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Assessing hybrid sterility in *Oryza glaberrima* × *O. sativa* hybrid progenies by PCR marker analysis and crossing with wide compatibility varieties

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Abstract Interspecific crossing of the African indigenous rice *Oryza glaberrima* with *Oryza sativa* cultivars is hindered by crossing barriers causing 100% spikelet sterility in F_1 hybrids. Since hybrids are partially female fertile, fertility can be restored by back crossing (BC) to a recurrent male parent. Distinct genetic models on spikelet sterility have been developed predicting, e.g., the existence of a gamete eliminator and/or a pollen killer. Linkage of sterility to the *waxy* starch synthase gene and the chromogen gene *C*, both located on chromosome 6, have been demonstrated. We selected a segregating BC_2F_3 population of semi-sterile *O. glaberrima* × *O. sativa indica* hybrid progenies for analyses with PCR markers located at the respective chromosome-6 region. These analyses revealed that semi-sterile plants were heterozygous for a marker (OSR25) located in the *waxy* promoter, whereas fertile progenies were homozygous for the *O. glaberrima* allele. Adjacent markers showed no linkage to spikelet sterility. Semi-sterility of hybrid progenies was maintained at least until the F_4 progeny generation, suggesting the existence of a pollen killer in this plant material. Monitoring of reproductive plant development showed that spikelet sterility was at least partially due to an arrest of pollen development at the microspore stage. In order to address the question whether genes responsible for F_1 sterility in intraspecific hybrids (*O. sativa indica* × *japonica*) also cause spikelet sterility in interspecific hybrids, crossings with wide compatibility varieties (WCV) were performed. WCV accessions possess “neutral” *S*-loci (S^n) improving fertility in intraspecific hybrids. This experiment showed that the tested S^n-loci had no fertility restoring effect in F_1 interspecific hybrids. Pollen development was completely arrested at the microspore stage and grains were never obtained after selfing. This suggests that distinct or additional *S*-loci are responsible for sterility of *O. glaberrima* × *O. sativa* hybrids.

Keywords Crossing barriers · Hybrid rice · *Oryza glaberrima* · *Oryza sativa* · Pollen

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Introduction

The African rice *Oryza glaberrima* (Steud.) is one out of two cultivated rice species and is traditionally found in diverse West African agro-ecosystems, e.g. in rainfed environments and deep water floating systems. *O. glaberrima* is today largely abandoned in favor of high yielding *Oryza sativa* cultivars due to its poor agronomic performance. However, rice cultivation in West Africa faces constraints specific to the region and *O. sativa* cultivars are often not sufficiently adapted. In contrast, *O. glaberrima* is resistant/tolerant to relevant diseases (e.g. African rice gall midge, rice yellow mottle virus) and stresses (e.g. drought, salinity, acidity). It is therefore a valuable source of useful genes (Jones et al. 1997a).

The introduction of the *O. glaberrima* desirable traits into *O. sativa* cultivars is hindered by reproductive barriers causing spikelet sterility of F_1 hybrids (Bougerol and Pham 1989; Sano 1990; Jones et al. 1997b). Since some embryo sacs are fertile, fertility can be restored by back crossing (BC) to the recurrent parent and subsequent selection of fertile progenies in successive selfing generations (Jones et al. 1997b; Heuer et al. 2003).

Hybrid sterility is a common phenomenon and has also been described for intraspecific crosses of *O. sativa* ssp. *indica* × ssp. *japonica* (e.g. Yanagihara et al. 1995; Xu et al. 1997; Ikehashi and Wan 1998). Intraspecific F_1 hybrids are, in contrast to *O. glaberrima* × *O. sativa* hybrids, only partially sterile (e.g. Ikehashi and Akari 1986). Numerous genomic sterility (*S*) loci and segregation distortion loci scattered over most of the rice chromosomes have been identified (for an overview see Xu et al. 1997). The discovery of wide compatibility varieties (WCV) possessing “neutral” sterility loci (S^n)

recently showed that individual S^n-loci (e.g. *S5*^{n}) are sufficient to restore fertility in *indica* × *japonica* hybrids (Ikehashi and Akari 1986). This facilitated a breakthrough in hybrid rice production in Asia (for a review see Ikehashi and Wan 1998). *S5*^{n} is located on chromosome 6, where it is linked to chromogen gene *C* and the *waxy* (*wx*) starch synthase gene (Yanagihara et al. 1995).

