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A bentazon and sulfonylurea sensitive mutant: breeding, genetics and potential application in seed production of hybrid rice

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Abstract The use of a thermosensitive genic male sterility (TGMS) system in two-line hybrid rice breeding is affected greatly by the sterility instability of TGMS lines caused by temperature fluctuation beyond their critical temperatures for fertility reversion. To prevent seed production from self contamination, we have developed a system to secure seed purity using a herbicide-sensitive TGMS mutant, M8077S, obtained by radiation. Genetic analysis, using the F_1, F_2 and F_3 populations derived from this mutant and other normal varieties, revealed that bentazon lethality/sensitivity was controlled by a single recessive gene, which was named *bel*. The mutant can be killed at the seedling stage by bentazon at 300 mg/l or higher, a dosage that is safe for its F_1 hybrids and all other normal varieties. This mutant is also sensitive to all the tested sulfonylurea herbicides. Response of segregating plants to these two types of herbicide indicated that sulfonylurea sensitivity was also controlled by *bel*. By crossing this mutant with Pei-Ai 64S, an F_2 population was developed for genetic mapping. Surveying the two DNA pools from sensitive and non-sensitive F_2 plants identified four markers that were polymorphic between the pools. The putative linked markers were then confirmed with the F_2 population. The *bel* locus was located on chromosome 3, 7.1 cM from the closest microsatellite marker RM168. Phenotypic analysis indicated that the *bel* gene had no negative effect on agronomic traits in either a homozygous or heterozygous status. The mutant M8077S is valuable in the development of a TGMS breeding system for preventing impurity resulting from temperature fluctuation of the TGMS. Several two-line hybrid rice crosses using this system are under development.

Keywords Herbicide sensitivity · Genetic mapping · Mutation · Seed production · Microsatellites · Hybrid rice (*Oryza sativa* L.)

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Introduction

Hybrid rice breeding has been very successful in China since the 1970s. With the development of photo-thermosensitive genic male sterility (P/TGMS) or environment-sensitive genic male sterility (EGMS) lines, a two-line breeding system has been developed as a simplified alternative to the traditional three-line breeding (Yuan 1992), that requires a male-sterile line, a sterility maintainer line and a fertility restorer. The two-line breeding system is much simplified since an EGMS line can serve as a sterile line under one environmental condition and can propagate itself under different environmental conditions. The ability to maintain sterility makes EGMS lines practicable as a female to cross with any other commercial rice variety. In recent years, a number of two-line hybrids have been commercialized in China, and several Asian countries have also established hybrid breeding programs using EGMS lines (Lu et al. 1994; Li and Yuan 2000).

However, most EGMS lines require a specific temperature to maintain their sterility. Abnormal weather could bring the temperature down below the critical level that is required for conversion of TGMS lines from sterility to fertility, simply called fertility conversion, which makes EGMS lines fertile or partially fertile in the location where they are supposed to be sterile in normal years. This results in a potential problem for seed production of two-line hybrid rice, i.e. an EGMS line producing seeds from selfing. The mixture of real hybrids with selfed seeds from the EGMS line cannot be used in

rice production, resulting in a great loss to seed producers or to rice producers once false hybrid seeds are used in rice production. As an insurance for seed production, marking the seeds from EGMS lines using genetic markers could help remove the false hybrids from the mixture.

Morphological and chemical markers have been investigated as genetic markers to identify specific seeds/plants from a mixture. In rice, several morphological markers such as pale leaves (Dong et al.1995) and purple leaves (Mou et al. 1995) have been employed for marking EGMS lines. These markers can be used to identify real F_1 hybrids from selfed seeds (false hybrids) at the seedling stage. However, removing false hybrid seedlings must be made by hand, which is labor-intensive, and cannot ensure that false hybrids have been completely cleaned up.

