A Gene Block Causing Cross-Incompatibility Hidden in Wild and Cultivated Rice

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ABSTRACT

Unidirectional cross-incompatibility was detected in advanced generations of backcrossing between wild (*Oryza rufipogon*) and cultivated (*O. sativa*) rice strains. The near-isogenic line (NIL) of T65*wx* (Japonica type) carrying an alien segment of chromosome 6 from a wild strain gave a reduced seed setting only when crossed with T65*wx* as the male. Cytological observations showed that abortion of hybrid seeds occurred as a consequence of a failure of early endosperm development followed by abnormalities in embryo development. The genetic basis of cross-incompatibility reactions in the female and male was investigated by testcrosses using recombinant inbred lines (RILs) that were established through dissecting the introgressed segments of wild and cultivated (Indica type) strains. The results revealed that the cross-incompatibility reaction was controlled by *Cif* in the female and by *cim* in the male. When the female plant with *Cif* was crossed with the male plant with *cim*, a failure of early endosperm development was observed in the hybrid zygotes. Among cultivars of *O. sativa*, *cim* was distributed predominantly in the Japonica type but not in the Indica type. In addition, a dominant suppressor, *Su-Cif*, which changes the reaction in the female from incompatible to compatible was proposed to present near the centromere of chromosome 6 of the Indica type. Further, the death of young F_1 zygotes was controlled by the parental genotypes rather than by the genotype of the hybrid zygote itself since all three genes acted sporophytically, which strongly suggests an involvement of parent-of-origin effects. We discuss the results in relation to the origin of a crossing barrier as well as their maintenance within the primary gene pool.

RECENT evolutionary studies have focused on how genes that cause isolating barriers can be fixed in a species during speciation in spite of the fact that such genes reduce fitness when they coexist within a population (Orr 1996; Lynch and Force 2000). Reproductive isolation is considered to result from a disharmonious interaction of genes from the parents, and complementary genes are frequently reported in various plant species (Stebbins 1958; Grant 1981). If one of the complementary genes were selectively neutral to another within a population, then it could become fixed with a minimal reduction in fitness. Cross-incompatibility is one of the most effective isolating barriers that restricts gene flow between diverged populations. Cross-incompatibility after pollination is classified into pre- and postfertilization barriers in plants (Stebbins 1958; Macnair 1989), with the former resulting mainly from pollen-pistil interactions and the latter from an arrest of the development of young zygotes. Sexual affinity or cross-compatibility has been widely surveyed in crops and their wild relatives since knowledge about the primary gene pool is a prerequisite for hybridization breeding (Harlan and de Wet 1971), but our present understanding of the genes involved in these phenomena and their distribution within the primary gene pool is limited.

The present study was carried out to examine the genetic basis of the unidirectional cross-incompatibility observed in hybrid derivatives between cultivated (*Oryza sativa*) and wild (*O. rufipogon*) rice strains. A domesticated plant and its progenitor generally belong to the same biological species, which consists of groups of potentially interbreeding populations, and the corresponding cultivated and wild forms of rice are regarded to be the *O. sativa-O. rufipogon* complex (Harlan 1975; Oka 1988). The unidirectional cross-incompatibility was detected after introducing an alien chromosomal segment of *O. rufipogon* into *O. sativa* and observing that in plants with the introduced (or introgressed) segment, abortion of hybrid zygotes occurred only when the plants were pollinated with pollen grains of the recurrent parent (Sano 1992). This provides a unique example in which genes for crossing barriers were present within the primary gene pool and a distinct isolating barrier resulted from hybridization and recombination, although no distinct crossing barrier has been reported within the rice species complex (Chu *et al.* 1969; Sitch *et al.* 1989). To examine the unidirectional cross-incompatibility, it was assumed that, as reported for maize (Rashid and Peterson 1992), the cross-incompatibility reactions in the female and male are determined by the parental genotypes and that a disharmonious interac-

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tion between parental reactions causes a crossing barrier. Cross-incompatibility between distantly related species frequently results from an arrest of endosperm development, which may be explained by a genomic imbalance showing parent-of-origin effects (Johnston *et al.* 1980; Scott *et al.* 1998), although few genetic studies have aimed at testing this. The present phenomenon found in closely related taxa should give us an opportunity to examine the genetic basis and the chromosomal localization of the genes responsible for the cross-incompatibility observed within species. We report here that three putative genes responsible for the cross-incompatibility are located on the introduced segments, and we propose a model for the genic interactions responsible for the cross-incompatibility reactions in the female and male. The distribution of these three genes in the genome is also discussed in relation to the origin of cross-incompatibility.

MATERIALS AND METHODS

Plant materials: The materials used were three near-isogenic lines (NILs): T65*wx* (Oka 1974), W593A (Sano 1992), and 868A (Dung *et al.* 1998). T65*wx* is a NIL of Taichung 65 (designated T65, Japonica type of *O. sativa* from Taiwan), in which the *wx* gene on chromosome 6 from Kinoshita-mochi (Japonica type from Japan) has been introduced by successive backcrosses (from BC14). The other two NILs, W593A and 868A, were established by successive backcrosses (from BC8) using T65*wx* as the recurrent parent. Those NILs were made originally to study the gene expression of *Wx* alleles responsible for grain quality (Sano 1984). The NILs grow normally, showing a high fertility like the recurrent parent; however, late-heading plants were detected in the segregating lines (Sano 1992; Dung *et al.* 1998). The late-heading plants carried most parts of the short arm of chromosome 6 and the homozygotes were used in the present study. W593A carries a segment that contains *Wx*, *Cif* (*Cross-incompatibility in the female*, formerly designated *Lcr*), *C* (*Chromogen for anthocyanin*), *Se1* (*Hd1*, *photoperiod sensitivity*; Yano *et al.* 2000), and *S6* (*hybrid sterility*) on chromosome 6 from W593 (*O. rufipogon* from Malaysia), and 868A carries a segment from *Wx* to *Se1* from Patpaku (designated 868, an Indica type of *O. sativa* from Taiwan). The *S6* gene caused F_1 hybrid sterility but showed no effect on cross-incompatibility (Sano 1992). All three NILs carry the cytoplasm of T65.