Crossing experiments performed with advanced back-crosses of *O. glaberrima* × *O. sativa* hybrid progenies led to the identification of tentative *S*-loci also in *O. glaberrima* (Sano et al. 1979; Sano 1986). The observed elimination of female and male gametes was best explained by the "one-locus sporo-gametophytic interaction" model. This model predicts that female and male gametes of genotype *Sa* abort in heterozygous *S*/*Sa* plants whereas *S*-gametes remain viable (Oka 1974; Sano et al. 1979). It is presumed that *O. glaberrima* is of genotype *S* and *O. sativa* of genotype *Sa* (Sano 1990). According to the model, *Sa* gametes abort in heterozygous F_1 plants and only *S*-gametes are represented in the F_2, which would accordingly be fully fertile. This was indeed shown for a BC_8F_1 and the corresponding F_2 generation of *O. glaberrima* × *O. sativa* ssp. *indica* hybrid progenies (Sano et al. 1979). An identical experiment with an *O. glaberrima* × *O. sativa* ssp. *japonica* back-cross population subsequently showed that female gamete abortion was absent or only partial in this plant material (Sano 1986, 1990). This led to the hypothesis that the gamete eliminator *S1* is essentially a pollen killer but can become a gamete eliminator due to the activity of modifiers and depending on the genetic background (*indica* or *japonica*; Sano 1990). The respective locus was shown to be linked to *C* and *wx* located on chromosome 6, as is the case for *S5*^{n} (see above). An *O. sativa* introgression line suggests that *S1* modifiers are located distally adjacent to the *wx* locus (Sano 1990).

Recent mapping analyses based on an interspecific *O. glaberrima* × *O. sativa* ssp. *indica* hybrid-progeny population showed a strong segregation distortion around the *wx* locus giving further evidence that a sterility locus (denoted *S10*) is located at this chromosomal region (Lorieux et al. 2000). Since *S10* was first described in intraspecific *indica*/*japonica* hybrids (Sano 1994) it remains to be determined whether *S10* is allelic to the *S1*-locus described by Sano et al. (1979).

Despite these crossing barriers, *O. glaberrima* × *O. sativa* hybrid progeny lines of agronomic value have been developed in Africa and demonstrated the potential of wide crossing for the improvement of local *indica* and *japonica* acccessions (Jones et al. 1997a, b; Dingkuhn et al. 1998; Heuer et al. 2003). However, access to *O. glaberrima* is still limited to few parental accessions and desirable traits are often lost in the fertility restoring process. In order to gain broader access to *O. glaberrima* it is therefore necessary to develop strategies to overcome or circumvent crossing barriers.

With the objective to better understand hybrid sterility, semi-sterile *O. glaberrima* × *O. sativa indica* hybrid progenies were selected, and analyzed at the molecular level and during plant development. Results obtained suggest that an arrest in early pollen development is at least partially responsible for spikelet sterility and showed that sterility was linked to a heterozygous *wx* promoter locus. Crossing experiments with wide compatibility varieties suggest that spikelet sterility of interspecific hybrids is caused by distinct or additional *S*-loci than those causing sterility in intraspecific hybrids.

Materials and methods

Plant material

Crosses were performed with *O. glaberrima* (Tog5681) as female parent and *O. sativa* ssp. *indica* (IR64) as male parent as described by Heuer et al. (2003). F_1 hybrid plants were completely self-sterile and backcrossing (BC) to IR64 as the male parent was performed to restore fertility. Three BC_1F_2 families and 17 BC_2F_2 families of 20–100 plants each were subsequently screened for spikelet fertility under field conditions in Northern Senegal (Ndiaye) during the dry season (DS) 2000. Semi-sterile plants (seed set <50%) and fertile control plants (seed set >50%) were selected, and F_3 progenies were cultivated under field conditions in the following wet season (WS) 2000. Only semi-sterile plants of similar plant type as fertile plants were selected to ensure that spikelet sterility was not due to a general weakness of the plant or environmental factors.

One BC_2F_3 family (#3–16) showing the most extreme range of spikelet fertility (12.4%–93%) was selected for more detailed analyses. For these analyses a different set of 33 BC_2F_3 plants (derived from the same semi-sterile BC_2F_2 progenitor as the BC_2F_3 field population) was cultivated in a greenhouse during the DS2001. A cooling system facilitated partial temperature control ensuring that temperatures stayed below 37 °C in order to prevent pollen sterility due to heat. Parental plants and F_3 progenies of a fertile #3–16 BC_2F_2 plant were cultivated as controls. Plants were monitored for various vegetative and reproductive characters (height, leaf characteristics, ligule length, straw weight, anthocyanin coloration, tiller and panicle number, panicle structure, duration, spikelet and pollen fertility, and grain weight) throughout development. BC_2F_4 progenies were cultivated in parallel under field conditions and monitored for spikelet fertility.