Selective responses of plants to a chemical product can be used to identify a specific type of plant from a mixture and thus this selective chemical can be exploited as a marker. Herbicides can be one of these selective chemicals. There are two different types of herbicide that can be used for removing false hybrids in rice and other crops, herbicides that can selectively kill rice and ones that are safe for rice. When a herbicide can selectively kill normal rice varieties, a mutant with dominant resistance to this herbicide will be free from killing. The herbicide can kill the seedlings from a normal variety but it does not work on a variety that has the resistance gene. Zhang et al. (1998) transferred the *bar* gene into a restore line of hybrid rice to make it resistant to the herbicide BASTA, which can be used to selectively kill false hybrids. When a herbicide is safe for normal rice varieties, recessive sensitivity to the herbicide can be used to selectively kill the seedlings from the selfed seeds of the female once it possesses sensitivity. Mori et al. (1984) obtained a herbicide-sensitive mutant through radiation, which was lethal to bentazon. The lethality was found to be controlled by a recessive gene. Maruyama et al. (1991) discussed the possibility of using this mutant in seed production by mixed planting.

We now report a herbicide-sensitive rice mutant obtained from an EGMS line that combines EGMS with bentazon-sulfonylrea sensitivity. The gene has been linked to microsatellite markers that can be used for marker-assisted breeding. Application of this mutant as an insurance for the purity of hybrid seeds in two-line hybrid rice production has been examined.

Materials and methods

Plant materials

A herbicide-sensitive mutant in rice, M8077S, used in this study, was obtained through a mutation breeding program. Seeds from a temperature-sensitive genic male-sterile rice variety, W6154S, were treated with 350 Rad Co^{60} in 1996, and plants from treated seeds (M_1) were planted in Badong, Hubei, China, at an elevation of 700 m with the temperature below the critical level required for fertility conversion of W6154S. M_1 plants were harvested in bulk as M_2 seeds, and M_2 plants were planted in the same location and harvested. M_3 plants were planted by families and some seeds from each M_2 plant were saved as seed sources since the sensitive mutant would be killed by herbicide. Six different herbicides, including NC-311, bentazon, Londax, molinate, facet and Weinong, which are safe to normal rice, were sprayed successively in the M_3 planting plot at one- to three-leaf stages. A recommended concentration rate for controlling weeds was used. Among 25,100 M_3 families tested, only one of them, numbered 8,077, was completely killed after spraying with bentazon and this mutant was named M8077S. The retained seeds from corresponding M_2 plants were then multiplied and used in further experiments.

Herbicide test

To screen a herbicide to which M8077S is significantly sensitive, a total of 29 herbicides belonging to 11 different chemical classes was tested using recommended rates for controlling weeds. The tested herbicides include bentazon and six sulfonylurea herbicides, i.e. Londax (bensulfuron), NC-311 (pyrazosulfuron), sulfometuron, metsulfuron, cinsulfuron and chlorsulfuron. Rice plants of M8077S, with the original variety W6154S and a commercial rice variety Ce64-7 as controls, were planted in 40×40×12-cm trays each containing 50 plants. Based on the response of plants to these herbicides, two of them, bentazon and Londax, were selected for further tests.

A field test was applied to find suitable growth stages and concentration rates for bentazon to kill M8077S. For the concentration test, M8077S and the two control varieties were planted in the field and sprayed with bentazon at the 3-leaf stage with eight different concentrations: 20, 39, 78, 156, 313, 625, 2,500 and 5,000 mg/l. For stage tests, plants were sprayed with 1,250 mg/l of bentazon at different growth stages including seedling, tillering and flowering. Since bentazon is absorbed by plant stems and leaves, the influence of plot size and bentazon concentration in the soil were not considered.

For the concentration test with Londax, M8077S and control varieties were planted in pots of 25-cm diameter, each with ten plants. Each pot was applied with 5 ml of Londax and six different concentrations, 3, 15, 30, 300, 1,500 and 3,000 mg/l, were tested.

