaf protoplasts of *Porteresia coarctata* (2n = 4x = 48), a saline-tolerant wild species. Fusion-treated protoplasts were plated on the surface of cellulose nitrate filter membranes, overlaying *Lolium multiflorum* nurse cells. The nurse cells were embedded in KPR medium containing 0.5 mg l^{-1} 2,4–dichlorophenoxyacetic acid and semi-solidified with SeaPlaque agarose. Putative somatic hybrid cell colonies were selected on the basis of their growth, whereby faster growing colonies were transferred preferentially to MS-based medium with 2.0 mg l^{-1} kinetin, 0.5 mg l^{-1} α-naphthaleneacetic acid, 30 g l^{-1} sucrose and 4.0 g l^{-1} SeaKem agarose to induce shoot regeneration. One hundred and nineteen regenerated plants were micropropagated clonally on MS-based medium containing 2.0 mg l^{-1} 6–benzylaminopurine, 50 g l^{-1} sucrose and 4.0 g l^{-1} SeaKem agarose, prior to DNA extraction of plant samples. Putative somatic hybrids were initially identified by RAPD analysis, and 8 plant lines were selected for further investigation by flow cytometric ploidy determination and cytology. Plants of one line had an allohexaploid chromosome complement (2n = 6x = 72) and, following examination of its vegetative clones by GISH, were confirmed as somatic hybrids containing full chromosome complements of both *O. sativa* and *P. coarctata*.

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Introduction

Somatic hybridisation provides a baseline for increasing genotypic variability in crop species effected, for example, by the transfer of gene(s) imparting resistance/tolerance to biotic and abiotic stresses from wild species. Somatic hybrids with novel agronomically important genes have been produced for several major crops; for example, in the genera *Brassica* (Gerdemann-Knoerck et al. 1995), *Lycopersicon* (Parokonny et al.1996) and *Passiflora* (Otoni et al. 1995).

Progress in developing efficient and reproducible systems for plant regeneration from protoplasts has facilitated the production of rice somatic hybrids following chemical and/or electrical fusion of protoplasts. Somatic hybrids involving the genus *Oryza* include interspecific crosses of the japonica rice *O. sativa* cv 'Nipponbare' with barnyard grass (*Echinochloa oryzicola*) (Terada et al. 1987), japonica rice cultivars with the four wild rice species *O. officinalis*, *O. eichingeri*, *O. brachyantha* and *O. perrieri* (Hayashi et al. 1988) and intraspecific crosses involving haploid cell suspensions of japonica cultivars (cvs 'Yamashoushi', 'Murasakidaikoka') following protoplast electrofusion (Toriyama and Hinata 1988).

Since several requisite crosses, involving rice and wild *Oryza* species have been produced following sexual hybridisation often combined with embryo rescue technology (D.S. Brar, personal communication), somatic hybridisation *per se* is clearly less relevant in the totality of rice breeding. The crucial exception to this is in the production of cybrids for the development of alternative germplasms for hybrid rice production (Akagi et al. 1989, 1995) and crosses which are extremely difficult to achieve by sexual hybridisation. Such crosses include the system described here involving *O. sativa* with *Porteresia coarctata*.

P. coarctata (Roxb.) Tateoka is a halophytic monotypic species which can withstand total submergence in sea water for periods of at least 10 h per day (Bal and Dutt 1986). It closely resembles species in the genus *Oryza*, but differs fundamentally in relation to embryo and leaf morphologies (Duistermaat 1987). Pre-zygotic incompatibilities have led to this species being considered effectively recalcitrant for sexual hybridisation with cultivated rices, although there have been reports of successful sexual crossing (Jena 1994; Farooq et al. 1996; Brar et al. 1997). In view of the limited genetic resources available to breeders in wild rice germplasms for increasing saline tolerance (Akbar et al. 1986), *P. coarctata* is, undoubtedly, one of the key source-species for elevating saline tolerance in cultivated rice.

Materials and method

Plant materials

Plants of *Porteresia coarctata* were propagated vegetatively, since seeds are inherently difficult to germinate (Aldridge and Probert 1993). *P. coarctata* plants [originally collected from the Bay of Bengal by the International Rice Germplasm Centre, International Rice Research Institute (IRRI), Philippines] were obtained from Dr. R.J. Probert (Royal Botanic Gardens, Wakehurst Place, Ardingly, UK) and were grown in 32 × 25-cm plastic tubs containing a 1:1 (v:v) mixture of Levington M3 soil-less compost (Fisons, Ipswich, UK) and John Innes No. 3 compost (Joseph Bentley, Barrow-on-Humber, UK) (Fig. 1 B). Plants were maintained with a 16-h photoperiod: night cycle (28 °C : 24 °C, respectively) with 70% relative humidity and high light intensity (55 µmol m^{-2} s^{-1}, Philips TLD 58W35 Daylight fluorescent tubes). Plants were fed 6 weeks after their initial potting and, subsequently, every 7 days with a 2% (v/v) solution of Bentley's No. 2 Liquid Fertilizer supplemented with 2% (v/v) Maxicrop Liquid Seaweed [with 2% (v/v) sequestered iron; Maxicrop, Corby, UK]. New shoots proliferated rapidly from submerged rhizomes, and these were repotted every 3 months.

