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Environmental Risk Assessment of Transgenic Melon in Japan

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Environmental risk assessment of transgenic melon plants, introduced with coat protein gene of cucumber mosaic virus was carried out in a closed and a semi-closed greenhouse and in an isolated field. Risk assessment of melon such as allogamous and entomophily plants was a first trial in Japan. In this risk assessment, the following evaluation items were compared between transgenic and non-transgenic melon plants; (1) morphological characteristics of plants and fruits maturation periods, (2) reproductive characters, e.g. pollen form and fertility, longevity of the pollen, pollen dispersal by artificial wind and under natural condition, (3) possibility of harmful impact on environment due to the presence of detrimental substances i.e. volatile compounds, allelochemical substances, (4) overwintering to predict increasing of weediness, (5) residual *Agrobacterium tumefaciens* used as a vector for the production of the transgenic melon. In conclusion, the result of the evaluation experiments conducted indicated that the influence of transgenic melon plants on the environment was not different from that of non-transgenic melon plants.

1. Introduction

The development of recombinant DNA (rDNA) technology has made possible the introduction of foreign genes from one organism to another. As the transformed organism due to the expression of a foreign gene may acquire new characteristics, the overall safty of the transformants in terms of other organisms must be evaluated carefully. In Japan, environmental risk assessment of transgenic plants is carried out in four stages; which are in closed and semi-closed greenhouses, isolated fields and later, in ordinary fields. The assessment within a closed greenhouse and semi-closed greenhouse conditions is carried out according to the "Guideline for Recombinant DNA Experiments" by the Science and Technology Agency [1]. Also, the environmental risk evaluation in an isolated field to an ordinary field is conducted by the "Guidelines for Application of Recombinant DNA Organisms in Agriculture, Forestry and Fisheries, the Food Industry and Other Related Industries" of the Ministry of Agriculture, Forestry and Fisheries [2].

New transgenic melon plants in which the coat protein gene of cucumber mosaic virus (CMV) was introduced were produced by Yoshioka et al. [3], and shown to exhibit CMV resistance [4]. The environmental risk of one line (designated M5) of these transgenic melons has already been evaluated in closed and semi-closed greenhouse conditions [5, 6, 7]. Some of the results of risk assessment in an isolated field were reported at the 4th International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plant and Microorganisums [8]. In this review, the results of experiments for environmental risk assessment under a closed greenhouse to an isolated field are summarized.

2. Risk assessment in a closed and a semi-closed greenhouse, and an isolated field

One line (M5) of transgenic melon plants that harbors the coat protein gene of CMV [3] and non-transgenic melon plants regenerated via shoot organogenesis from non-transformed explants were used as experimental plant materials. In the experiment on pollen dispersal, *Fusarium* resistant melon cv. 'Ooi' (old pure-bred line in Japan) was used as control for non-transgenic melon plant.

The items used in evaluating the environmental risks of transgenic melon are summarized in Table 1. Environmental risk assessment of transgenic melon was determined by comparing the following characteristics between transformed and non-transformed plants: (1) morphology and maturation period; (2) reproductive traits; (3) harmful impact on other plants; (4) overwintering (5) influence on soil microflora; and (6) residual *Agrobacterium* as vector. The layout of the isolated field and greenhouse is depicted in **Fig. 1.**

2.1 Morphology and maturation period (experiment in closed and semi-closed greenhouse)

Sixteen morphological traits selected from the seed and seedlings law [9] and the fruit maturation period as a growth characteristic were compared. The morphological characteristics of the transgenic plants were not different from those of non-transgenic plants. The fruit maturation period of the transformed plants (44.7 days) did not differ significantly from that of the non-transformed plants (43.0 days).

2.2 Reproductive traits

2.2.1 Pollen form and fertility (experiments in a closed greenhouse)—The size, form and fertility of pollens were compared between transgenic and non-transgenic melon plants. The size of all the pollens were about 50–60

Table 1 Items of environmental risk evaluation on transgenic melon

Evaluation items	Closed greenhouse	Semi-closed greenhouse	Isolated field
1. Confirmation and expression of the introduced gene			
1) Expression of NPT-II*	○		
2) Confirmation of CMV-CP*	○		
3) Expression of CMV-CP*	○		
4) Resistance to CMV*	○		○
2. Morphological and growth characteristics			
1) Morphological characteristic	○		
2) Fruit maturation period		○	
3. Reproductive characteristics			
1) Pollen form	○		
2) Fertility of pollen	○		
3) Longevity of pollen	○		
4) Pollen dispersal by wind	○		
5) Pollen dispersal by insect			○
6) Kinds of flower visiting insect			○
4. Evaluation of harmful impact to other plants			
1) Phenolic acids produced in leaves and stems		○	
2) Phenolic acids released from root	○		
3) Production of volatile compounds	○		
4) Influence of dry powder to another plant		○	
5) Influence on succeeding crop		○	
5. Overwintering			
1) Plant body			○
2) Seeds in fruits put on the ground			○
3) Seeds in fruits buried under the ground			○
6. Influence on soil microflora		○	○
7. Residual *Agrobacterium* as vector	○		

* Results were reported by Yoshioka et al. (1992, 1993).