Analysis of genetic pattern and gene effects

M8077S was used as a female parent, and crossed with the original W6154S and five other indica varieties, Ganghui 2, Ce64-7, R1073, R1074 and R6175, respectively. Each panicle in F_1 and F_2 populations was bagged to prevent outcrossing. At the 2-leaf stage, all plants were sprayed with 1,250 mg/l of bentazon. Plants were then scored as normal or dead based on their reaction to the herbicide 7 days after spraying. The segregation ratio of normal to dead plants was calculated for each cross. To investigate multiple effects of herbicide sensitivity on other agronomic traits, two crosses, M8077S/Ganhui 2 and W6154S/Ganghui 2, were compared agronomically.

To determine whether reactions of the mutant to two different herbicides, Bentazon and Londax, are controlled by an identical gene, two experiments were designed. (1) *The segregating population test*: F_2 seeds from two crosses, M8077S/R1073 and M8077S/R1074, were planted in 40×40×12-cm trays. At the one-leaf stage, each tray was sprayed with 20 ml of 30 mg/l of Londax. Seedlings were evaluated for growth inhibition 5 days after spraying, and then sprayed with 1,250 mg/l of bentazon. The seedlings were scored for death 7 days later. The correlation between the inhibited growth by Londax and the plant death by bentazon was investigated. (2) *The interaction test*: 30 M8077S seeds were planted in a pot. At the one-leaf stage, seedlings in the pot were sprayed with 5 ml of 30 mg/l of Londax, and followed by 5 ml of 1,250 gm/l of bentazon, 1, 3, 6, 12, 18, 24 and 48 hours respectively, in seven different pots, after spraying with Londax. Interaction between these two herbicides was evaluated by comparison with a control in which only bentazon was sprayed.