Leaf protoplasts of *P. coarctata*

Newly emerged shoots of *P. coarctata* were used for protoplast production following the method of Finch et al. (1990), with enzyme digestion increased to 10–12 h. Released protoplasts were washed once with CPW salts solution (Frearson et al. 1973) supplemented with 604 m*M* sorbitol (CPW11 S solution) prior to two washes and a final resuspension, at a density of 10^5 protoplasts per milliliter, in electrofusion solution (713 m*M* mannitol with 0.5 m*M* $CaCl_2$).

Cell suspension protoplasts of *O. sativa* cv 'Taipei 309'

Embryogenic cell suspension cultures were initiated from mature, seed scutellum-derived calli of *O. sativa* cv 'Taipei 309' using seeds supplied by IRRI (Finch et al. 1991). Protoplasts isolated from cell suspension cultures using an 18-h enzymatic incubation period (Abdullah et al. 1986) were washed once with CPW salts solution supplemented with 713 m*M* mannitol (CPW13 M solution) prior to suspension in electrofusion solution at a density of 10^5 protoplasts per milliliter.

Electrofusion and culture of protoplasts

The electrofusion apparatus was as described by Jones et al. (1994). Parental protoplasts were mixed [1:1 (v:v) ratio] and aliquots (1.5 ml) dispensed into 25-well plastic dishes (Bibby-Sterilin, Stone, UK). Aliquots of protoplasts of each parental species were also dispensed separately into wells for self-fusion (homokaryon production) and to act subsequently as culture response controls. Protoplasts were allowed to settle (1 min) before the electrode was progressively introduced into each well of the dish for electrofusion as described previously (Blackhall et al. 1994). Protoplasts were aligned as "pearl chains" using a 1 Mhz AC field (280–430 V cm^{-1}) for 2–4 s followed by a 0.5-msec DC pulse (1 kV cm^{-1}) to induce fusion. Immediately after fusion, 0.75-ml aliquots were added to each well of liquid KPR culture medium [K8P medium (medium 8P of Kao and Michayluk, 1975, as modified by Gilmour et al. 1989) supplemented with 0.3 mg l^{-1} 2,4–dichlorophenoxyacetic acid]. Protoplasts were incubated, at room temperature, for 1 h. This dilution of the electrofusion solution with KPR medium was repeated twice. Subsequently, treated protoplasts [as well as un-fused (viability controls) and self-fused controls] were pelleted by centrifugation (100 *g*, 7 min) and cultured over nurse cells of *Lolium multiflorum* (Jain et al. 1995), the latter being embedded previously in KPR medium semi-solidified with 1.2 g l^{-1} SeaPlaque agarose (FMC BioProducts-Europe, Vallensbaek Strand, Denmark). A minimum of ten such fusion experiments were undertaken, each utilising 2.6×10^6 protoplasts of both parental species.

Putative somatic hybrid (SH) colonies were progressively and preferentially selected from the dishes of fusion-treated mixed protoplasts based on an initially presumed heterosis in the somatic hybrid, as confirmed subsequently, based on comparisons to control dishes. Colonies, taken 5–6 weeks post-fusion (each 0.5–1.0 mm in diameter) and which developed in the fused mixed species protoplast cultures were transferred individually to the surface of MS-based shoot regeneration medium (Murashige and Skoog 1962) (1.5 ml per well; 25 well plastic dishes) supplemented with 2.0 mg l^{-1} kinetin and 0.5 mg l^{-1} (α–naphthaleneacetic acid (NAA) and semi-solidified by the addition of 4.0 g l^{-1} SeaKem agarose (FMC BioProducts), pH 5.8. A total of 1,493 protoplast-derived colonies sampled from all the fusion experiments were transferred to shoot regeneration medium. The resulting and putatively somatic hybrid shoots were transferred to MS-based micropropagation medium with 2.0 mg l^{-1} 6–benzylaminopurine, 50 g l^{-1} sucrose and 4.0 g l^{-1} SeaKem agarose under continuous illumination (55 µmol m^{-2} s^{-1}, Daylight fluorescent tubes) at 27 °C ± 1 °C. Cloned shoots were sub–cultured every 30 days. Selected plants (of ultimately 119 separate lines/fusion events) were transferred to MS-based medium with 1.5 mg l^{-1} NAA and 4.0 g l^{-1} SeaKem agarose for a period of 21 days to promote root formation, prior to their transfer to the glasshouse. Acclimation of plants to glasshouse conditions was undertaken over a 21-day period as described (Blackhall et al. 1999).

Random amplified polymorphic DNA (RAPD) analysis

For all selected plant lines, including control plant materials, DNA was extracted from 0.2 g (f. wt.) leaf tissue of *in vitro*-grown clones (Dellaporta et al. 1983) taken 15–21days after sub-culture. The DNA concentration was measured by a micro-assay technique based on the fluorescence of Hoechst 33258 using a Hoefer TKO 100 DNA Fluorometer (Amersham-Pharmacia Biotech, Amersham, UK) in accordance with the manufacturer's instructions. RAPD analysis utilised the basic protocol of Otoni et al. (1995), with 2 pg of target DNA per sample. Four primers were utilised; OPAA-06, OPAA-07, OPAA-08 and OPAA-10 (Operon Technologies, Alameda, Calif.). Based on the results from these analyses, a subset of regenerated plants (lines) was identified as being putative somatic hybrids, and individuals (plus controls) were subjected to further analyses.