Crossing with wide-compatibility varieties

The WCV accessions Aus373, Dular, Ketan Nangka, Kinandang Patong, N22 and Peta were kindly provided by the Genebank of the International Rice Research Institute (IRRI, Manila, the Philippines). They possess one or combinations of three to six neutral *S*-loci (AUS373: *S5*^{n}; Dular: *S5*^{n}, *S7*^{n}, *S8*^{n}, *S9*^{n}, *S16*^{n}, *S17*(t)^n; N22: *S5*^{n}, *S7*^{n}, *S8*^{n}, *S15*^{n}, *S16*^{n}; Ketan Nangka: *S5*^{n}, *S15*^{n}, *S17*(t)^n; Kinandang Patong: *S5*^{n}; Peta: *S5*^{n}; Ikehashi and Wan 1998; IRRI, personal communication). The *O. glaberrima* accessions Tog5681, Tog5674, Tog5672 and CG17, as well as the *O. sativa* accessions IR64 and IR67013-58 are available at WARDA. Crosses were performed in November/December 2000 using *O. glaberrima* as female and WCV accessions as male parents as described by Heuer et al. (2003). Reciprocal (N22 × CG17; CG17 × N22) and WCV × *O. sativa* crosses (Aus373 × IR64; Kinandang Patong × IR67013-58), as well as Dular × Dular crosses, were performed as controls. Representative F_1 plants were cultivated in the greenhouse during DS2001 as described above.

Sampling of spikelets and pollen analyses

Spikelets 1–2 days before anthesis were dehydrated in an ethanol series (30%–70%) and stored in 70% ethanol at 4 °C for pollen analysis. Spikelets were washed in H_2O before anthers were squeezed on a slide and pollen-stained with J_2KJ solution. About 300 pollen grains from three spikelets were counted in independent

experiments for an estimation of the percentage of microspores, bicellular and mature pollen. For monitoring of temporal endosperm and embryo development, spikelets were labeled at anthesis to facilitate documentation of grain development at defined stages (days after anthesis) until plant maturity.

DNA isolation and PCR analyses

Genomic DNA from young flag leaves frozen in liquid nitrogen was extracted with extraction buffer (1% Laurylsarcosine, 100 mM of Tris-HCl pH 8, 100 mM of NaCl and 10 mM of EDTA) and phenol:chloroform:isoamylalcohol (25:24:1) following the protocol of Pallotta et al. (2000). Amplification of DNA fragments by a hot-start PCR (5 min at 94 °C; 35 cycles: 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C; and final elongation for 5 min at 72 °C) was performed as described by Heuer et al. (2000), except that 200 nM of primers, 200 μM of each dNTP and 1 U of *Taq*-DNA polymerase (GibcoBRL) were used. The primer pairs RM204 (Chen et al. 1997) and OSR19 (Akagi et al. 1996) were kindly provided by Dr. M. Lorieux (CIRAD, Montpellier, France). OSR25 primers (for: 5′-CAGAAACCACACACCACC-3′; rev: 5′-CTCCGCTCCGTT-CTTTTC-3′) were designed according to the sequence published by Akagi et al. (1996). RG213 primers (for: 5′-CTGGATAA-GAGTTTCATTGCA-3′; rev: 5′-GCACGTATTCTGAACTGCTA-3′) were derived from the sequence published under GenBank accession AQ074249 (McCouch et al. 1988). The primer pair MRG5263 (for: 5′-ATCTCCTGCCACTGCACA-3′; rev: 5′-AG-GAGGAGGTGTCGATCT-3′) was designed from the sequence published by Monsanto (http://www.monsanto.com) stating that the marker closest to MRG5263 is C1084 (Harushima et al. 1998).

Amplified DNA fragments were separated on 3.5–4% agarose gels in order to determine polymorphism. Representative samples were subsequently separated on 8% polyacrylamide gels and DNA fragments were silver-stained.

Data analyses

Data were analyzed with Microsoft Excel 97 software. Simple linear correlation (Pearson *r*) of plant traits was calculated with the STATISTICA 5 software package.

Results

Spikelet sterility is maintained in *O. glaberrima* × *O. sativa* F_3 and F_4 hybrid progenies

Spikelet fertility of Tog5681 × IR64 interspecific hybrid progenies (BC_1/BC_2F_2–F_4) was monitored during three seasons under field and greenhouse conditions. In the F_2 generation, progeny families developed a variable number (11%–70%) of semi-sterile plants (<50% seed set), with the exception of two BC_2F_2 families developing only fertile plants (data not shown). F_3 families derived from semi-sterile progenitors developed either a high (32%–64%) or very low percentage of semi-sterile plants (0%–20%). F_3 families of fertile BC_2F_2 progenitors developed 4%–15% semi-sterile plants. Tog5681 and IR64 control plants were always fertile. Plants were monitored with the objective to identify progeny families segregating into semi-sterile and fertile plants of similar plant type. This criteria was applied in order to exclude the possibility that sterility was due to a general weakness of the plant or to environmental factors. One BC_2F_3 line (#3–16) was finally selected for more in-depth studies and a second set of #3–16 BC_2F_3 plants was cultivated in a greenhouse. Out of a total of 33 BC_2F_3 progenies, 15 plants showed >50% seed set (range: 56–88%; average 70.4%) and 14 plants were semi-sterile (range: 1%–36%; average 19.6%). Four plants were photosensitive and were excluded from further analyses. The number of semi-sterile plants was lower in the BC_2F_3 field population (13 semi-sterile: 26 fertile plants), which might be due to cross-pollination. A 1:1 segregation for spikelet fertility (24 semi-sterile:22 fertile plants) was again observed in BC_2F_4 progenies of a semi-sterile #3–16 F_3 plant cultivated under field conditions. Spikelet fertility was on average 36.3% (range 15.9%–49%) in the semi-sterile F_4 progenies and 71.5% (range: 51%–92%) among fertile F_4 progenies.