To examine the cross-incompatibility or crossability with W593A carrying *Cif*, 12 cultivated strains (7 of Japonica type, 1 of Javanica type, and 4 of Indica type; Table 1) were used. Although Asian cultivated rice strains are often treated differently in the literature, the three varietal groups, Indica (continental), Javanica (tropical insular), and Japonica (temperate insular) were used in the present study, according to Oka (1953) and Chang (1976). The parental seeds were obtained from the National Institute of Genetics, Mishima, Japan.

Cultivation and hybridization: Seeds were germinated in petri dishes at 30° in late April, and each of the 4-week-old seedlings was transplanted in a plastic pot in a greenhouse. The plants were grown in a short-day field (10.5 hr) after 8 weeks from sowing due to photoperiod sensitivity. For genetic analysis of the unidirectional cross-incompatibility, the incompatible reactions in the female and male were investigated through hybridization with different lines. The female reaction of a plant is testable by pollination with the pollen grains of T65*wx*, while the male reaction is testable by pollination to the pistils of W593A. For crossing, the female parent was emasculated before anthesis in hot water at 42° for 7 min and then used for cross-pollination. At maturity, the numbers of plump and aborted seeds were counted for each cross, and crossability was assessed as the rate of seed setting (100 × the number of plump seeds obtained/the number of florets pollinated). Plants grown in a short-day field were used for hybridization to avoid low temperatures. All the tested plants had a high seed setting on selfing (>80%). In the present experiments, incompatible crosses always gave a high frequency of aborted or shriveled seeds (Figure 1), which was regarded as a good indication of seed arrest after fertilization.

Cytological observations: For cytological observations of the growth of pollen tubes, florets were sampled ~4 hr after pollination. The samples were fixed in a solution (3:1 ethanol:acetic acid) and were stored at 4° in 70% ethanol until use. The dissected pistils and ovaries were washed twice with distilled water and then incubated in a solution of 8 N NaOH for 1 hr. Then the samples were stained in 0.1% aniline blue in K_3PO_4 buffer and examined under UV illumination to visualize the callose of pollen tubes. To examine the development of seeds after fertilization, florets were collected and fixed in FAA (1:1:18 formalin:glacial acetic acid:70% ethanol) at 2, 4, and 8 days after pollination (DAP). After overnight incubation, the samples were stored at 4° in a solution of 70% ethanol until use. For paraplast sections, dissected pistils were dehydrated in a graded n-butanol series, embedded in paraplast, and then sectioned at 10 μm. The sections were stained with safranin and counterstained with fast green.

Fragmentation of alien chromosomal segments: The introduced chromosomal segment was dissected by repeated self-pollination after hybridization to examine the location of the genes of interest. Recombinant inbred lines (RILs) were made thereby from crosses of T65*wx* × W593A (R lines), T65*wx* × 868A (P lines), and W593A × 868A (RP lines). Since W593A and 868A were NILs of T65*wx*, the resultant RILs were expected to have the same genetic background except for the region of chromosome 6 noted above. Of the three sets of NILs, 16, 16, and 5 lines were used after genotyping the alien segments in the R, P, and RP lines, respectively.

Genotyping of alien segments in the NILs: Genomic DNA was isolated from 2-month-old seedlings by the cethyltrimethylammonium bromide method according to Murray and Thompson (1980). The length of the chromosome segment introduced from alien strains was surveyed using 20 molecular markers on chromosome 6. Thirteen of these markers (RZ516, RZ398, RZ588, RG264, RZ192, C764, S1520, G200, C235, R538, R111, R32, and G2028) were restriction fragment length polymorphism (RFLP) markers provided by S. McCouch, Cornell University, and T. Sasaki, Rice Genome Research Program, National Institute of Agrobiological Resources, Tsukuba, Japan. For RFLP analysis, after digestion with restriction enzymes the DNAs were subjected to electrophoresis on 1% agarose gels and transferred to BIODYNE B membranes (Pall). Southern blotting was performed by using ECL direct nucleic acid labeling and detection systems (Amersham). The remaining markers were PCR-based markers from the *Wx* gene (Mikami *et al.* 2000a), the *OsC1* gene (Mikami *et al.* 2000b), and three microsatellite markers RM204, RM253 (Chen *et al.* 1997), and RM136 (Temnykh *et al.* 2000). In addition, the following gene-specific primers were designed from the database (DDBJ accession nos. AB041837 and AP000399): 5′-gtcag tgcttacacagattccatc-3′ and 5′-ccttcttcttctccctgtacctgag-3′ for *Hd1* and 5′-atggggatgctgaatctgatg-3′ and 5′-gacagaagagagcatg ggaaat-3′ for E12. To detect polymorphisms for E12, the amplified products were digested with *Hin*fI.

TABLE 1

The rates of seed setting observed in crosses between 12 cultivated strains (*O. sativa*) and W593A (*O. rufipogon*) carrying *Cif*

		Crossed with male W593A				Crossed to female W593A			
Strains	Type	No. of florets pollinated	No. of seeds obtained: Normal	No. of seeds obtained: Aborted	Seed setting (%)	No. of florets pollinated	No. of seeds obtained: Normal	No. of seeds obtained: Aborted	Seed setting (%)
T65*wx*	Japonica	120	80	3	66.7	331	40	177	12.1[a]
T65	Japonica	61	43	2	70.5	79	9	45	11.4
Koshihikari	Japonica	46	34	3	73.9	88	23	29	26.1
Nipponbare	Japonica	42	35	5	83.3	32	1	22	3.1
A58	Japonica	56	25	3	44.6	59	10	30	16.9
Shiokari	Japonica	46	19	3	41.3	69	6	41	8.7
Kitaake	Japonica	47	26	6	55.3	42	1	29	2.4
Yukara	Japonica	23	16	2	69.6	38	2	27	5.3
221	Javanica	62	39	1	62.9	55	4	32	7.3
IR36	Indica	45	33	3	73.3	37	23	2	62.2
868	Indica	54	27	2	50.0	63	32	1	50.8
Acc27593	Indica	26	19	1	73.1	33	22	2	66.7
108	Indica	46	22	3	47.8	51	33	4	64.7

[a] Underlined numerals show incompatible crosses that were judged from the rates of seed setting and aborted seeds.