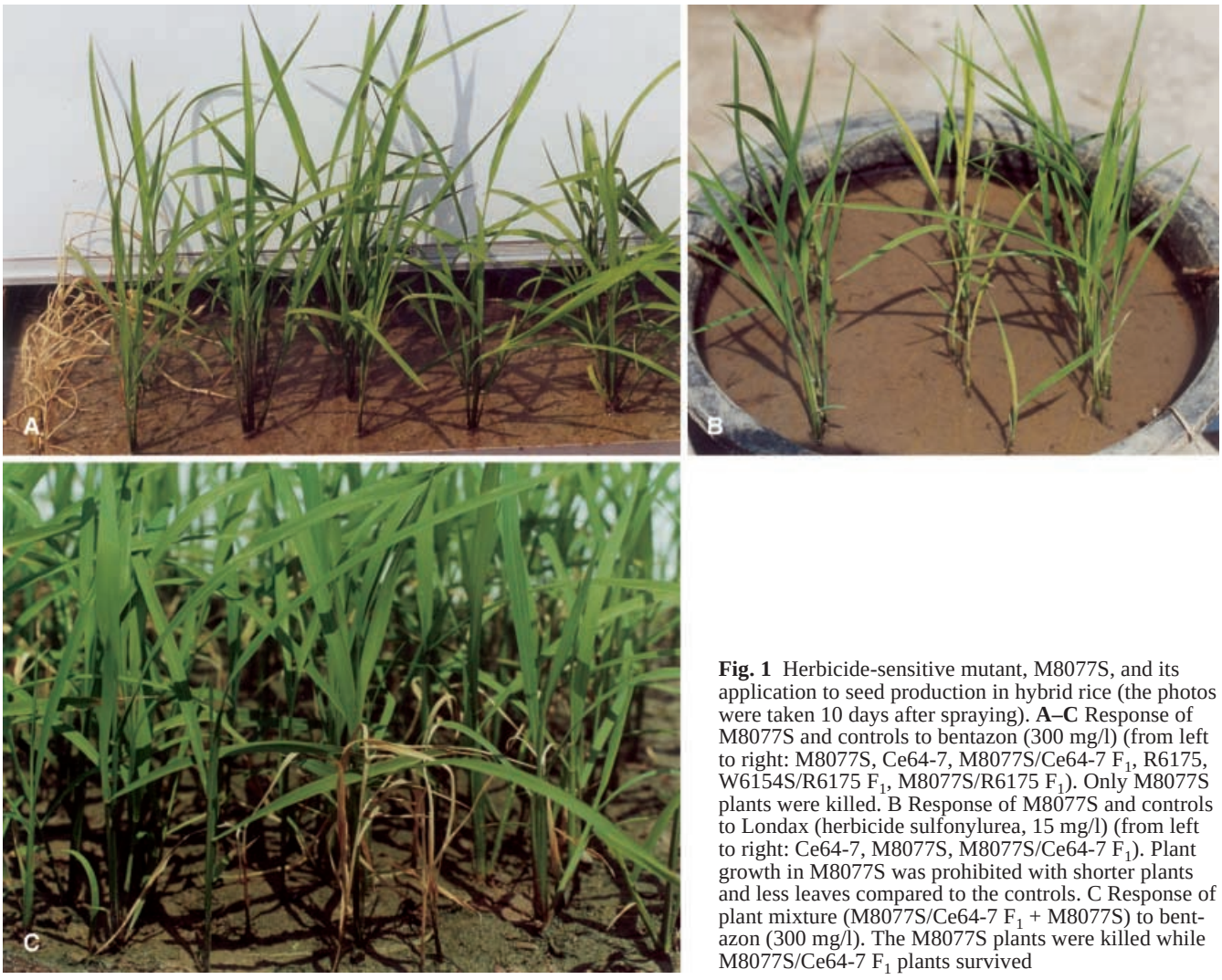

Fig. 1 Herbicide-sensitive mutant, M8077S, and its application to seed production in hybrid rice (the photos were taken 10 days after spraying). **A–C** Response of M8077S and controls to bentazon (300 mg/l) (from left to right: M8077S, Ce64-7, M8077S/Ce64-7 F_1, R6175, W6154S/R6175 F_1, M8077S/R6175 F_1). Only M8077S plants were killed. B Response of M8077S and controls to Londax (herbicide sulfonylurea, 15 mg/l) (from left to right: Ce64-7, M8077S, M8077S/Ce64-7 F_1). Plant growth in M8077S was prohibited with shorter plants and less leaves compared to the controls. C Response of plant mixture (M8077S/Ce64-7 F_1 + M8077S) to bentazon (300 mg/l). The M8077S plants were killed while M8077S/Ce64-7 F_1 plants survived

Genetic mapping

An F_2 population was derived from a cross between M8077S and a commercial EGMS variety, Pei-Ai 64S. The population was planted in 1999 at the experimental station of Hubei Academy of Agricultural Sciences, Wuhan, China. Herbicide sensitivity was scored for each F_2 plant 7 days after treatment with 1,250 mg/l of bentazon at the tillering stage. Only two leaves on each plant were treated and used for scoring; the rest of the leaves were used for DNA extraction. The F_3 families from these F_2 plants were then planted for the seedling test using 1,250 mg/l of bentazon to confirm the genotype of F_2 plants. Total DNA was extracted as described by McCouch et al. (1988) with DNA precipitated using isopropanol instead of 95% ethanol.

Microsatellite markers developed at Cornell University (Chen et al. 1997; Temnykh et al. 2000) were used for genetic mapping. A total of 240 markers that cover the whole rice genome were selected for the parental survey. The markers polymorphic between the parents were used to detect differences between two DNA pools, one from ten sensitive F_2 plants and the other from ten insensitive plants. The markers polymorphic between the pools were confirmed to be associated with herbicide sensitivity using all the F_2 plants. Polymerase chain reaction (PCR) amplification and radioautography were performed as described in Wu and Tanksley (1993).

Results

Selective herbicides

Among all the tested herbicides, only bentazon selectively killed the seedlings of M8077S but was safe to the original variety, W6154S, the tested varieties Ce64-7 and R6175, and hybrids (Fig. 1A). M8077S was sensitive to bentazon at all growth stages from seedling to flowering. The mutant was also sensitive to all the sulfonylurea herbicides tested in this study. The typical injury resulting from sulfonylureas included termination of leaf development, inhibition of plant and leaf growth, leaf yellowing and, occasionally, plant death. The variety W6154S and the control variety Ce64-7 had nearly normal growth and development with the applied herbicides. In some cases, the control varieties showed a certain degree of damage, but was significantly less than the mutant. For all other herbicides, M8077S had a normal response without any visible injury. There was no significant difference in responses to other herbicides between the mutant and the control varieties. Therefore, the mutant M8077S is selec-

tively sensitive to the herbicides bentazon and sulfonylureas.