Correlation of plants traits to spikelet fertility

Monitoring of the BC_2F_3 greenhouse population during vegetative and reproductive development revealed that spikelet sterility was significantly correlated to the number of secondary branches and spikelets per panicle, as well as to plant height and straw weight (Table 1). Semi-sterile and fertile plants differed in height (average: 1.18 m semi-sterile, 1.08 m fertile) and straw weight (average: 154 g semi-sterile, 123 g fertile). Semi-sterile plants developed on average 185 spikelets and 40 secondary branches per panicle, both numbers significantly higher than in fertile plants (average: 157 spikelets, 35 secondary branches). The same trend was also observed in BC_1F_2 and BC_2F_2 families, as well as in the #3–16 BC_2F_3 field population (data not shown). No correlation to spikelet sterility was observed for anthocyanin coloration (data not shown). Other plant characters monitored were very similar or identical in fertile and semi-sterile plants (number of primary branches: 10; ligule length: 27 mm; width of flag leaf: 18 mm; panicle number: 60), indicating a similar plant type (Table 1).

Analysis of pollen and grain development in hybrid progenies

Spikelets of BC_2F_3 hybrid progenies were morphologically normal and all reproductive organs were developed. Detailed microscopic analysis of anthers at anthesis revealed that semi-sterile plants developed a high number of immature pollen (Table 2). With the majority of immature pollen arrested at the one-cellular microspore stage, pollen appeared heterogeneous in terms of size and starch-filling status. The percentage of microspores (MS) was inversely correlated to the percentage of seed set. In semi-sterile plants up to 59% (average 44.9%) of the pollen was arrested at the microspore stage, whereas fertile plants showed on average 7.9% MS (Table 2). BC_2F_3 progenies of fertile progenitors and the *O. sativa* parent IR64 developed 90% and 97.5% mature pollen, respectively (Table 2). The Tog5681 parent is photosensitive and did not develop panicles under long-day conditions.

Table 1 Pearson correlation matrix of vegetative and reproductive traits monitored in BC_2F_3 hybrid progenies. Traits showing significant correlation to spikelet sterility are indicated by bold numbers. Pan = panicle; br = branches; prim = primary; sec = secondary

Item	Panicles plant^{-1}	Height (cm)	Straw (g)	Tassel (g)	Flag leaf (mm)	Fertility (%)	prim. pan. br.	sec. pan. br.	Ligule (mm)	% Microspores
Panicles plant^{-1}	1.0									
Plant height [cm]	–0.13	1.0								
Straw weight [g]	0.38	0.64	1.0							
Tassel weight [g]	0.60	–0.38	–0.05	1.0						
Flag leaf width [mm]	–0.03	0.50	0.24	–0.09	1.0					
Spikelet fertility [%]	0.15	**–0.67**	**–0.48**	**0.78**	–0.32	1.0				
prim. pan. br.	–0.08	0.42	0.47	–0.13	0.10	–0.27	1.0			
sec. pan. br.	–0.12	0.57	0.50	–0.21	0.11	**–0.41**	0.80	1.0		
Ligula length [mm]	0.34	0.15	0.37	0.24	0.15	–0.14	0.40	0.35	1.0	
Microspores [%]	–0.18	0.57	0.50	–0.67	0.40	**–0.85**	0.25	0.25	0.13	1.0
Spikelets pan^{-1}	–0.17	0.57	0.45	–0.25	0.17	**–0.46**	0.74	0.97	0.31	0.26

Table 2 Percentage of spikelet and pollen fertility, and PCR marker pattern of fertile and semi-sterile BC_2F_3 hybrid prognies. Fertile and semi-sterile *O. glaberrima* × *O. sativa indica* BC_2F_3 showed a different number of pollen grains arrested at the microspore stage and a distinct PCR marker pattern. Whereas fertile plants were homozygous for the *O. glaberrima* (glab) allele of the marker OSR25 (*waxy* promoter), semi-sterile plants were heterozygous (sat/glab). For the marker OSR19 (*waxy* gene) all plants except three were homozygous for the *O. glaberrima* allele. [1]For the microsatellite marker RM204, a 110 base-pair (bp) and a 150-bp fragment not represented in the parents was amplified. BC = back cross