A linkage map of the markers was made using 165 F_2 plants of T65*wx* × IR36 and 99 F_2 plants of T65*wx* × W593A. Recombination values were calculated by the maximum-likelihood method (Allard 1956) and converted to centimorgans using the Kosambi function (Kosambi 1944).

RESULTS

Cytological observations: Unidirectional cross-incompatibility was observed between a specific type of female (W593A) and a specific type of male (T65*wx*) parent (Table 1). W593A showed a low seed setting (12.1%) when crossed with pollen grains of T65*wx* in spite of the fact that the reciprocal cross gave a high seed setting (66.7%). A previous study revealed that the dominant gene *Cif* caused abortion of hybrid zygotes, although it showed low expressivity (Sano 1992). Cytoplasmic differences were ruled out as the causal factor because both parental lines had the cytoplasm of T65. The incompatible crosses frequently gave aborted seeds, suggesting that it was a postfertilization barrier (Figure 1A). To examine when and how the unidirectional cross-incompatibility took place, histological observations were carried out. The pollen tubes from the incompatible male parent (T65*wx*) penetrated immediately after pollination into the styles of the incompatible female parent (W593A) and reached the region around the micropyle ~4 hr after pollination (Figure 1C). Double fertilization seemed to be accomplished normally since each of two sperm cells fused with the egg cell and the binucleated central cell.

The development of the embryos proceeded normally morphologically until 2 DAP in the incompatible cross, as in the compatible cross (Figure 1, D and F). However, at 4 DAP the embryos in the incompatible cross began to overgrow in comparison with those in the compatible cross (Figure 1G), and at 8 DAP giant embryos were formed with defective formation of differentiated tissues (Figure 1I). On the other hand, the triploid endosperm began to deteriorate within a few days after pollination in the incompatible cross (Figure 1F). This suggests that abortion of seeds occurred as a consequence of the failure of early endosperm development followed by abnormalities in embryo development.

Responses of cultivated strains to W593A carrying *Cif*: To examine whether or not T65*wx* responds uniquely to W593A, 12 strains belonging to three varietal groups of *O. sativa* were crossed reciprocally with W593A (Table 1). All of the eight strains of the Japonica and Javanica types gave a high seed setting (41.3–83.3%) when pollinated with the female W593A; however, the reciprocal cross gave a low seed setting (2.4–26.1%). In addition, the reciprocal cross frequently produced aborted seeds, suggesting that the seed abortion was caused by the *Cif* gene. Thus, the distinct difference in the reciprocal crosses revealed that the eight strains responded to W593A in a similar manner to T65*wx*. On the other hand, all four strains of Indica type always showed a high seed setting (47.8–73.3%), without differences between the reciprocal crossings, suggesting that the response to W593A was different among the varietal groups of rice.

Mapping of *Cif* by using RILs: In a previous study, it was concluded that *Cif* was loosely linked to *Wx*. To locate *Cif* more precisely, 16 RILs (R lines) derived from T65*wx* × W593A were further investigated. On the basis of the 16 molecular markers on chromosome 6, these

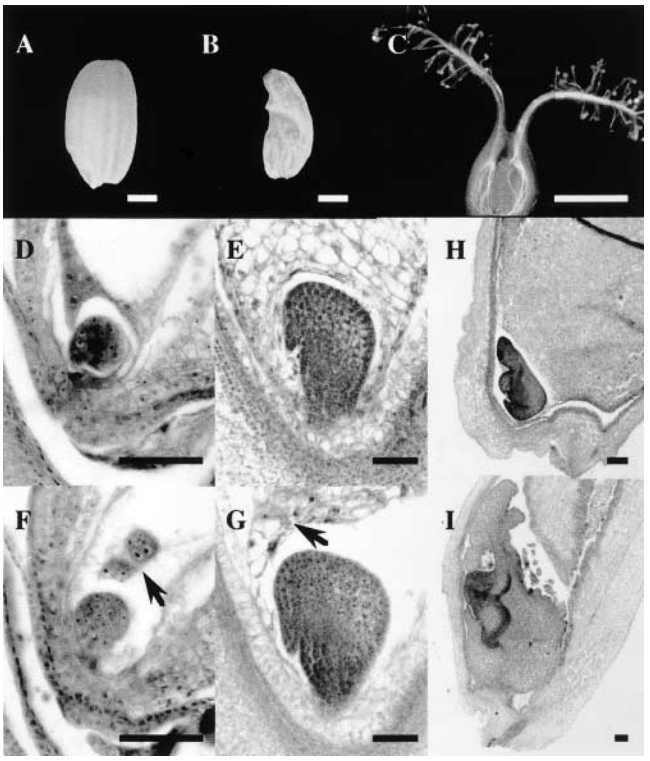

FIGURE 1.—Processes of pollination and seed development in the compatible and incompatible crosses. Plump (A) and shrunken (B) seeds resulted from compatible and incompatible crosses, respectively. Pollen tubes of T65*wx* reaching the micropyle in the incompatible cross (C) showed no arrest of pollen-tube growth. The development of embryos and endosperms was observed in the compatible (D, E, H) and incompatible (F, G, I) crosses. At 2 DAP, globular embryos with normal and abnormal endosperms in the compatible (D) and the incompatible (F) crosses, respectively, were observed. At 4 DAP, a coleoptilar-stage embryo in a compatible cross (E) and the overgrowth of an embryo with a degenerated endosperm in an incompatible cross (G) were observed. A first-leaf-stage embryo with a developing endosperm from a compatible cross (H) and a giant embryo without endosperm from an incompatible cross (I) at 8 DAP are shown. Arrows indicate degenerated endosperms (F and G). Bars: 1 mm for A and B; 0.5 mm for C; 50 μm for D, E, F, and G; and 100 μm for H and I.