Flow cytometric analysis

Leaf samples were collected from seed-derived (control) plants and from the somatic hybrids (initially identified by RAPD analysis) that had been sub-cultured for 2–3 weeks on micropropagation

medium. Flow cytometric analysis was performed as described previously (Otoni et al. 1995).

Genome *in situ* hybridisation (GISH)

Preparation of root-tip squashes

Fresh roots were harvested from *in vitro* and glasshouse-grown target plants and root tips placed in 2 m*M* 8-hydroxyquinoline (90–120 min) at room temperature. Root tips were immersed in freshly prepared Carnoy's fixative [6:3:1 (v:v:v) ethanol: ethanoic acid: chloroform] and placed at –20 °C for a minimum of 48 h. After this period, root tips were washed briefly in 10 m*M* citrate buffer (6 m*M* trisodium citrate, 4 m*M* citric acid, pH 4.8) and placed in an enzyme solution [20 gl^{-1} Cellulase 'Onozuka' R-10 (Yakult Honsha, Tokyo, Japan), 10 gl^{-1} Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), 6 m*M* trisodium citrate, 4 m*M* citric acid, pH 4.8] and incubated (37 °C) for 10–12 min. Tips were transferred to a watch glass containing a hypotonic solution (75 m*M* KCl; 16 h, room temperature). Roots were examined to determine whether the apices and, hence, the root meristems, were intact.

Roots, for conventional staining, were hydrolysed in 100 *mM* HCl (10 min) at 65 °C, stained in Feulgen (Schiff's reagent, 60 min) and counterstained on a glass slide in ethanoic orcein (5 min) before being macerated with a brass rod and squashed beneath a coverslip (Fukui and Iijima 1992). The coverslip was sealed with vulcanising solution prior to examination.

Slides were treated with a tissue mordant when preparing chromosome squashes for *in situ* hybridisation using either 10% aqueous poly-L-lysine or silane (amino alkyl silane). Root tips were redigested briefly in the aforementioned enzyme solution (37 °C, 10 min) and macerated in 45% ethanoic acid before squashing beneath a coverslip. The preparations were scanned under phase contrast to determine whether the material was suitable for *in situ* hybridisation.

DNA extraction and denaturation of probes

DNA was extracted from leaves of *O. sativa* and *P. coarctata* using methods described previously (Doyle and Doyle 1990) and resuspended in TE buffer (10 m*M* TRIS-HCl, pH 7.4, 1 m*M* EDTA) to a final concentration of 1 µg μl^{-1}. This extracted DNA, to be used as probe, was sheared by vortexing (5 min) and autoclaving (121 °C, 1 bar, 6 min). DNA fragment size was checked by agarose gel electrophoresis and labelled using a nick translation kit (Oncor, Chester-le-Street, UK) with biotin-16-dUTP according to the manufacturer's instructions. The labelled DNA was mixed with blocking DNA in various ratios to produce the best GISH probe, the blocking DNA having been extracted in a similar manner from the other parent species. The hybridisation solution (35 ml formamide, 7.0 g dextran sulphate, 1.23 g NaCl, 618 mg sodium citrate, 2 ml reverse-osmosis water together with 1 µg of probe) was denatured by incubation at 75 °C (15 min in a water bath), allowed to cool (10 min) and then applied to the target slide.

Denaturation of target material

In some cases, it was found necessary to treat slides with a RNAse A solution [Sigma, Poole, UK; 100 mg ml^{-1} in 2 x SSC buffer (3 *M* NaCl, 300 m*M* sodium citrate)] at 37 °C for 60 min when preliminary phase-contrast observations suggested that chromosome spreads (or nuclei) were masked with cytoplasm and other cell debris. Slides were dipped in a fixative solution [3:1 (v:v) ethanol: ethanoic acid] and allowed to air dry in an incubator at 37 °C for a minimum of 18 h. Preparations were denatured in a Coplin jar containing 70% (v/v) formamide in 2 x SSC buffer at 72 °C for 3 min and then plunged progressively into ice-cold 70% (v/v), 85% (v/v) and 100% ethanol, each for 2 min. Slides were air-dried in an incubator (37 °C) before transfer to a humidified box, followed by the application of the previously denatured hybridisation solution. Plastic coverslips were applied, sealed with vulcanising solution and incubated at 37 °C for 12–20 h.

Visualisation of in situ *hybridisation*

After hybridisation, the coverslips were removed and the slides were given three stringency washes in 50% (v/v) formamide (in 2 x SSC buffer) for 5 min each (45 °C) and placed in 2 x SSC buffer for a further 5 min. Slides were transferred to a humidified box and subjected to the following treatments.

1 Labelling, with 200 µl per slide, of fluorescein isothiocyanate (FITC)-conjugated avidin diluted 1:500 (v:v) in blocking buffer [500 ml 4 x SSC buffer/0.5% (v/v) Tween 20, 25 g non-fat milk powder, 0.1 g sodium azide];
2 Three washes in 4 x SSC buffer/0.5% (v/v) Tween 20, each for 5 min;
3 Signal amplification with 200 µl per slide of biotinylated goat anti-avidin antibody (Cambio, Cambridge, UK) diluted 1:250 (v:v) in blocking buffer;
4 Three washes in 4 x SSC buffer/0.5% (v/v) Tween 20, each for 5 min;
5 Re-labelling with FITC conjugated avidin as per step (1);
6 Three washes in 4 x SSC buffer/0.5% (v/v) Tween 20, each for 5 min.

