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Evaluation of Crossability, Seed Dormancy and Overwintering Ability in Glufosinate Ammonium-Resistant GM Rice and Their Hybrids with Non-GM and Weedy Rice

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ABSTRACT : This study was conducted to investigate the crossability, seed dormancy and overwintering ability of rice plant in GM (glufosinate ammonium-resistant lines, Iksan 483 and Milyang 204) and non-GM (their parents) or red rice (Andongaengmi). Seed-setting rate was not significantly different between GM and non-GM rice varieties. Iksan 483 and Milyang 204 showed the similar level of seed germination rate from 30 to 50 days after heading as compared to non-GM rice varieties. After overwintering in paddy field, seed germination rate of GM and non-GM rice varieties ranged from 14.3 % to 57.6 % in dry soil condition, but there was no germination in wet-soil except red rice. The result in wet-soil condition may help to set up a strategy for reducing the risk of gene flow of transgene via dispersal of seeds of GM plants. The crossability, seed dormancy and seed overwintering of Iksan 483 and Milyang 204, herbicide resistant GM rice varieties, were not significantly different compared to non-GM rice varieties. The results might be helpful to reduce the risk of transgene dispersal from GM crop via seeds and pollens.

***Keywords*:** crossability, herbicide resistance, *Oryza sativa*, seed dormancy, seed overwintering

During the last 30 years, the rapid development of plant biotechnology has created a number of genetically modified (GM) crops. The molecular breeding using biotechnology revolutionized traditional plant breeding programs. Nowaday, development of molecular breeding with functional genomics will provide the increase of yield, nutritional quality and tolerance to biotic and abiotic stresses. During the last 15 years, many kinds of GM crops have been developed by individual scientists and many companies such as Monsanto, Syngenta, Bayer, etc, and GM crops have been reported in more than 100 plant species including maize, wheat, soybean, tomato, potato, cotton, rice, etc (Mohan Babu *et al.*, 2003). GM crops were grown by approximately 8.25 million farmers in 17 countries in 2004, and the estimated global area of approved GM crops was 81.0 million hectares in 2004 (James, 2004). Commercial cultivation of major GM crops was increased as following: soybean (48.4 million hectares), maize (19.3 million hectares), cotton (9.0 million hectares), and canola (43.3 million hectares) in 2004.

Rice(*Oryza sativa* L.) is an leading crop as a staple food source in the world, and it is also the most important economically crop in Korea. The improvement of rice for yield, nutritional quality, diseases and insects resistance, and tolerance to environmental stresses by traditional breeding has been stagnated. Recently, there were many reports on the development of commercially important GM rice such as Bt rice (Tu *et al.*, 2000), provitamin A-enriched rice (Datta, 2003; Ye *et al.*, 2000), and iron-enriched rice (Vasconcelos *et al.*, 2003). Among GM rice, the development of golden rice provides the belief that biotechnology is a key to solve world food and nutrition problems (Ye *et al.*, 2000). Futhermore, China has launched a project that will create potential economic gains of $4 billon from Bt rice in 2010 (James, 2004). In Korea, GM rice still has not been grown in field, commercially, but a global trend to produce GM crops will be inevitably accepted by Korean farmers. The GM rice improved nutraceutical value can be popular for rice farmers in recent. However, GM rice variety has not been commercialized worldwide yet. If the cultivation of GM crops becomes more common, the scientists working in the fields related to the GM rice have to estimate the potential risk of GM rice to health, ecosystem and agricultural environment. Before release of GM rice, the risk assessment should be conducted and the management strategy for the risk should be developed.

Especially, the farmers of conventional or organic rice farms will be feared by the contamination from GM rice, because consumers do not want to purchase the rice contaminated with transgene from GM rice. The contamination of GM rice can actually be found in non-GM conventional rice farms and organic rice farms as the results of gene flow via seeds or pollen from neighboring GM rice and post harvest contamination. In non-GM rice farms, the contamination of GM rice can also lead to the subsequent serious economic risks at last. Accordingly, the efforts to minimize the contamination of GM rice, and to eliminate potential risk should be necessary before releasing the GM rice.