Suitable herbicide concentrations

The results for the concentration test with bentazon are listed in Table 1. M8077S started to show injury when the concentration was at 39 mg/l. It could be killed 2–7 days after spraying when the concentration was higher than 300 mg/l. However, the controls were not affected significantly even when concentration was up to 5,000 mg/l.

The concentration test with Londax indicated that no negative effect was observed for the mutant and the controls when 5 ml of 3 mg/l (equivalent to 3 g/h) of Londax was applied to each pot. When the concentration increased to 15 mg/l (equivalent to 15 g/h), the mutant was severely injured. Ten days later, the mutant was only about half as tall as the controls (the other parental variety, Ce64-7, and their hybrid, 8077S/Ce64-7 F_1) (Fig. 1B). When the concentration increased to 300 mg/l, plant growth of the mutant was severely inhibited and seedlings died eventually. However, this concentration was still safe for the controls. Control varieties could maintain normal growth and tillering, although they showed some injury on their leaves with a color slightly darker than normal.

Genetic patterns and gene effects

M8077S has so far been by selfed for eight generations. The plants in the M_2 to M_8 generations could be killed with 1,250 mg/l of bentazon. No segregation in response to bentazon was found among plants in any of the generations from M_3 to M_8, indicating that the mutated herbicide lethality was genetically stable.

When M8077S was used as a female parent to cross with other varieties, all F_1 plants were insensitive to bentazon with concentrations even up to 5,000 mg/l. This indicates that bentazon sensitivity of the mutant is genetically recessive.

When four F_2 populations derived from crosses M8077S/Ce64-7, M8077S/R6175, M8077S/R1073 and M8077S/R1074 were treated with 500 mg/l of bentazon at about the 3-leaf stage, the numbers of dead and normal plants, scored 7 days after spraying, fitted very well with the 1:3 ratio expected for single-locus segregation (Table 2). When backcrossed to the mutant, BC_1 populations showed 1:1 segregation for dead and normal plants (data not shown). The genetic pattern for single genes was also confirmed by two F_3 families where the numbers of killed families, segregating families and normal families fitted a 1:2:1 ratio (Table 3). This single bentazon lethality locus was named *bel*.

As a TGMS line, M8077S had an identical fertility conversion pattern and very similar agronomic traits compared to its donor variety W6154S (Table 4). When crossed with Ganhui 2, the mutant produced hybrids very similar to those produced with the original variety W6154S (Table 5). M8077S also showed the same performance as the original variety in characteristics such as plant type, photo-thermo response and combining ability. This indicated that the *bel* gene did not have a significant effect on the agronomic traits of the mutant and its hybrids, except for bentazon sensitivity.

Table 1 Response of M8077S and controls to bentazon at different concentrations

Concentration (mg/l)	M8077S	Controls (W6154S, Ce64-7, M8077S/Ce64-7 F_1)
20	Normal[a]	Normal
39	Leaf-tip rolling	Normal
78	Leaf yellowish	Normal
156	Leaf dropped	Normal
313	Plant died 5 days after spraying	Normal
625	Plant died 4 days after spraying	Normal
2,500	Plant died 2 days after spraying	Normal
5,000	Plant died 2 days after spraying	Normal

[a] Normal: no visible injury

Table 2 Response to the herbicide bentazon in F_2 populations derived from crosses involving M8077S

Cross	Dead plants	Normal plants	*P* (1:3)
M8077S/Ce64-7	130	398	0.75–0.90
M8077S/R6175	73	233	0.50–0.75
M8077S/R1073	37	116	0.75–0.90
M8077S/R1074	35	128	0.30–0.50