Excess fluid was gently tapped from the slide and specimens counterstained with 4,6-diamidino-2-phenylindole (DAPI). The aqueous DAPI solution (2 µg ml^{-1}) was diluted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK) to a final concentration of 0.1 µg ml^{-1}. Preparations were observed with an epifluorescence microscope using either UV (DAPI) or blue (fluorescein) excitation. Photographs of each chromosome preparation were taken under dual excitation conditions with 35 mm film (Fujichrome Provia 400; ASA 1600) and by a low-light (CCD) camera linked directly to a dye sublimation colour video printer (Hitachi VY300; Micro Instruments, Witney, Oxon, UK).

Morphological characteristics of regenerated plants

Putative somatic hybrid plants, seed-derived plants of *O. sativa* cv 'Taipei 309' and micropropagated plants of *P. coarctata* were compared morphologically under glasshouse conditions for a range of characteristics (see Table 2).

Results

Culture of fusion products and plant regeneration

Protoplast yields for *P. coarctata* and *O. sativa* were 3.48 ± 1.21 and 7.01 ± 1.65×10^6 g^{-1} f. wt. with viabilities of 79% and 92%, respectively. Electrofusion gave a heterokaryon formation frequency of 3.18% coupled with an overall protoplast plating efficiency for fusion-treated protoplasts of 0.18 ± 0.02% after 28 days. Protoplasts of *P. coarctata* (unfused controls, homokaryons or protoplasts simply mixed 1:1 with protoplasts of *O. sativa*) did not divide. Microcolonies were visible after 21 days, whereupon the larger microcalli in the fusion-treatment dishes were transferred from day 21 onwards to shoot regeneration medium. Plants were regenerated progressively over a subsequent 3–4 week period. For ten independent replicate experiments, a total of 1,493 calli were selectively transferred to regeneration medium, whereby 119 calli (8%) gave 1 (or more) green regenerant plants with 42 (3%) of the remaining calli giving albino regenerants. The overall plant regeneration frequency from calli was therefore 11% (161 plants from 1,493 calli). The

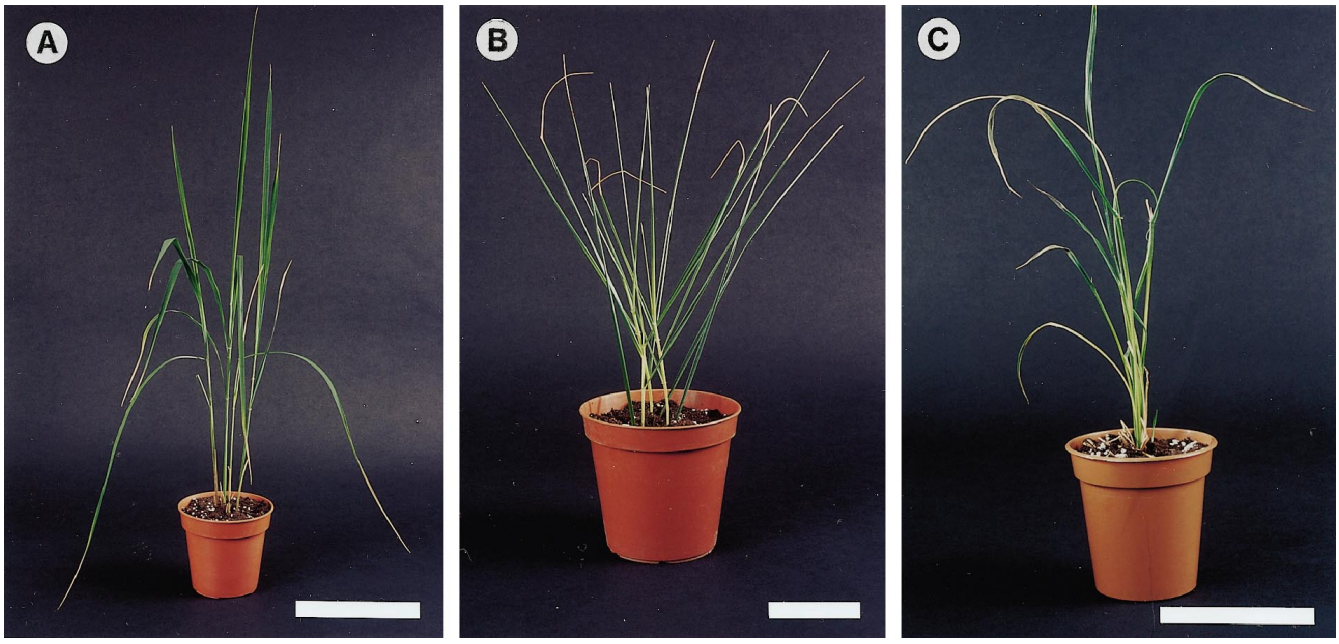

Fig. 1 A-C Glasshouse-grown plants of *Oryza sativa* (**A**), *Porteresia coarctata* (**B**) and the somatic hybrid *Oryza sativa* (+) *Porteresia coarctata* SH61 (**C**). Bar: 9 cm