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The objective of this study was to gain the basic informations associated with seed contamination and weediness of GM rice. The crossability, seed dormancy, and seed overwintering ability were investigated using two glufosinate ammonium-resistant GM lines, and compared to the non-GM rice varieties.

MATERIALS AND METHODS

A total of seven rice varieties, two phosphinothricin (Glufosinate ammonium) herbicide resistant GM lines (Iksan 483 and Milyang 204 containing *bar* gene), non-GM rice varieties (Sindongjinbyeo, Anjungbyeo, Junambyeo and Donjinbyeo), and one Korean red rice (Andongaengmi) were harvested at biosafety rice field (Honam Agricultural Research Institute, NICS, RDA, Korea) in the Autumn of 2003. Iksan 483 and Milyang 204, herbicide resistant GM lines, contained one and two stably incorporated copies of the *bar* gene, and expressed tolerance to 0.3% Bastar (Bayer Crop Science Korea Co.).

Crossability for hybrid seed production

For comparison of the crossability between herbicide GM rice and non-GM rice (their parents) or Korean red rice, artificial pollination was examined during the summer of 2004. Andongaengmi and non-GM rice varieties were used as female parents, and GM rice varieties were used as pollen parents. Thirty to forty spikelets per panicle from female parents were emasculated by clipping method at the day before flowering, and pollinated heavily with pollen grains of GM rice at the next day. The crossability was estimated based on seed-setting rate to the total spikelets pollinated. F_1 seeds were harvested, and were utilized to the experiment of seed overwintering.

Seed dormancy and shattering

Seed dormancy was evaluated by determining the germination percentage of immature seeds, because the degree of dormancy showed the greatest value at 40 to 50 days after heading in most rice varieties. Tested materials was seeded on April 25, and transplanted with 30×15 cm space on May 30, 2004. Five panicles were collected with five replications from each variety at 30, 40, 50 and 60 days after heading. Collected panicles placed on plastic plate lined with paper towel, and then incubated for 6 days at 25. Seed dormancy was estimated by counting the germinated seeds. Grain shattering was investigated by hand grasping method at 50 days after heading. The degree of shattering (1~9) was recorded by the standard investigation system of Rural Development Administration of Korea (RDA, 1993) as the following; 1: below 1%, 3: 1~5%, 5: 6~25%, 7: 26~50% 9: over 51%.

Seed overwintering

To know seed overwintering ability, the seeds of parents and F_1s was sown immediately after harvesting on seedling trays filled with the commercial nursery soil (PUNONG-SANGTO Co., Korea). The number of tested seeds of GM rice and their parents was 2,500 per replication, and that of F_1s was 1,000 or 500 per replication. The seeded trays were maintained under water saturated soil (hereafter wet soil) and relatively dry soil (snowy and rainy condition, hereafter dry soil) conditions with and without irrigation. Mean values were compared using a complete randomized design with five replications. In the spring of 2005, germinated seeds were counted December 12, 2004, and May 10, 2005. The overwintering ability was estimated based on the germination percentage. To conform the herbicide resistance of F1s from the crossing with the GM rice varieties after overwintering, the germinated F_1 plants were treated two times by spraying 0.3% Bastar® (PPT ammonium glufosinate content, 18%), and then total DNA for Polymerase chain reaction (PCR) analysis was isolated from young leaves of randomly 10 individuals among the survived F_1 plants using a QIAGEN DNeasy Plant Maxi Kit (Qiagen, Inc.). PCR was completed in a T-gradint 96 Thermocycler (Biometra®, Imprint) using primers (forward 5'-GGTCTGCAATCGT-CAACC-3' and reverse 5'-TCAGATCTCGGTGACGGGC-A-3') and Accupower PCR premix (Bioneer Co., Ltd.) The amplification was set followed; 94 ℃ pre-denaturing for 5 min, 36 cycles of 94 ℃ denaturing for 30 sec, 58 ℃ annealing for 30 sec, 72 ℃ extension for 1 min, and 72 ℃ post-extension for 5 min. PCR products were separated by electrophoresis at 50 V for 1 hour (Mupid-2, Cosmo Bio. Co., Ltd.) on 1.5% agarose gels containing ethidium bromide, and recorded under UV illuminator.