Table 3 Response to the herbicide bentazon in F_3 populations derived from crosses involving M8077S

Cross	Dead families	Partially dead families	Normal families	*P* (1:2:1)
M8077S/Ce64-7	41	96	40	0.50–0.75
M8077S/R6175	55	106	47	0.50–0.75

Table 4 Comparison of agronomic traits between M8077S and the original variety W6154S

Traits	M8077S	W6154S	*t*-test
Plant height (cm)	73.2	74.6	NS[a]
Panicle length (cm)	18.8	18.4	NS
Grains per panicle	76.2	79.8	NS
1,000-grain weight (g)	28.3	28.3	NS
Fertility (%)[b]	60.5	60.3	NS
Sterility duration[c]	Jul. 8–Aug. 30	Jul. 8–Aug. 30	

[a] NS, no significant difference
[b] Observed in Lingshui, Hainan, China, March 1999
[c] Observed in Wuhan, China, 1998

Table 5 Comparison of agronomic traits between two F_1 hybrids, M8077S/Ganhui 2 and W6154S/Ganhui

Traits	M8077S/ Ganhui 2	W6154S/ Ganhui 2	*t*-test
Plant height (cm)	90.0	89.5	NS[a]
Flag leaf length (cm)	33.5	34.3	NS
Upmost internode length (cm)	31.8	32.1	NS
Panicle length (cm)	22.9	23.4	NS
Panicles per plant	17.0	16.5	NS
Florets per panicle	135	137	NS
Fertility (%)	64.2	64.3	NS
1,000-grain weight (g)	29.85	29.80	NS

[a] NS, no significant difference

Two of the F_2 populations listed in Table 2, M8077S/R1073 and M8077S/R1074, were also used for a test of their response to Londax, a sulfonylurea herbicide. Because the mutant could survive from injury after treatment with the relatively low concentration of Londax, as indicated previously, and could recover 2 weeks after spraying, the same F_2 plants could be tested using two different herbicides at different growth stages. In each F_2 population, as expected, some plants showed terminated leaf growth and yellow new leaves, but were still alive after spraying with 30 mg/l of Londax at the one-leaf stage. All the F_2 plants were then sprayed with 1,250 gm/l of bentazon, and scored for bentazon lethality 7 days later. The result indicated that the F_2 plants inhibited by Londax at the one-leaf stage were killed later by bentazon, while the plants that were not inhibited were still alive after bentazon treatment. Therefore, the reaction of the mutant to sulfonylureas and bentazon appeared to be controlled by the same genetic system or closely linked genes.

When different herbicides were sprayed on the same plants, it was found that the plants became less-sensitive to bentazon if they were pretreated with sulfonylureas, i.e. the plants gained resistance to bentazon. An interaction test indicated that plant resistance to bentazon could be induced promptly by the application of Londax (Fig. 2). The induced resistance increased and became strongest 3 h after pretreatment, and then decreased gradually and disappeared completely 18 h after pretreatment. Although sulfonylurea pretreatment could induce resistance to bentazon and help the plants live longer, it could not prevent the plants from final death. The interaction test in this study provided evidence that the plant reaction to these two types of herbicide might be controlled by an identical genetic system rather than closely linked genes.

Chromosomal location of the bentazon lethality gene, *bel*

Among 240 microsatellite markers used for the parental survey, 90 of them detected polymorphism between two parents, M8077S and Pei-Ai64S. Four of these markers, RM55, RM186, RM168 and RM49, were also polymorphic between the two DNA pools that consisted of sensitive and insensitive F_2 plants, respectively. For each of these markers, two different alleles were identified to distinguish the DNA pools. According to Chen et al. (1997) and Temnykh et al. (2000), all these markers were from chromosome 3, with cosegregation between RM55 and RM186. Linkage between these markers and the herbicide lethality gene *bel* was confirmed using 91 F_2 plants. The *bel* gene was linked to RM168 at a distance of 7.1 cM (Fig. 3).