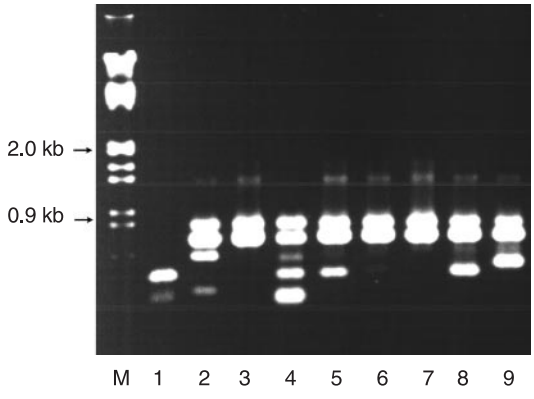

Fig. 2 Representative electropherogram showing products from *O. sativa*, *P. coarctata* and regenerated plants after DNA amplification with primer OPAA-7. *Lane M Eco*R1/*Hin*dIII-digested λ-DNA. *Lanes 1–9* DNA from *P. coarctata* (*1*), *Oryza sativa* (*2*) and plants regenerated from tissues derived from the fusion of protoplasts of *O. sativa* with *P. coarctata* (*3–9*). *Lanes 4, 5* and *8* were considered to contain amplification products from both parents

morphology of a somatic hybrid plant compared with the morphologies of parental plants is shown in Fig. 1 A-C.

RAPD analysis

DNA extracted from 1 plant of each of the 119 green regenerant lines was analysed and compared with extracts from the parent species. Amplified fragments varied in size from 200 to 2,200 bp; a representative example is shown in Fig. 2. For each sample/primer combination, the number of parental species-specific (*O. sativa* or *P. coarctata*) or novel bands was recorded. Eight independently arising plants and, hence, lines, were thus identified as putative somatic hybrids (SH) (Table 1). The presence of novel amplification products (bands) indicated possible somatic recombination of parental genomes, thus eliminating the likelihood that regenerants were chimaeric plants. These plant lines were arbitrarily coded SH26, SH28, SH40, SH41, SH61, SH66, SH90 and SH137 (Table 1) and, following vegetative propagation, cloned offspring were subjected to further analyses.

Flow cytometric and cytological analyses

The DNA contents for the 8 selected somatic hybrid plants and parents reflecting their ploidy status are given in Fig. 3. The relative mean linear fluorescence values for *P. coarctata* and *O. sativa* cv 'Taipei 309' were 218 and 347, respectively. Interphase nuclei of tetraploid *P. coarctata* (2n = 4x = 48) contained 63% of the total DNA content of cultivated rice (*O. sativa* cv 'Taipei 309'). Lines SH40, SH66 and SH90 had a tetraploid DNA content. Interestingly, SH61 had a hexaploid DNA content and the other putative somatic hybrid lines, SH26, SH28, SH41 and SH137, all had notional diploid DNA contents when compared to 'Taipei 309'.

Counts of somatic chromosomes in cells of three to five separate root tips per sample plant are also given in Fig. 3. Plants which were selected as positive by RAPDs had differing root chromosome complements. All were diploid (24 chromosomes) except for lines SH40 and SH90 (tetraploid) and SH61, which approached a hexaploid value of 2n = 6x = 72. Based on this analysis alone, SH40 and SH90 were considered as possible amphi-diploids, whilst SH61 with a chromosome count consistent with the summation of both parental genomes was the symmetric somatic hybrid. Overall, ploidy lev-

Table 1 Origin (and numbers) of RAPD bands used to determine hybridity status of the selected plant lines

Putative SH regenerant (Code number)	Origin [a] of RAPD bands/primer															
	OPAA-06				OPAA-07				OPAA-08				OPAA-10			
	P	T	N	X	P	T	N	X	P	T	N	X	P	T	N	X
SH26	1	1	1	1	0	0	2	2	0	0	0	2	3	0	1	1
SH28	1	1	1	1	0	1	1	2	0	0	0	2	3	0	1	1
SH40	1	1	1	1	0	0	6	0	0	2	0	2	0	4	0	4
SH41	0	2	0	2	0	0	6	0	0	1	0	1	0	4	0	4
SH61	0	2	2	2	0	0	1	3	0	2	0	0	0	0	4	1
SH66	1	0	1	0	0	0	2	3	1	1	0	2	0	1	3	1
SH90	1	0	2	1	0	0	3	2	0	0	0	2	1	4	1	2
SH137	2	0	1	0	0	0	3	1	0	0	1	2	0	1	1	2

[a] **P**, unique to *P. coarctata*; **T**, unique to *O. sativa*, cv 'Taipei 309'; **N**, novel; **X**, expressed in both parental lines