RILs were grouped into 11 types. Each of the lines was crossed with the male parent of T65*wx* to determine whether the *Cif* gene was present or absent. Different lines with the same graphical genotype showed similar rates of seed setting, and therefore their data were pooled (Figure 2). A low seed setting was always associated with the production of aborted seeds. The crossing experiments suggested that *Cif* is located between RZ516 and RM204, because only the lines carrying the region between these two markers showed a distinct reduction in seed settings (10.5–18.6%). The results also revealed that no other region on the short arm of chromosome 6 was associated with a reduced seed setting.

Since W593A itself was self-fertile, the difference in the reciprocal crosses could not be explained only by the *Cif* gene, and thus it was considered likely that additional gene(s) on the introduced segment were involved. The cross-incompatibility reaction in the female could be investigated by pollinating T65*wx*. When T65*wx* was used as the male parent (Table 2), the W593A × T65*wx* F_1 plant (*Cif/cif*) showed crossability as low (8.1%) as that of the homozygote (*Cif/Cif*). This suggests that *Cif* acts sporophytically, which is consistent with the previously reported finding of 3:1 Mendelian inheritance (SANO 1992).

Unidirectional cross-incompatibility reaction in the male: W593A had a high seed setting upon selfing in spite of the presence of the *Cif* gene as mentioned. One possible explanation is that an additional gene(s) suppressing the effect of *Cif* is present on the introduced segment. To examine this possibility, three different RILs (R-1, R-5, and R-6) were used to pollinate W593A (Figure 3). All crosses gave a high seed setting, showing that R-5 and R-6 without the *Cif* gene were compatible with the *Cif* female. This suggests the presence of a gene(s) that modified the male reaction of T65*wx* and was located near the centromere. The above observation led us to consider the possibility that the unidirectional cross-incompatibility might be regulated not only by the female reaction but also by the male reaction.

As mentioned before, all four strains of Indica type were compatible with W593A, whereas the eight strains of Japonica and Javanica types were not (Table 1), suggesting that the Indica strains might carry the same gene for the male reaction as W593A. To confirm this, 868A was used as the male parent to pollinate W593A, since 868A carried the short arm of 868 (Patpaku) in the genetic background of T65*wx* (Figure 3). The cross gave a high seed setting (64.9%), showing that a gene(s) that modified the male reaction of T65*wx* was present on the introduced segment. The segment introduced from 868 was then segmented by repeated selfing of T65*wx* × 868A F_2 plants. Sixteen of the resultant RILs (P lines) were used in the present experiments and were divided into 10 groups by genotyping with 14 molecular markers. When the lines were used to pollinate the female W593A, they were clearly classifiable into compatible and incompatible lines (Figure 3). R-6, P-4, and P-10 gave high seed settings (76.0, 63.6, and 55.0%), indicating that a gene(s) modifying the male reaction of T65*wx* was present between R111 and G2028. On the basis of the estimated position in W593A and 868A, it was suggested that both of these lines had the same gene for the male reaction. Accordingly, the causal gene near the centromere was tentatively designated *cross-incompatibility in the male reaction* (*cim*).

When the F_1 hybrid of T65*wx* × 868A was crossed to the female W593A, the seed setting was as high as 55.9%, and few aborted seeds were produced (Table 2). This indicates that the gene carried by 868A is dominant and that it acts sporophytically since the crossability would