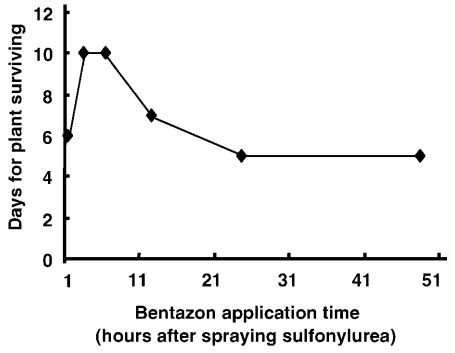

Fig. 2 Induced bentazon resistance in M8077S by pre-application of Londax (the herbicide sulfonylurea). CK (application of bentazon without pre-application of Londax.) died 4 days after bentazon was sprayed

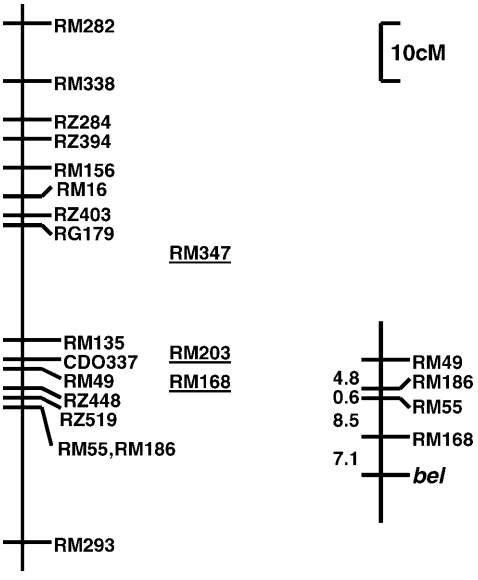

Fig. 3 Location of the bentazon lethality gene bel on rice chromosome 3 (right), compared with a reference map (left) from Temnykh et al. (2000). *The underlined markers* in the reference map are located roughly based on a different mapping population. The *numbers* on the left of chromosome bar are the genetic distance (cM), and on the right are the molecular markers

Discussion

Herbicide lethal/sensitive mutation

The mutant discovered by Mori et al. (1984) was lethally sensitive to bentazon with no report on its response to

Table 6 Seed mixtures and clean-up by spraying with bentazon

Mixture components	M8077S in mixture (%)	Plants in mixture		Plants killed by bentazon	Killing (%)
		Total	M8077S		
M8077S/Ce64-7 F1 + M8077S	20	1,000	200	200	100
	10	1,000	100	100	100
Ce64-7 + M8077S	10	2,000	200	199	99.5
	5	2,000	100	100	100
Total	10	6,000	600	599	99.9

other herbicides. We have presented here the discovery of a herbicide-mutant in rice that was not only lethal to bentazon but also sensitive to sulfonylurea herbicides, which were identified from more than 20 herbicides tested. Both lethality to bentazon and sensitivity to sulfonylureas were found to be controlled by a single recessive gene or, most likely, by an identical gene.

Differential bentazon responses were also found in cowpea (*Vigna unguiculata*) (Harrison and Fery 1993). Most cowpea accessions tested were intermediate in bentazon tolerance while the most-susceptible accessions were killed or severely injured by bentazon at 2 kg/ha. In maize, Green et al. (1999) reported a breeding line that was sensitive to bentazon and four sulfonylurea herbicides. Since both types of herbicide, bentazon and the six sulfonylureas, investigated in this study, are safe to most graminaceous crops, the same mutation discovered in the two species, rice and maize, provides a clue that these herbicides may have an identical or similar mechanism in the de-toxification of target sites.

Marker-assisted selection for bentazon sensitivity

Because the M8077S mutant discovered in this study is lethal to bentazon, homozygous plants at the mutated locus will be killed by the herbicide and selection in breeding programs must be made without application of the chemical. Using microsatellite markers, the bentazon sensitivity has been mapped and closely linked to several microstellite markers on chromosome 3. Marker-assisted selection will help select these homozygous plants without the need to spray the herbicide. These markers can also be used to identify heterozygous plants during continuous backcrossing procedures for gene transfer from one genetic background to another. Combined with other markers located around this locus, marker-assisted selection will help greatly reduce linkage drag for the genetic introgression of this gene. This technique has been used in our breeding programs to transfer this gene to a widely used EGMS variety, Pei-Ai 64S. After two cycles of marker-assisted selection, a new line with bentazon sensitivity was obtained and is ready for seed production.