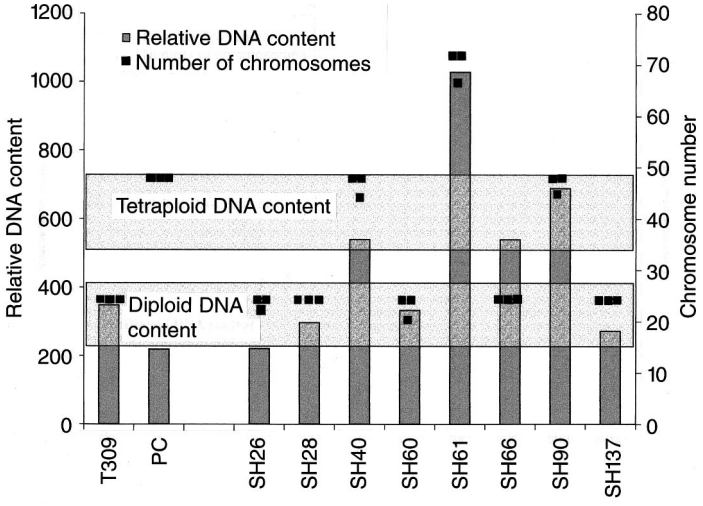

Fig. 3 Relative DNA content as determined by flow cytometry and individual chromosome numbers (▬; three counts for each plant) for *O. sativa* cv 'Taipei 309' (*T309*), *P. coarctata* (*PC*) and putative somatic hybrid plants SH26, SH28, SH40, SH60, SH61, SH66, SH90, SH137. The range of DNA content measurements representing diploid and tetraploid values for *O. sativa* are indicated by *shaded regions*

els determined by flow cytometry were consistent with somatic chromosome counts (Fig. 3). Micropropagated plants of SH61 were thus subjected to detailed GISH analysis.

GISH analysis

Chromosome preparations for plants of line SH61 confirmed that all 72 chromosomes could be counted (Fig. 4 A). Their extremely small size and large number made karyotyping impossible, but some homologues could be matched, adding to earlier preliminary evidence that these plants were composed of diploid sets of chromosomes. As a result of GISH, colorimetric discrimination was observed on the squash-prepared chromosome spreads corresponding to the two genomes (Fig. 4 B,C). The best ratio of probe:blocking DNA was 1:5. A 5-min denaturation in 70% formamide was optimum for probe uptake, although this did result in some morphological degradation of the chromosomes. In line SH61, labelled *O. sativa* DNA bound only to 24 of the 72 chromosomes (Fig. 4 B), representing the complete *O. sativa* diploid genome in the somatic hybrid. These 24 chromosomes were distinguishable from the *P. coarctata* chromosomes (Fig. 4 C) when viewed under UV light. Based on information from Fig. 4 B, the brightly labelled chromosomes in Fig. 4 C are indicated in a separate diagram (Fig. 4 D) to allow identification of the *O. sativa* chromosomes. In Fig. 4 C, the fluorescence from 48 of the DAPI-stained chromosomes is considerably weaker than the fluorescence of the 24 *O. sativa* chromosomes (as identified in Fig. 4 B,D). These 48 chromosomes have a dark-blue fluorescence and are presumed to represent the genome from the *P. coarctata* parent. GISH, where labelled *P. coarctata* DNA blocked with unlabelled *O. sativa* DNA was used (not shown), confirmed that line SH61 contained the *P. coarctata* genome as well as the genome derived from *O. sativa*.

Characterisation of somatic hybrid plants

Under *in vitro* growth conditions, putative somatic hybrid plants (as screened by RAPDs) were more or less phenotypically uniform, except for plants of line SH61 (subsequently confirmed as somatic hybrids by GISH) which were slow growing, a characteristic of *P. coarctata*. Plants of these lines and seed-derived (control) plants of rice were transferred to the glasshouse 2–3 months after rooting alongside vegetatively propagated plants of *P. coarctata*.