Plant name	Spikelet fertility (%)	Microspores [%]	Number of plants with allele	Marker OSR19	OSR25	RM204	MRG5263
BC_2F_3 fertile progenies	Average: 70.4	Average: 7.9	glab	15	14	0	0
	Range: 55.9–88.1	Range: 4.1–23.5	sat	0	0	0	15
			sat/glab	0	0	0	0
			sat/150 bp[1]	–	–	10	–
			glab/150 bp	–	–	3	–
			sat/110 bp	–	–	0	–
			150 bp	–	–	2	–
			110 bp	–	–	0	–
			150/110 bp	–	–	0	–
BC_2F_3 semi-sterile progenies	Average: 19.6	Average: 44.9	glab	11	0	0	0
	Range: 1.0–36.4	Range: 36.5–59.0	sat	3	0	1	14
			sat/glab	3	14	0	0
			sat/150 bp	–	–	4	–
			glab/150 bp	–	–	0	–
			sat/110 bp	–	–	1	–
			150 bp	–	–	1	–
			110 bp	–	–	0	–
			150/110 bp	–	–	6	–
O. glab				180 bp	160 bp	112 bp	130 bp
O. sativa				190 bp	180 bp	169 bp	220 bp

Monitoring of temporal grain development showed that at 3 days after pollination endosperm development was detectable in fertile spikelets, whereas sterile spikelets remained undistinguishable from unfertilized spikelets until plant maturity. This indicates that either no fertilization takes place or that reproductive development in sterile spikelets is arrested before 3 days after pollination.

PCR analysis of the chromosome-6 region adjacent to a tentative *S*-locus

Genetic data give consistent evidence that genomic loci located on chromosome 6 are of importance for spikelet sterility in rice hybrids. A scheme of the respective chromosomal region, as was deduced from published molecular maps is given in Fig. 1 (Kinoshito 1993; Causse et al. 1994; Yanagihara et al. 1995; Chen et al. 1997; Harushima et al. 1998; Lorieux et al. 2000). The putative sterility locus *S1* (or *S10*) is located on the short arm of chromosome 6 between the *wx* locus (at approximately 7.9 cM) and the marker *RZ398* (at 25 cM). The markers *OSR19* and *OSR25* represent the *wx* gene and *wx* promoter, respectively. The *S5* locus is located further downstream (at approximately 39 cM) and closely linked to *C* (at approximately 34 cM), *Est-2* (at approximately 37.4) and *RG213* (at approximately 43 cM). The marker *MRG5263* is reported to be located close to *C1084*, the latter located at approximately 1.4 cM from *wx*. The

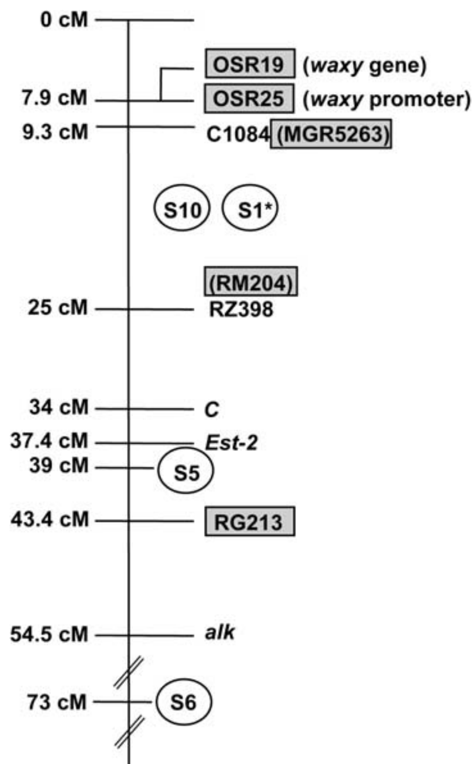

Fig. 1 Genetic map of the region of the short arm of rice chromosome 6. The position of molecular makers, major genes (*in italics*), and sterility loci (*in circles*) was estimated from maps published by Kinoshito (1993), Causse et al. (1994), Yanagihara et al. (1995), Chen et al. (1997), Harushima et al. (1998), and Lorieux et al. (2000). PCR markers tested in a BC_2F_3 *O. glaberrima* × *O. sativa* hybrid progeny population are indicated by *gray boxes*. (*) The *S1* locus is linked to *wx* and *C* (Sano 1990), but may be upstream or downstream of RM204; cM, centi Morgan

microsatellite marker *RM204* is located approximately 7.5 cM from the *wx* locus and flanks *S1* downstream. The RFLP marker *RG213* was shown to detect distinct alleles in *O. sativa indica* and *japonica* accessions, and in WCV accessions (Yanagihara et al. 1995). With the exception of the RG213 PCR-marker designed for this study, all markers were polymorphic in the parental accessions and were subsequently tested in the BC_2F_3 greenhouse population.