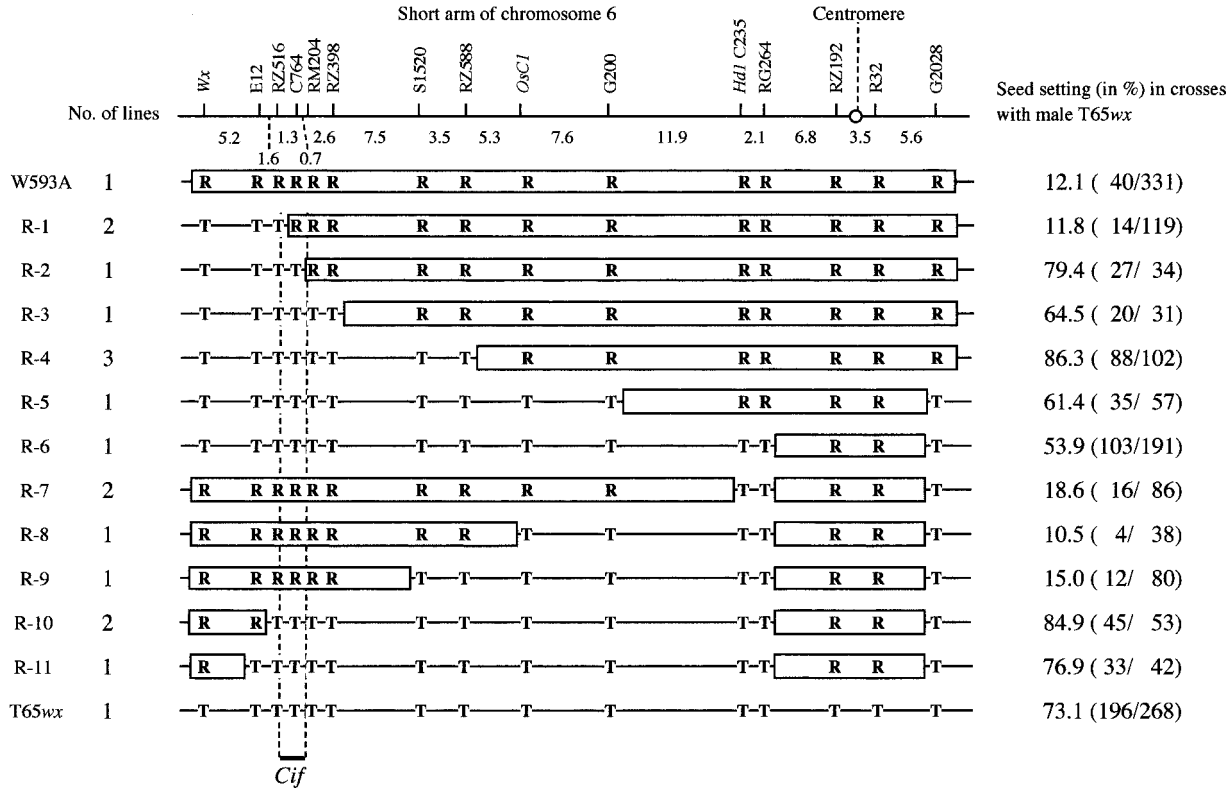

FIGURE 2.—Testcrosses to determine the location of *Cif* by using RILs. T and R indicate the markers from T65*wx* (Japonica) and W593 (*O. rufipogon*), respectively. The possible range of the alien segment is shown by boxes. The location of *Cif* is shown by a solid bar. The number of plump seeds obtained/number of florets pollinated is shown in parentheses.

be reduced by half, owing to the generation of aborted seeds if it acted gametophytically in the heterozygote. Therefore, 868 and T65*wx* were assumed to carry *Cim* and *cim*, respectively. *Cim* seemed to have no effect on the cross-incompatibility reaction in the female, because W593A carried *Cim* as well as *Cif*.

Suppressor of *Cif* in the female reaction: Regarding the female reaction, the following unexpected result was obtained when the F_1 hybrid of W593A × P-4 was pollinated with the male T65*wx* (Table 2). The genotype of the F_1 hybrid was expected to be *Cif Cim/cif Cim* on the basis of the graphical genotype of P-4 (Figure 3). The cross gave a high seed setting (61.9%) in spite of the fact that it should have given a reduced seed setting since *Cif* was dominant. This revealed that the segment introgressed from 868 carried a dominant gene(s) that changed the incompatible reaction in the female. No such segments modifying the female reaction were detected in RILs from W593A and T65*wx*. The high seed setting with few aborted seeds indicated that the dominant gene acted sporophytically, too. The suppressor of *Cif* was tentatively designated *Suppressor of Cif* (*Su-Cif*).

To determine the location of the suppressor more precisely, the segment introgressed from 868A was dissected in the derivatives of W593A × 868A hybrids. In the F_4 generation, five different RILs (RP lines) were selected using 12 markers, and each was pollinated with the male T65*wx* to determine the female reaction (Figure 4). Four of the five lines, *i.e.*, all except RP-1, carried *Cif* from W593A together with varying lengths of segments from 868A. All lines tested gave a high seed setting (50.0–92.7%), as high as that of the F_1 of W593A × P-4. Although R32 and G2028 were monomorphic between 868A and W593A, the present results demonstrated that the suppressor is located between *Hd1* and G2028.

The assumed location of *Su-Cif* suggested that NILs with a segment from 868A would not have *Su-Cif* (P-1 and P-2; Figure 3). Since the two lines were highly crossable to the male T65*wx* (57.2 and 64.7%), 868A seemed not to have *Cif*. Thus, the haploid genotypes of T65*wx*, 868A, and W593A were assumed to be *cif su-Cif cim, cif Su-Cif Cim*, and *Cif su-Cif Cim*, respectively, with those genes forming a gene block on chromosome 6.

DISCUSSION

Genic interactions among three genes that appear to account for the unidirectional cross-incompatibility in rice: Cross-incompatibility after fertilization is manifested as F_1 inviability and is caused by the failure of young F_1 zygotes to develop, especially by failure of endosperm development in plants (GRANT 1981; BERGER 1999). It is well documented in plants that hybrid inviability is of-

TABLE 2

Testcrosses to examine the genic expressions in the heterozygotes for *Cif cim*, and *Su-Cif* responsible for controlling the cross-incompatibility reactions in the female and male

Cross		Genotype								No. of florets	No. of F_1 seeds		Seed setting (in %)
Female	Male	Female				Male					Normal[a]	Aborted	
T65*wx* × W593A	T65*wx*	*wx*	+	+	*cim*	*wx*	+	+	*cim*	186	15	98	8.1
		+	*Cif*	+	+	*wx*	+	+	*cim*				
W593A	T65*wx* × 868A	+	*Cif*	+	+	*wx*	+	+	*cim*	68	38	4	55.9
		+	*Cif*	+	+	+	+	*SuCif*	+				
W593A × P-4	T65*wx*	+	*Cif*	+	+	*wx*	+	+	*cim*	105	65	7	61.9
		wx	+	*Su-Cif*	+	*wx*	+	+	*cim*				

The cross-incompatibility reaction in the female was assumed to be controlled by *Cif* and *Su-Cif*, while that in the male was assumed to be controlled by *cim*. The alien segments of chromosome 6 in the four parental strains (T65*wx*, W593A, 868A, and P-4) are shown in Figure 3.