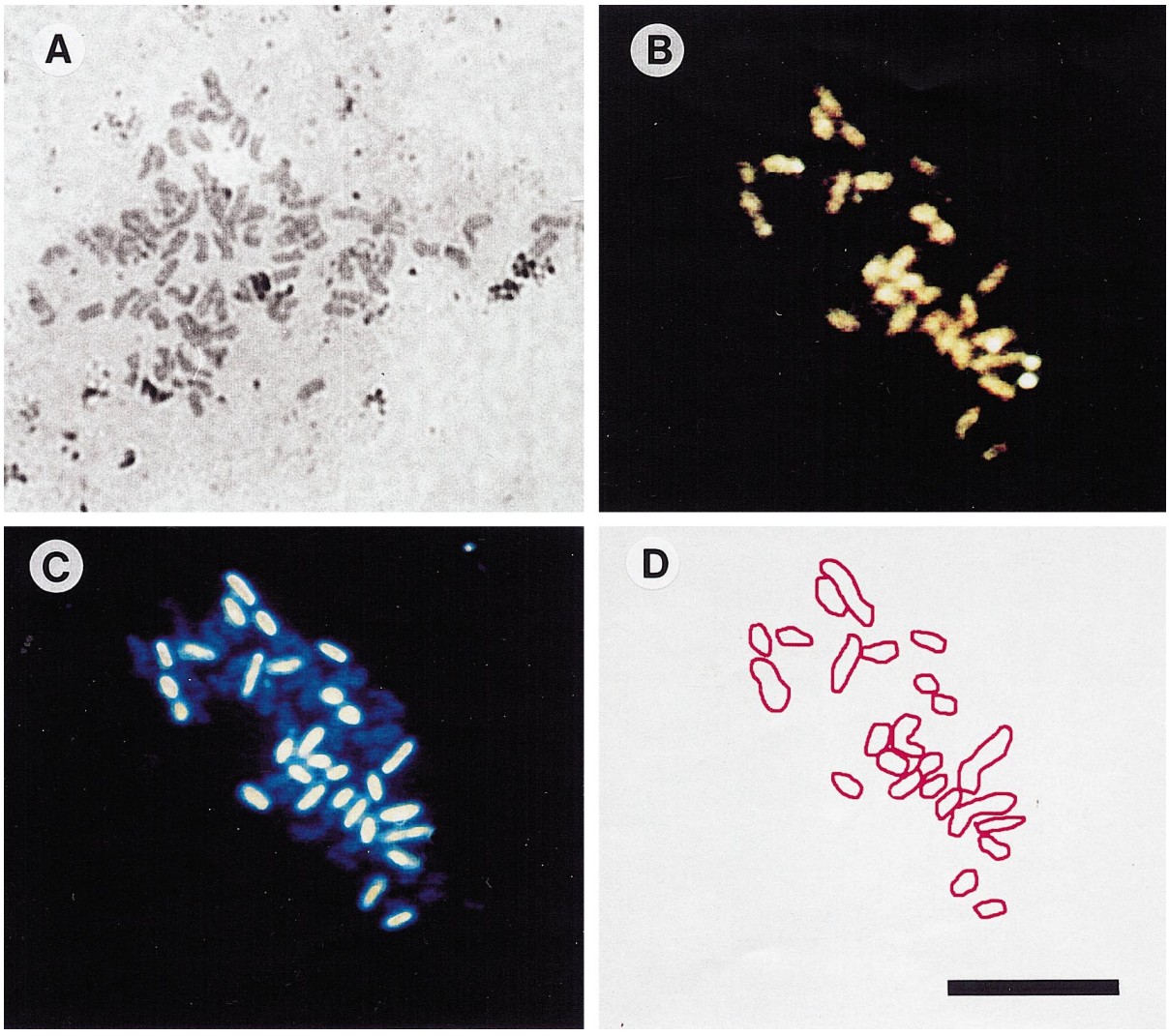

Fig. 4 A—D Cytological preparations of the somatic hybrid *O. sativa* (+) *P. coarctata* SH61. **A** Root tip squash stained with Feulgen and counterstained with orcein, showing 72 chromosomes. **B** GISH to chromosomes of SH61 visualised with a fluorescein emission filter. Biotin-*labelled O. sativa* DNA (after blocking with *P. coarctata* unlabelled DNA) was hybridised to the chromosome spread and incubated with fluorescein-conjugated avidin. **C** GISH to chromosomes of SH61 with a DAPI filter. The 24 *Oryza* chromosomes fluoresce much brighter than the 48 chromosomes of the *Porteresia* parent. The *Porteresia* chromosomes exhibit less DAPI fluorescence, reflecting both the smaller genome size of *Porteresia* and the relatively greater attrition by the formamide denaturation. **D** Outline of the 24 *Oryza* chromosomes, as detected by the FITC filter, to be used in conjunction with **Fig. 4 C**. *Bar*: 10 µm

In the glasshouse, phenotypic differences became apparent (Table 2). Leaves of *P. coarctata* had a purple tint, whereas those of 'Taipei 309' were green, as were plants of all the selected lines, including SH61. *Porteresia coarctata* is rhizomatious, whereas rice has a normal root structure as did plants of all selected lines. The growth habit of *P. coarctata* is more prostrate than rice; amongst the selected lines only those of line SH66 demonstrated this trait. Of the 8 putative hybrid lines, 2 lines (SH40 and SH66) flowered, were fertile and set awnless seeds consistent with rice. Conversely, *P. coarctata* sets seeds bearing awns. Plants of line SH61 have yet to flower.

Discussion

To date, all successful reports of somatic hybridisation involving cereals and grasses have employed embryogenic cell suspensions as the source of protoplasts. However, *P. coarctata* is not responsive to *in vitro* procedures. Consequently, leaves are the sole source of protoplasts. In the present study, cell suspension-derived protoplasts of *O. sativa* cv 'Taipei 309' (2n = 2x = 24; japonica rice) were fused with non-dividing leaf mesophyll protoplasts of *P. coarctata* (2n = 4x = 48); the failure of *Porteresia* protoplasts to undergo mitotic division in culture provided one-half of a selection strategy. It was assumed that potential somatic hybrid tissues could be identified, by heterosis (Otoni et al. 1995) following appropriate nurse culture of fusion-treated protoplasts. Plants identified by RAPD analysis as being different from the two parental species were further characterised by flow cytometric analysis and cytology. Based on

Table 2 Morphological comparisons of selected fusion-derived plants and their parental species (*w* leaf blade with midrif, *wo* leaf blade without midrib)

Plant characteristics						
Regenerant/ parental species	**Plant growth**	**Leaf midrib**	**Leaf-sheath pigmentation**	**Tillering**	**Awn**	**Root or rhizome**
Taipei 309[a]	Fast	w	Green	Erect	Awnless	Root
P. coarctata[b]	Slow	wo	Purple	Spreading	Awned	Rhizome
Regenerants						
SH26[c]	Fast	w	Green	Erect	–[e]	Root
SH28[c]	Fast	w	Green	Erect	–[e]	Root
SH40[c]	Fast	w	Green	Erect	Awnless	Root
SH41[c]	Fast	w	Green	Erect	–[e]	Root
SH61[c]	Fast	w	Green	–[d]	–[d]	Root
SH66[c]	Fast	w	Green	Spreading	Awnless	Root
SH90[c]	Fast	w	Green	Erect	–[e]	Root
SH137[c]	Fast	w	Green	Erect	–[e]	Root

[a,b] Parental species
[c] Putative SH plants which were selected by RAPD analysis
[d] Plant was not established under glasshouse conditions and died during *ex vitro* aclimation although this accession is vegetatively propagated *in vitro*
[e] Putative SH plants were sterile and did not produce seeds

these studies, 1 plant was analysed further by GISH and confirmed as being a somatic hybrid.