An overview of the size and different combinations of amplified DNA fragments is given in Table 2 and representative samples analyzed by acrylamide-gel electrophoresis are illustrated in Fig. 2. Progeny plants classified as fertile were exclusively homozygous for the *O. glaberrima* allele of the *wx* gene (marker OSR19) and promoter (marker OSR25). In contrast, all semi-sterile plants were heterozygous for OSR25. Three out of 14 semi-sterile plants were also heterozygous for the *wx* gene, whereas all remaining plants carried the *O. glaberrima* allele (Table 2, Fig. 2). The pattern obtained with the primer pair RM204 was complex. A 150-bp and a 110-bp DNA fragment not represented in either parent appeared in the analysis, which might represent variable numbers of CT-repeats constituting this microsatellite. The majority of fertile plants showed a combination of the 150-bp DNA fragment with either the *O. sativa* (169 bp) or the *O. glaberrima* (112 bp) allele (Table 2, Fig. 2). Most semi-sterile plants carried either the *O. sativa* allele in combination with the 150-bp DNA fragment, or a combination of the 110-bp and the 150-bp DNA fragments. The former was predominantly represented in semi-sterile plants with less than 25% spikelet fertility. The *O. glaberrima* allele was not represented in any combination. Both, fertile and semi-sterile plants, were homozygous for the *O. sativa* allele of MGR5263 (Table 2, Fig. 2).

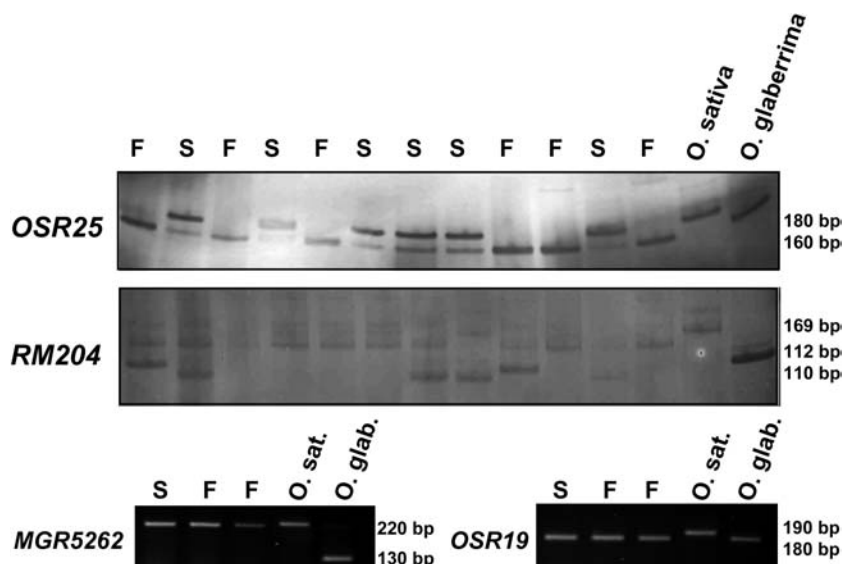

Fig. 2 PCR marker analysis of the chromosome-6 region adjacent to a putative sterility locus. Analysis was performed with genomic DNA of fertile (*F*) and semi-sterile (*S*) interspecific progenies (BC_2F_3), and the two parents (*O. sativa*, IR64; *O. glaberrima*, Tog5681). Representative samples were separated by polyacrylamide-(OSR25, RM204) and agarose gel-electrophoresis (MGR5262, OSR19). The size of DNA fragments is indicated in base pairs (bp)

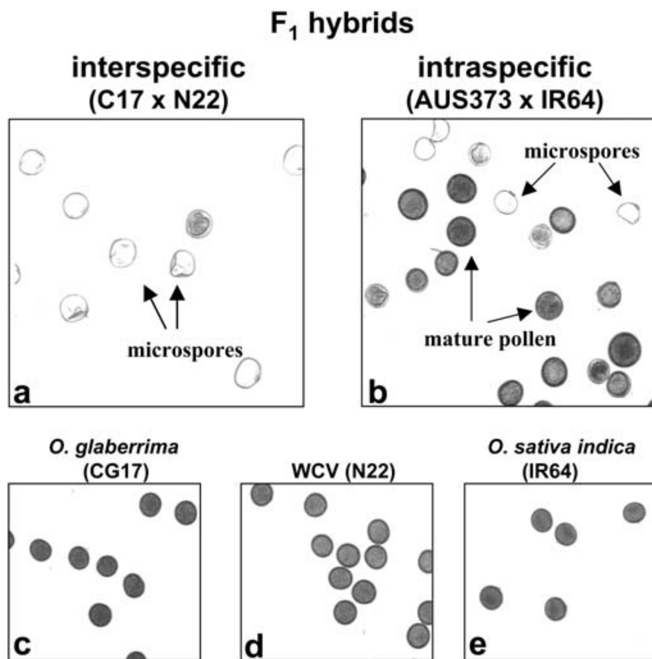

Fig. 3a–e Pollen grains of F_1 hybrids derived from crosses with wide compatibility varieties (WCV). Pollen of *O. glaberrima* × WCV and WCV × *O. sativa* F_1 hybrids was stained with iodine solution about 2 days before anthesis. In interspecific hybrids (**a**) pollen development was completely arrested at the microspore stage. Intraspecific hybrids were semi-sterile, and pollen at all developmental stages was represented (**b**). Pollen of parental accessions was fertile (**c–e**)