[a] The genotypes of *Wx/wx* and *wx/wx* were segregated into 12:17 and 30:35 in the crosses of W593A × (T65*wx* × 868A) and (W593A × P-4) × T65*wx*, respectively, showing no distorted segregation for *wx*.

ten controlled by a set of complementary genes as well as by nucleo-cytoplasmic interactions and the occurrence of abnormalities in F_1 and F_2 depends on the degree of dominance of the responsible genes (Oka 1988; Macnair 1989). Thus, cross-incompatibility after fertilization is genetically different from internal barriers that appear in the later stages of development such as F_1 sterility and F_2 breakdown. In most cases, it is assumed that the death of a young F_1 zygote depends on the genotype of itself and is caused by a set of complementary dominant genes derived from the parents. This assumption suggests that differing gene dosages in triploid endosperm could give rise to differences between the reciprocals (Chu and Oka 1970). Cross-incompatibility was frequently observed in wide hybridization but not within the primary gene pool (Chu *et al.* 1969; Sitch *et al.* 1989), although numerous genes for isolating barriers except for crossability were reconfirmed to appear in crosses between cultivars of *O. sativa* (Li *et al.* 1997; Harushima *et al.* 2002). The present results showed that the unidirectional cross-incompatibility was detected in the *O. sativa-O. rufipogon* complex and that this cross-incompatibility was controlled by at least three genes, as shown in Figure 5, although further studies are needed since two of these genes (*cim* and *Su-Cif*) were not recombined in the present study. The simple inheritance observed for each of these three presumptively responsible genes ruled out nucleo-cytoplasmic interactions as the causal factor. These three genes control whether the incompatible or compatible reactions occur in the females or males. The *Cif* and *cim* genes are responsible for the incompatible reactions (Cif and cim) in the female and male, respectively. In addition, the *Su-Cif* gene that was detected only from an Indica strain was proposed to change the incompatible reaction (Cif) to the compatible reaction (+) in the female. All three genes act sporophytically, so that, for example, the heterozygote of *Cim/cim* produces only male gametes with the compatible reaction (+) even if half of the gametes carry *cim*. The deterioration of endosperm took place only when female gametes with the incompatible reaction (Cif) were fertilized with male gametes with the incompatible reaction (cim). On the basis of the molecular mapping in rice, the locations of the three genes differ from those of *S5* (Yanagihara *et al.* 1995) and *esa1* (Liu *et al.* 2001), which cause abortion of female gametes. The introduced segment carries *S6* near the centromere and their interactions on cross-incompatibility are under investigation.

Furthermore, W593A is self-fertile because it carries *Cif* and *Cim*; however, it undergoes seed abortion when crossed with male T65*wx*, indicating that maternally inherited *Cim* has no effect on rescuing the F_1 zygote and paternally inherited *Cif* has no effect on the deterioration of endosperm. Whether tissue-specific expression of *Su-Cif* occurs is uncertain, since no recombinant with *Su-Cif* and *cim* was obtained here, as mentioned. On the basis of the genic interactions proposed, if *Cim* were replaced by *cim* in W593A, the plant would be expected to be self-incompatible. This means that cross- and self-incompatibility could be convertible, depending on the combination of genes participating.

Sex-specific expression: All three genes for cross-incompatibility found in the present experiments acted sporophytically in spite of the fact that the degeneration took place after fertilization. The paternally derived *cim* from the heterozygote (*Cim/cim*) had no effect in seed abortion, suggesting that seed abortion is determined