RAPD analysis, which was used to identify initially putative hybrids within the regenerated population, showed that amplification with three distinct primers produced an average of 17 bands per plant, of which approximately 11 were either unique to one of the parents or novel. The number of markers required to identify hybrids is dependent upon the extent of chromatin introgression from each of the parental species. No information is available concerning the location of the RAPD markers within the plant genome. It is therefore conceivable that asymmetric hybrids, which would contain only a small quantity of chromatin from one of the parental species, would have been missed by this selection system.

An amphiploid somatic hybrid produced from *O. sativa* and *P. coarctata* would be expected to carry 72 chromosomes, this being the sum of the somatic chromosome complements of the respective parents. Numerically, 72 chromosomes could be attributed, theoretically, to polyploidisation, particularly of rice regenerants, during the electro-fusion of protoplasts (homokaryon formation) resulting in a hexaploid *O. sativa*, or remotely, mixaploid *P. coarctata*. Plants regenerated from such experiments, i.e. polyploids, are likely to require a lengthy period for flowering. Thus, meiotic analysis to determine whether multivalents are formed during prophase I would not be readily possible. Consequently, genome *in situ* hybridisation (GISH) of mitotic chromosomes was developed to discriminate between the two genomes. GISH has been used recently, to identify the 24 rice D-genome chromosomes in *O. latifolia* (Fukui et al. 1997), and such genome discrimination was also extended to *O. minuta*. Published information together with the results described here confirm that GISH technology can be extended to rice, with its small chromosomes, as a means to distinguish specific genomes at both the intra- and inter-generic levels.

When the chromosome preparations were counterstained with DAPI, there was a marked difference in the intensity of the fluorescence staining of the two sets of parental chromosomes. It is conceivable that this may be related to the comparative genome sizes of *P. coarctata* and *O. sativa*. Flow cytometry indicated that *P. coarctata* has only 63% of the total DNA content per nucleus of *O. sativa*, yet there are twice as many chromosomes in each *P. coarctata* nucleus. Similarly, an unwanted side effect of the formamide denaturation is DNA loss (Leitch et al. 1994), and this effect is exacerbated by the significantly greater surface area to volume ratio of *P. coarctata*. This means that the *Porteresia* component of the genome is denatured significantly faster than the rice component and that more DNA is lost, thus accentuating the poor stain uptake and adding significantly to the ease by which the two parental genomes may be distinguished. Forty-eight *Porteresia*-like chromosomes were identified in spreads prepared from SH61. Fluorescently labelled DNA from *O. sativa* bound to 24 of the chromosomes of SH61, indicating the presence of a rice diploid genome.

In a sexual hybridisation study, Farooq et al. (1996) used *Porteresia* as both the male and female parent in crosses with wild [*O. rufipogon* (2n = 2x = 24, AA), *O. punctata* (2n = 4x = 48, BBCC), *O. officinalis* (2n = 2x = 24, CC)] and cultivated [*O. sativa* cvs 'Pokali', 'Jhona-349', 'Nonabokra', 'Basmati-198', 'Basmati-Pak', 'Basmati-385', 'Kashmir-Basmati' (2n = 2x = 24, AA)] rice varieties. In their report, hybrid plants were produced and hybridity was confirmed by isozymes. Chromosome analysis of root tips collected from hybrids of *P. coarctata* x *O. punctata* exhibited 48 chromosomes, while hybrids between *P. coarctata* x *O. sativa* cv 'Jhona-349' showed 24 instead of 36 chromosomes. Also, intergeneric sexual hybrids between *Porteresia* and *O. sativa* exhibited 36 chromosomes and were male sterile (Jena 1994). Jena (1994) and Farooq et al. (1996) also reported slow growth of their hybrids, as was observed in the present study with SH61.

Recently, sexual hybrids of *O. sativa* x *P. coarctata* were reported, following recovery by embryo rescue (Brar et al. 1997). Consistent with the somatic hybrid of these two species described here, the F_1 hybrid counterpart was also slow growing and was propagated vegetatively, since it has been shown to be male sterile. Interestingly, the somatic hybrid (2n = 6x = 72) containing double the chromosome complement of the F_1 hybrid (2n = 3x = 36) (Brar et al. 1997) may be more likely to be both male and female fertile, since in the F_1 sexual hybrid there was evidence of a lack of pairing between chromosomes of *O. sativa* and *P. coarctata*, – 36 univalents were observed at metaphase I. Evidence from other high ploidy status somatic hybrid systems (Parokonny et al. 1997) containing a doubled chromosome complement compared to the F_1 hybrid counterpart, as in the rice system described here, would suggest that somatic hybrids of sexually incompatible or difficult-to-cross species combinations are the preferred and realisable route leading to gene/chromosome introgression in a spectrum of allodiploid individuals following backcrossing. The potential of the novel somatic hybrid [*Oryza sativa* (+) *Porteresia coarctata*] will now be assessed in breeding programmes as a basis for the introduction into rice of genes for salinity tolerance.

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