Statistical analyses

For all experiments, data analyses were performed using the SAS statistical software (V 6.12, SAS Institute Inc.). All data was completed on treatment mean values. Means were separated using Duncan's multiple range test (DMRT) at 5%.

RESULTS AND DISCUSSION

Crossability for hybrid seed production

The hybrid seed production between herbicide GM rice and non-GM rice was examined (Fig. 1). In six cross combination, a total of 11,518 grains was produced from 22,594 pollinated spikelets, and mean seed-setting rate was 49.3%. Seed-setting ratio ranged from 40.8 to 53.5%, and that of Andongaengmi/Milyang 204 was slightly as low as 40.8% compared to other cross combinations. Seed-setting rate was not significantly different among GM and non-GM rice varieties. This result may due to the relatively similar crossability between cultivars belonged to japonica type rice. Iksan 483 and Milyang 204 showed the similar levels of crossability to non-GM rice and Andongaengmi (Korean red rice), and this result meant that the opportunity of pollen-mediated gene flow from the GM rice plants to the surrounding red rice plants could frequently occurred in the rice field. In cultivation of herbicide resistant GM rice, the gene flow to red rice was one of serious problems, and the progeny of hybrids between herbicide resistant GM rice and red rice could become a super weedy rice. If the weedy rice can not be controlled, seed contamination through this weedy rice also causes the reduction of yield, deterioration of quality as well as the contamination of GM rice in non-GM conventional rice fields and organic rice fields (Noldine 1998; Pantone and Baker 1991).

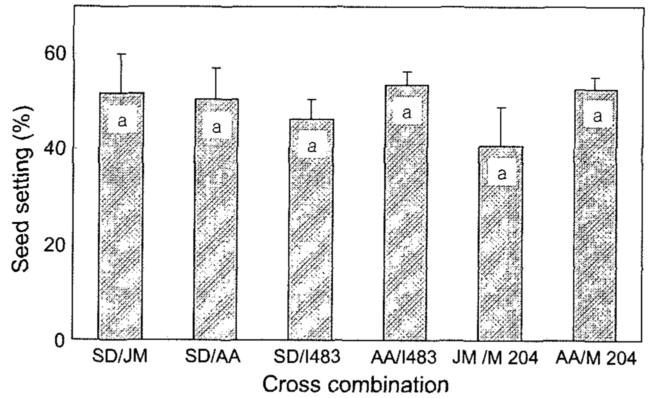

Fig. 1. Ability of hybrid seed production through artificial pollination in GM (Iksan 483 and Milyang 204) and non-GM rice. SD, Shindongjinbyeo; JM, Junambyeo; AA, Andongaengmi; I 483, Iksan 483; M 204, Milyang 204. Bars followed by the same letter were not significantly different at the 5% level based on Duncan's multiple range test.

Seed dormancy and shattering

The germination potential of immatured seeds has the closest relationship with the seed dormancy of rice varieties. For comparison of seed dormancy, the variation of seed germination according to the days after heading was measured in GM (Iksan 483 and Milyang 204) and non-GM rice (Table 1). Seed germinations at 30, 40, 50 and 60 days after heading ranged 11.7-16.3%, 37.4-50.0%, 58.5-66.1% and 66.9-98.6%, respectively. Seed germinations at 30, 40 and 50 days after heading were not significantly different between GM and non-GM rice. Especially, the seeds from 50 days after heading showed high germination percentage over 58% in both GM and non-GM rice. This result may due to the low seed dormancy in the tested varieties belonged to japonica type rice. At 60 days after heading, seed germination percentage of Donjinbyeo was the highest (98.6%) among tested varieties, but those of Iksan 483 and Milyang 204 were significantly low (66.9% and 71.3%) compared to other varieties. The result showed that Iksan 483 and Milyang 204 could be turn into the secondary dormancy as seeds matured in field. The assessment of seed dormancy is an important factor which is closely related to the weediness of GM rice. Seed dormancy is one of the important traits in rice breeding because it is associated with pre-harvest sprouting (Seshu and Sorrells, 1986). Seed dormancy is usually estimated by germination rate, but com-

Table 1. Variation of seed germination percentage according to the days after heading in GM (Iksan 483 and Milyang 204) and non-GM rice.