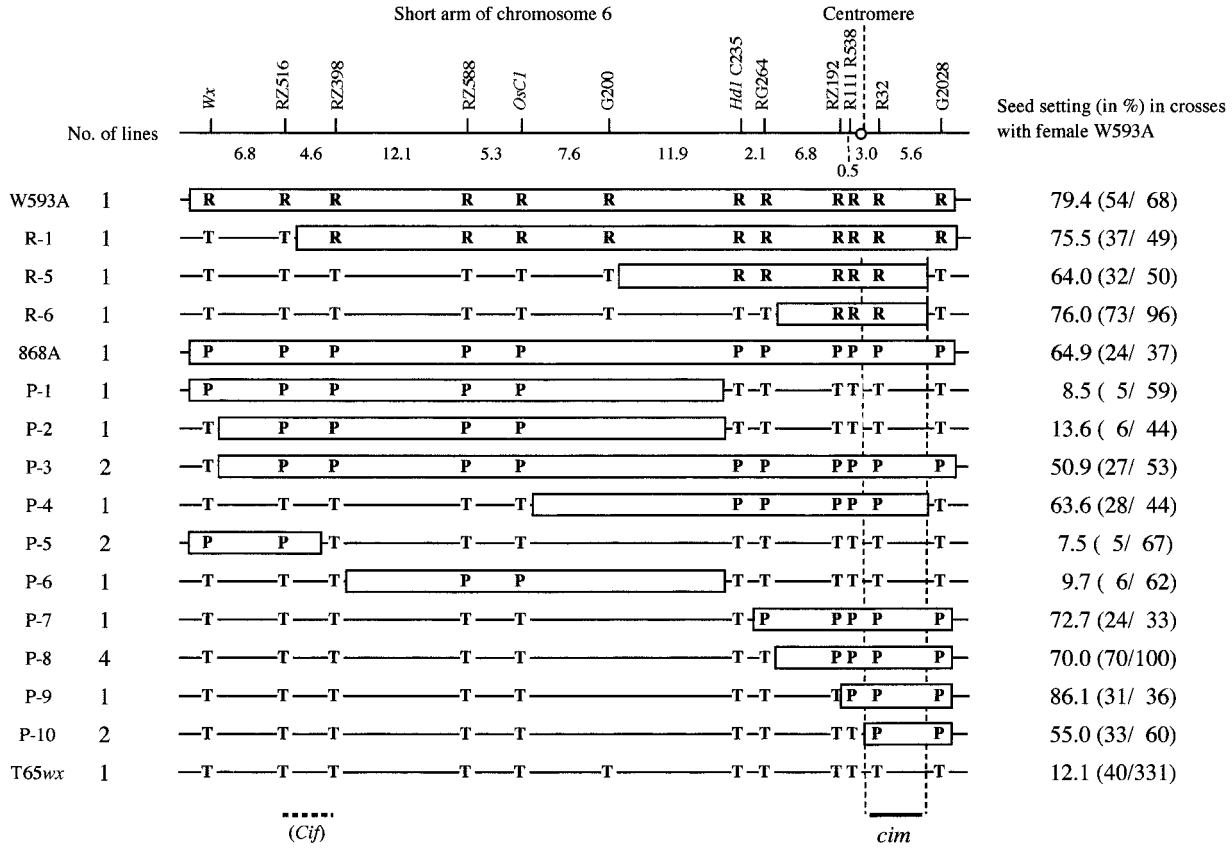

FIGURE 3.—Testcrosses to determine the location of *cim* by using recombinants. T, R, and P indicate the markers from T65*wx* (Japonica), W593 (*O. rufipogon*), and 868 (Indica), respectively. The possible range of the alien segment is shown by boxes. The location of *cim* is shown by a solid bar. The number of plump seeds obtained/number of florets pollinated is shown in parentheses.

not by the genotype of the zygote but by the genotypes of the parents. Therefore, it is not due to zygotic lethality. Sporophytic expression means that the reactions are determined before meiosis in the parent. Our cytological observations suggested that the degradation results from an arrest in early endosperm development, as is frequently observed in interspecific hybrids of plants. Numerous genes regulate the formation of gametes and the seed development in plants (FREELING and WALBOT 1993; GOLDBERG *et al.* 1994) and maternally and paternally derived factors also play significant roles in early seed development (EVANS and KERMICLE 2001b; BERGER 2003).

The sporophytic expression detected in this study might possibly be explained by mechanisms such as a transmission of some products and signals from gametes into hybrid zygotes or their epigenetic modifications depending on the parental genotypes. In one known example of the first case, a paternally contributed factor is actually transmitted to the fertilized egg and takes part in the early development of the embryo in *Caenorhabditis elegans* (HILL *et al.* 1989; BROWNING and STROME 1996). In an example of the second case, the paternal copy of the gene is silenced via a mechanism with the features of imprinting in the Arabidopsis mutant *medea* (GROSSNIKLAUS *et al.* 1998; KINOSHITA *et al.* 1999).

Genetic comparisons between pre- and postfertilization barriers: Regarding prefertilization barriers, in intergeneric hybridizations, including those among bread wheat, rye, and *Hordeum bulbosum*, cross-incompatibility or crossability is regulated by three *Kr* genes that cause an arrest of pollen-tube growth at the base of the stigma, thereby preventing the subsequent penetration of the ovary wall (SNAPE *et al.* 1980). The recessive alleles promote crossability and act additively. Genetic mechanisms for prefertilization barriers have also been reported in maize and its relatives. Within maize, the phenotype of a spontaneous mutant showing unidirectional cross-incompatibility was explained by three recessive genes (RASHID and PETERSON 1992). One of them controls the cross-incompatibility reaction in the female and the others control the cross-incompatibility reaction in the male, suggesting that these reactions reflect altered affinities in the style and pollen, respectively. If these recessive genes were accumulated in an individual, the plant would be expected to be self-incompatible. It was also reported that between maize and teosinte, cross-incompatibility was controlled by a series of alleles of the *Ga1* gene, which was originally detected as a gametophyte gene causing a distorted segregation through certation or pollen competition within maize (KERMICLE and ALLEN 1990; EVANS and KERMICLE

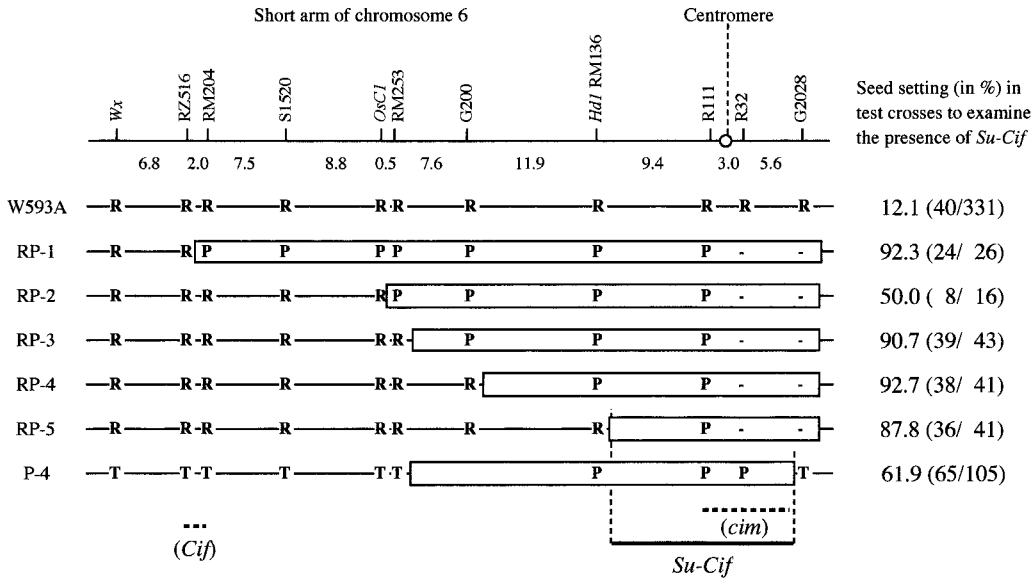

FIGURE 4.—Testcrosses to determine the location of *Su-Cif* by using recombinant inbred lines. T, R, and P indicate the markers from T65*wx* (Japonica), W593 (*O. rufipogon*), and 868 (Indica), respectively. A dash indicates monomorphism between W593 and 868. The possible range of the alien segment is shown by boxes. To determine whether the alien segment carried *Su-Cif*, all the lines except for P-4 were crossed with T65*wx*. In the case of P-4 (not carrying *Cif*), the F_1 of W593A × P-4 was crossed with the male T65*wx* (see Table 2). The location of *Su-Cif* is shown by a solid bar. The number of plump seeds obtained/number of florets pollinated is shown in parentheses.