Testing neutral-sterility loci by interspecific crossing with wide compatibility varieties

A total of 112 grains were obtained from 165 individual crossings performed with six WCV and four *O. glaberrima* accessions. With few exceptions, grains germinated normally and representative F_1 plants developed into mature, morphologically normal plants. Individual plants showed a strong positive heterosis effect with respect to growth vigor, early tillering ability and duration (data not shown). Monitoring of hybrid plants at flowering showed that spikelets appeared normal, but that pollen development was completely arrested at the microspore stage (Fig. 3a). Plants were accordingly 100% male-sterile and grains were never obtained after selfing in any of the cross-combinations tested. Likewise, F_1 hybrid plants derived from a reciprocal WCV × *O. glaberrima* cross (N22 × CG17; CG17 × N22) were completely self-sterile giving evidence that cytoplasmic factors are not responsible for spikelet sterility in this material. Intraspecific WCV × *O. sativa indica* F_1 hybrids (Patong × IR67013-58; Aus373 × IR64) developed about 40% mature pollen (Fig. 3b) and showed partial seed set (data not shown). Parental accessions were fully fertile and representative pollen is shown in Fig. 3c–e.

Discussion

Segregation of spikelet sterility in hybrid progenies suggests the existence of a "pollen killer"

In the present study it was shown that semi-sterility was maintained until the F_4 generation in interspecific hybrid progenies. This is in agreement with the "pollen killer" model on hybrid sterility since semi-sterility can only be maintained after selfing if female (*Sa*) gametes remain viable (Oka 1974; Sano 1990). The basic assumption for both the "pollen killer" and the "gamete eliminator" models is that parental plants are of genotype *S*/*S* and S^a/S^a, respectively, and that S^a gametes abort in heterozygous plants. In the case of a gamete eliminator, all male and female *Sa* gametes abort. Subsequent selfing of a heterozygous plant would produce exclusively F_2 progenies of genotype *S*/*S*, which would accordingly be fully fertile. In the case of a pollen killer, pollen of genotype *Sa* abort whereas female *Sa* gametes remain viable. Semi-sterile, heterozygous *S*/*Sa* plants can therefore be obtained after selfing by combining *S* pollen and *Sa* embryo sacs. The semi-sterility of BC_1F_2 and BC_2F_2 to BC_2F_4 hybrid progenies reported here is accordingly in agreement with a putative pollen killer, but not with a gamete eliminator. The observed 1:1 segregation into semi-sterile and fertile progenies in the analyzed F_3 and F_4 progeny families is additionally expected if female *Sa* gametes remain viable.

According to the literature, there are two putative chromosomal locations for a pollen killer locus. One locus (*S3*) was extracted from BC_8F_2 interspecific progenies and was shown to be tightly linked to *lazy growth habit* (*la*) on chromosome 11 (Sano 1986). In our experiments, semi-sterile and fertile control plants were of similar plant type and duration, and a linkage to *la* was therefore not evident.

A second putative pollen killer is represented by *S1* located on chromosome 6 (see below). This locus is complex in its effect on spikelet sterility since it can behave as a gamete eliminator or a pollen killer depending on the activity of modifiers and genetic background (Sano 1990). A region distal to *S1* encompassing the *wx* locus was critical for such a conversion, as was shown in an *O. sativa indica* introgression line carrying *O. glaberrima* chromosome-6 segments of different size (Sano 1990). Female sterility was significantly higher in plants carrying the *O. glaberrima* distal segment than in plants carrying the *O. sativa* segment, suggesting that *O. glaberrima* carries a *S1* modifier that enhances the abortion of female *Sa* gametes.

Semi-sterile plants are heterozygous for the OSR25 marker

The molecular analysis presented here showed that semi-sterile plants were heterozygous at the *wx* promoter (OSR25), whereas fertile progenies were homozygous.