Varieties	Days after heading[y]				Degree of seed shattering (1-9)[z]
	30	40	50	60	
Iksan 483	11.7a	47.0a	58.5a	66.9c	1
Shindongjinbyeo	12.2a	40.0a	65.4a	87.8b	1
Anjungbyeo	12.0a	37.4a	64.1a	84.7b	1
Milyang 204	18.3a	50.0a	66.1a	71.3c	1
Junambyeo	16.3a	43.8a	60.9a	88.3b	1
Dongjinbyeo	12.2a	50.0a	61.6a	98.6a	5
Mean	13.9	44.7	62.8	82.9	

[y]Panicles were germinated under saturated water condition using paper towels for 6 days at 25 °C incubator. [z]Data were investigated by hand grasping method at 50 days after heading, 1, below 1%; 3, 1~5%; 5, 6~25%; 7, 26~50%; 9, over 51%. Data followed by the same letter in each column are not significantly different at the 5% level based on Duncan's multiple range test.

plex inheritance of seed dormancy has been noted by breeders and geneticists (Takahashi, 1997). In rice, the inheritance of seed dormancy is complex and affected by environmental interaction, and it was controlled by dominant two genes (Seshu and Sorrels, 1986; Takahashi, 1997). During the recent years, the identification of molecular markers for seed dormancy and pre-harvest sprouting resistance was reported by some research groups (Cai and Morishima, 2000; Kato *et al.*, 2001; Miura *et al.*, 2002).

On the other hand, the shattering degree of Iksan 483 and Milyang 204 (non-shattering) was similar with the non-GM rice varieties except Dongjinbyeo (medium-shattering). It seems that the degree of seed shattering is the characteristics of rice cultivars without regard to the introgression of *bar* gene. The degree of seed shattering is also related to seed dispersal because more brittle spikes shed seeds on rice field more easily. The results showed that the risk of weediness potential of Iksan 483 and Milyang 204 not critical with respect to the seed dormancy and shattering. Although these factors are problem in the dispersal of GM seeds, the GM rice developed in the future will be overcome the risk of weediness through molecular breeding.

Seed overwintering

In the temperate regions, overwintering ability of GM rice seeds closely related to the likelihood of gene flow into non-GM rice field. The germination property of GM (Iksan 483 and Milyang 204) and non-GM rice or their F_1s' seeds was compared under wet soil and dry soil fields before and after overwintering (Table 2). Under dry soil condition, no seeds germinated before overwintering, but the seed germinations of six varieties had ranged from 16.4% to 54.6% after overwintering. Andongaengmi, red rice, showed the highest ger-

Table 2. Variation of seed germinability between GM and non-GM rice or their F_1s under wet-soil and dry-soil fields before and after overwintering.

Varieties	No. of tested seeds	Germination (%)	
		Before overwintering	After overwintering
Dry-soil condition			
Iksan 483	2,500	0.0	29.2bcd
Shindongjinbyeo	2,500	0.0	23.5cd
Anjungbyeo	2,500	0.0	38.1a-d
Milyang 204	2,500	0.0	22.8cd
Junambyeo	2,500	0.0	25.3cd
Dongjinbyeo	2,500	0.0	34.5a-d
Andongaengmi	2,500	0.0	54.6a
Shindongjinbyeo/Iksan 483	500	0.0	18.2cd
Andongaengmi/IKsan 483	1,000	0.0	48.9ab
Junambyeo/Milyang 204	500	0.0	16.4d
Andongaengmi/Milyang 204	1,000	0.0	40.1abc
Wet-soil condition			
Iksan 483	2,500	8.1b	0.0
Shindongjinbyeo	2,500	3.1b	0.0
Anjungbyeo	2,500	4.8b	0.0
Milyang 204	2,500	3.3b	0.0
Junambyeo	2,500	0.7b	0.0
Dongjinbyeo	2,500	4.0b	0.0
Andongaengmi	2,500	0.0b	37.9
Shindongjinbyeo/Iksan 483	500	36.0a	0.0
Andongaengmi/IKsan 483	1,000	30.6a	0.0
Junambyeo/Milyang 204	500	28.2a	0.0
Andongaengmi/Milyang 204	1,000	30.3a	0.0

Data followed by the same letter in each column are not significantly different at the 5% level based on Duncan's multiple range test.