Use of the *bel* gene as insurance for the purity of hybrid seeds

One of the most important applications of the *bel* gene in hybrid rice is to maintain seed purity. It has been about 20 years since Shi (1981) discovered the phenomenon of photoperiod-sensitive genic male sterility in rice. Zhang et al. (1992) proposed a model to explain the male sterility induced by changing photoperiod and temperature. They considered the fertility conversion of EGMS as a gradual changing procedure caused by an interaction between photoperiod and temperature. Based on this theory, rice breeders in China are now aiming at breeding new EGMS lines with a relatively low critical temperature for fertility conversion. Considering that hybrid rice has been planted across China and other Asian countries, so that the lowest temperatures that could happen varies widely from one place to another, it is very difficult (if not impossible) to breed a new EGMS that can be as widely planted as the currently commercialized three-line hybrid rice. Breaking low-temperature records could happen from time to time at a specific rice region even if we can breed an EGMS line based on the temperature records currently available. On the other hand, the lower critical temperature an EGMS has, the more limitation it has in its seed multiplication. Use of the bentazon sensitivity gene in EGMS breeding provides great flexibility in the critical temperature requirements for two-line hybrid rice breeding.

The technique for the use of the *bel* gene in seed-purity management has also been developed in our breeding program. A field test was done to examine the effect of bentazon on all traits of rice plants. M8077S was mixed with M8077S/Ce64-7 F_1 and Ce64-7 respectively, at different mixture ratios. Bentazon at a 0.1% concentration was prayed at the two-leaf stage. Seedlings were evaluated 7 days later. From a total of 6,000 mixed plants, all 600 plants from M8077S were killed, except one (Table 6). Figure 1C shows that M8077S plants were selectively killed when they were planted with the M807S/Ce64-7 F_1.

The *bel* gene can also be used for purity tests in seed production. Hybrid rice seeds must be tested for purity before their release to rice producers. Traditionally, seed samples must be planted and wait until they flower, or they can be employed from the false hybrid plants based on distinct agronomic traits. In order to obtain purity test results before the next planting season, seed samples are usually sent to a location where rice can be planted in the winter, like Hainan, China. Nevertheless, this is labor-intensive and also very expensive. Using bentazon sensitivity, false hybrids can be detected by killing at the 2- to 3-leaf stage. A seedling tray in a greenhouse or in an incubator will be enough for a purity test required for any sample of hybrid seeds.

The herbicide mutant and mixed planting for seed production

In seed production, sterile plants are currently planted with pollinators in alternative rows. This system requires a large manpower number for transplanting and harvesting if planting is not mechanized. It is very difficult to use manpower to produce F_1 seeds in countries where hands are limited and/or very expensive. Mixed planting of sterile plants with pollinators could make seed production to be mechanized more easily. This could also ensure a higher ratio for seed-set on male-sterile plants because the average distance between the pollinators and male-sterile flowers becomes closer than under alternative-row planting. As discussed by Maruyama et al. (1991), incorporating a herbicide-sensitivity gene to a pollinator, which could be killed by spraying a specific herbicide just after pollination, could ensure that all the harvests are F_1 seeds only.

Combining use of this herbicide-sensitive mutant with a herbicide-resistant mutant could be used not only for mixed planting in hybrid seed production but also for selfed-seed removal. When a pollinator has a sensitivity gene for herbicide A and a resistance gene for herbicide B, it can be killed after pollination by spraying with herbicide A while the plants from false hybrids can be killed by spraying with herbicide B. M8077S, reported in this study, can be used as a gene donor for breeding herbicide sensitivity, while the *bar* gene can be used for breeding a herbicide-resistance pollinator as reported by Zhang et al. (1998).

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