This is in agreement with the basic assumption that hybrid sterility is correlated to a heterozygous *S*-locus and its linkage to *wx* (Sano 1990). Surprinsingly, such a correlation was not observed for the adjacent *wx* gene (OSR19), expected to be tightly linked to its promoter. Only three semi-sterile plants were heterozygous for the OSR19 marker, whereas the remaining semi-sterile and fertile plants were homozygous for the *O. glaberrima* allele. This reduced number of heterozygous plants and the absence of plants homozygous for the *O. sativa* allele, for both the *wx* promoter and the *wx* gene, suggest selection against the *O. sativa* allele. This would be in agreement with the assumption that *O. glaberrima* is carrying the *S*-allele inducing abortion of the *O. sativa Sa* allele in heterozygous plants (Sano et al. 1979). Analysis of a higher plant number is necessary in order to exclude that absence of the *O. sativa* allele is due to a low sample number. No selection against the *O. sativa* allele was observed for the microsatellite marker RM204 distantly adjacent to *wx*. Instead, neither the *O. glaberrima* allele nor the heterozygous *O. sativa* × *O. glaberrima* combination was represented. For the marker MGR5263 it can not be excluded that it is not located at the presumed position close to *wx*, and the exact chromosomal localization of MGR5263 should therefore be determined before interpreting this result. Despite the fact that the linkage of *C* and S_1 with a recombination value of less than 0.05 has been reported for some *O. glaberrima* × *O. sativa* hybrids (Sano et al. 1979; Sano 1990), no such linkage was observed in our plant material, as was determined by the monitoring of anthocyanin coloration.

Analysis of a larger plant population of segregating hybrid progenies is needed in order to determine linkage of the tested markers to spikelet sterility. With the sequencing of the rice genome, detailed information on the respective chromosome-6 region is now available facilitating highly saturated marker analysis.

Hybrid sterility is correlated to total spikelet number and plant height

Semi-sterile hybrid progenies developed about 8% more spikelets per panicle than fertile plants and therefore had a higher-sink organ size. This might cause a sub-optimal sink/source ratio of the plant, i.e. the spikelet number might exceed the capacity of the plant to provide sufficient metabolites for grain filling, thereby partially accounting for the observed spikelet sterility. However, the high straw-weight of semi-sterile plants suggests that sufficient metabolites are provided but remain in vegetative organs instead of being translocated into grains. This was also observed in another context in otherwise fertile rice accessions when spikelet sterility was exceptionally high due to, e.g., heat sterility (Haefele et al. 2002).

A correlation of plant height and spikelet sterility observed in our plant material has also been reported from *O. sativa indica* × *japonica* crosses, where it has been attributed to a linkage of the *gametophytic lethal 2* locus and the semi-dwarf gene *d60* located on chromosome 7 (Tomita 1996).

Pollen development in BC_2F_3 hybrids is arrested at the microspore stages

Pollen or male sterility of interspecific hybrids has been described as one of the major components of crossing barriers in rice (e.g. Sano et al. 1979; Bougerol and Pham 1989; Zhang and Lu 1996). Our observations showed that sterile pollen mainly consists of microspores (often referred to as "small, empty" pollen) and pollen at the bi-cellular stage (often referred to as intermediate pollen or small stainable pollen). This indicates that pollen sterility is not due to a degeneration of pollen but to an arrest of pollen development before and shortly after the first pollen mitosis.

Despite a high percentage of immature pollen grains, the number of mature pollen grains was theoretically sufficient for the fertilization of the spikelet. This was also reported from advanced backcrosses of *O. glaberrima* × *O. sativa japonica* and *O. rufipogon* × *O. sativa* interspecific hybrids (Sano 1986, 1992). As was shown for intraspecific hybrids, the low seed-set observed, despite of a 50% pollen stainability, was due to impaired in vitro pollen germination since only about 10% of the pollen grains germinated (Lin et al. 1992). Pollen-germination tests should now be performed in order to determine whether this is also the case for interspecific hybrids, or whether the low seed set is due, e.g., to anther indehiscent or female sterility. Some genes triggering pollen meiosis and mitosis are known (see Heuer et al. 2000, and references therein) and might be putative target genes for functional genomics on pollen sterility in hybrids.

O. glaberrima × WCV F_1 hybrids are male-sterile

The complete spikelet sterility of *O. glaberrima* × WCV F_1 hybrids showed that the S^n-loci tested by this approach had no fertility restoring effect in interspecific hybrids. This suggests that sterility is caused by genes distinct from those causing sterility in *O. sativa indica* × *japonica* hybrids, or that an additional sterility gene(s) dominates in the F_1.

Complete pollen sterility in the F_1 can not be explained by the gamete eliminator or pollen-killer model, since *S* gametes are expected to be fertile in both models (see above). Therefore, at least one additional sterility gene or modifying factor causing an arrest of pollen development in the F_1 must be predicted. This tentative *S*-gene would be eliminated by back-crossing thereby facilitating partial pollen fertility. Since female F_1 gametes are at least partially fertile (about 30%, Chu et al. 1969) this gene might have no, or a less severe, effect on female gametophytes. Since reciprocal crosses indicate that

cytoplasmic factors are not generally involved in F_1 hybrid sterility (this study; Bougerol and Pham 1989; Sano et al. 1979) it is likely to be a nuclear gene/locus.

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