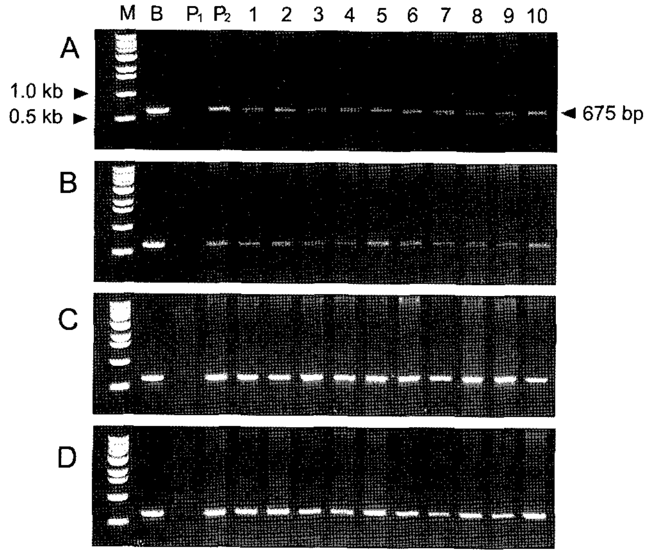

Fig. 2. PCR products of the germinated individuals in four F_1s crosses to the GM rice varieties after overwintering. M, 1 kb DNA ladder; B, *bar* positive; P_1, female parents P_2, male parents (Iksan 483 and Milyang 204); Lanes1-10, F_1 plants; A, Shindongjinbyeo/Iksan 483; B, Andongaengmi/IKsan 483; C, Junambyeo/Milyang 204; D, Andongaengmi/Milyang 204. Bands indicate the *bar* gene (675 bp).

mination rate (54.6%). The seed germinations of two F_1s from the crossing between GM rice and Andongaengmi were higher than other F_1s. In four F_1s crosses to the GM rice varieties, the germinated individuals after overwintering were resistant to 0.3% Bastar (ammonium glufosinate). The PCR analyses showed that the randomly selected 10 individuals among the survived F_1 plants contained the *bar* gene (Fig. 2).

Under wet soil condition, the seed germination of six varieties ranged from 0.7 to 8.1% before overwintering, and that of four F_1s crosses to the GM rice varieties were significantly high compared to their parents. The seeds of Andongaengmi (red rice) before overwintering were no germinated, but that after overwintering was increased as 37.9%. After overwintering, other varieties were not germinated under wet soil condition. This result guessed that Andongaengmi had a strong seed dormancy. Before overwintering, the increase of seed germination in four F_1s from the crossing with Iksan 483 and Milyang 204, seemed to caused by the expression of F_1 heterosis related to low temperature germination ability. Andongaengmi (red rice) had strong seed dormancy, but two F_1s from the crossing with the GM rice varieties were not also germinated under wet soil condition.

In present work, the hybrid seed production, seed dormancy and seed overwintering of Iksan 483 and Milyang 204 were not significantly different from the non-GM rice varieties. Especially, it guessed that Iksan 483 and Milyang 204 were low the risk of weediness potential via seed dispersal, because they had the low seed dormancy, and they showed similar degree of shattering (non-shattering) for the non-GM rice varieties except Dongjinbyeo (medium-shattering). These results guessed that GM rice transformed with *bar* gene will not increase or decrease the fitness for traits such as seed dormancy, shattering, overwintering or crossability that are associated with the risk of weediness potential compared to non-GM rice. In the future, the GM rice varieties having enriched nutrients, Bt and herbicide resistance will be cultivated in a large acreage of Asia and Africa (Datta, 2003; James, 2004; Tu *et al.*, 2000; Vasconcelos *et al.*, 2003; Ye *et al.*, 2000). We guessed that weediness potential was different by the introduced genes in crops, the risk of weediness potential should be assessed under various field conditions according to the transgenes before release of GM rice. This results might be help to assess the potential risk of transgene dispersal by GM seeds.

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