2001a). Regarding pollen-pistil interactions, the breakdown of pollen-tube growth occurs between different genotypes in cross-incompatibility whereas it occurs between the same genotypes in self-incompatibility. Recent studies have revealed that an *S* allele is formed in a gene complex in which different elements are responsible for the incompatible reactions in the stigma or pollen, suggesting that a gene complex might play a role in self-incompatibility (SCHOPFER *et al.* 1999; TAKAYAMA *et al.* 2000). The complex nature of the genetic organization around the *Ga1* alleles was also shown in maize and teosinte (KERMICLE and ALLEN 1990; EVANS and KERMICLE 2001a); however, it is not known to what extent this complex organization is associated with the preservation of the *Ga1* alleles within the primary gene pool.

In contrast to prefertilization barriers, postfertilization endosperm developmental arrest has been reported for a number of interspecific hybrids, and the present case was one such example. To explain failures of endosperm formation, it was proposed that normal development requires a proper balance of the female and male genome sets (NISHIYAMA and YABUNO 1979; JOHNSTON *et al.* 1980). The importance of the ratio of the parental genomes was proved by using an indeterminate gametophyte (*ig*) mutant of maize. A ratio within the endosperm of two chromosome sets of maternal origin to one of paternal origin is required for normal development in maize (LIN 1984). The failure of interspecific crosses can be well explained by assuming that the effective ploidy levels are determined in a species-specific manner (JOHNSTON *et al.* 1980; SCOTT *et al.* 1998). Although the genetic basis thereof is unknown, it has been suggested that the two parental genomes are not equivalent and that genomic imprinting might be related to the mechanism of parental conflict (HAIG and WESTOBY 1989; KONDOH and HIGASHI 2000).

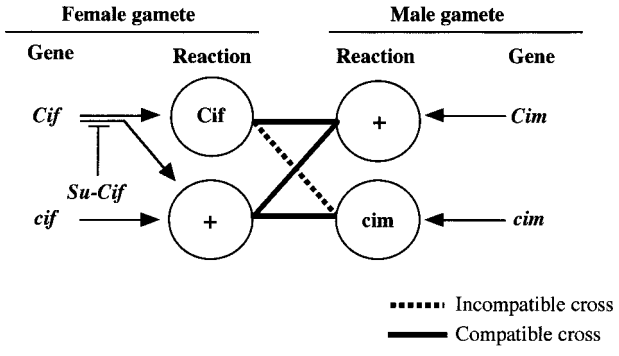

FIGURE 5.—Putative genetic model by which the three genes *Cif*, *cim*, and *Su-Cif* are involved in unidirectional cross-incompatibility. Cross-incompatibility occurs when gametes with the cross-incompatibility reactions in the female (Cif) and the male (cim) are used for fertilization. "+" indicates the cross-compatibility reaction.

Assuming that genomic imprinting is a possible mechanism for cross-incompatibility does not negate the possibility of zygotic lethality due to complementary genes. If imprinting is involved in the present case, all three genes might be modifiers for imprinting because the imprinted gene should behave in an allele-specific manner, but they all act sporophytically. Whatever the causal factor, the present results have confirmed that the two parental genomes are not equivalent for normal seed development and that sex-specific regulation is used for recognizing an appropriate partner through the cross-incompatibility in rice.

Origin of cross-incompatibility barriers: Deleterious genes reducing fitness are eliminated due to segrega-

tional loads within populations. A simple genetic mechanism for the origin of reproductive isolation was proposed by Bateson, Dobzhansky, and Muller (Dobzhansky 1970; Orr 1996). Assuming two loci for simplicity, one daughter species becomes fixed for an allele at one locus, whereas the other daughter species becomes fixed for a second allele at another locus. Hybrid inviability would be established without reducing fitness if both these mutations were neutral (or advantageous) within the population in which they arose; however, they cause hybrid inviability when expressed together in the hybrid. Varietal groups of rice such as Indica and Japonica as well as wild rice tend to be more or less isolated from each other geographically and artificially (Oka 1988; Harushima *et al.* 2002). The wild relatives of rice are predominantly cross-pollinated; therefore, it is possible that the unidirectional cross-incompatibility might be a partial remnant phenomenon. However, this was ruled out by the fact that only prefertilization barriers were responsible for self-incompatibility minimizing the reduction of fitness. It is possible the *cim* found in the Japonica type is a recently derived recessive mutation that disturbs normal seed development when expressed together with *Cif*. Although *cim* is recessive, it affects the F_1 zygote through parent-of-origin effects, as mentioned above. Since Japonica-type rice carries *cif*, *cim* has no adverse effects among Japonica-type rices, supporting the above idea that fixation of neutral genes could give rise to isolating barriers.

The question of why genes causing crossing barriers are present within the primary gene pool then arises. Although the dominant suppressor (*Su-Cif*) seems to be carried often by the Indica type, no crossing barrier appears in hybridization between the Indica type and wild strains. Furthermore, during the procedure of backcrossing between W593 and T65*wx*, the cross-incompatibility became more marked in later generations, suggesting that another suppressor(s) has to be involved in the wild strain. Perhaps such suppressors could maintain genes for crossing barriers as a hidden variation in closely related taxa, which suggests that the use of alien genes might change their sexual affinities during wide-hybridization breeding. Thus, the present results show that a variety of crossing barriers could be established by the combination of genes that determined the sexual reactions for cross-incompatibility. The gene block detected here on chromosome 6 might maintain the established sexual reactions against a breakdown due to recombination, or the recombined genes might generate the diversified sexual affinities actually observed in nature.

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