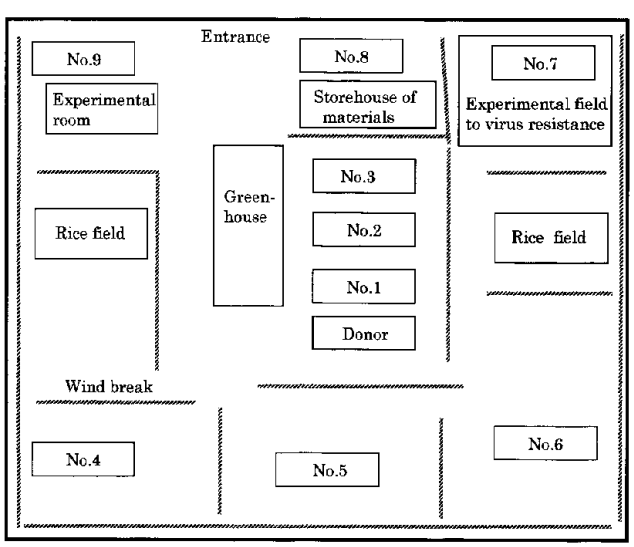

Fig. 1 Layout of an isolated field for environmental risk assessment of transgenic melon No. 1–3, 4–6, 8, 9: recipent plants of dispersed pollen from donor plants. No. 7: experimental field for virus resistance. Donor: cultivation area of transgenic melon and non-transgenic melon with *Fusarium* resistance.

μm, and fertility was about 97%. The pollens from both melon plants were not different for these traits.

2.2.2 Longevity of pollen (experiments in a closed greenhouse)—Logevity of pollen viability from transgenic and non-transgenic melon plants was examined on sunny days in May 1992. The pollens were collected from plants at 9:30, 11:30, 13:30, 15:30, 17:30 and sowed onto pollen germination medium. Most of the pollens collected at 9:30 germinated, but germination frequency of pollen collected at 11:30 was lower. A few pollen grains collected at 13:30 germinated, but the pollen grain collected at 15:30 did not germinate at all. Therefore, the viability of the pollen grain of both transgenic and non-transgenic melon plants seems to last until about 13:30 in a closed greenhouse on a sunny day.

2.2.3 Pollen dispersal by wind (experiments in a closed greenhouse)—The wind pollination of transgenic and non-transgenic melon plants was investigated under an artificial wind generated by an electric fan in a closed greenhouse. Vessels of pollen germination medium were placed at 0, 5, 10, 15, 50, 100, 200, and 300 cm from the plants. The wind was blown from 10:00 to 15:30 at a velocity of 0.5–4.0 m/sec. The pollen grains from the transgenic or non-transgenic plants were not detected on

any of the germination medium. We concluded that pollen of melon, an entmophilous plant, is not dispersed by wind and is generally dispersed only by insects.

2.2.4 Pollen dispersal by insects in an isolated field (experiment in an isolated field)—Recipient melon plants which did not harbor the kanamycin resistance gene (NPT-II) and were not *Fusarium* wilt were planted around the donor (transgenic and non-transgenic *Fusarium* wilt resistant cv. 'Ooi') melon plants (**Fig. 1**). *Fusarium* resistant progenies of the recipients were observed at a distance of 15 m from the donor, while progenies harboring the kanamycin resistance gene were observed at a distance of 10 m from the donor. Since cv. 'Ooi' harbors the homozygous *Fusarium* wilt resistance gene and transgenic melon harbors the heterozygous NPT-II gene, more progenies exhibiting *Fusarium* wilt resistance at a distance from donor were observed than that of progenies harboring NPT-II gene in No. 1, No. 2 and No. 3 areas. However, progenies resistant to *Fusarium* wilt and progenies with the NPT-II gene were not detected at a distance of 25 m from the donor (**Table 2**). These results indicated that the degree of pollen dispersal was not different between transgenic melon plants (M5) and non-transgenic melon plants (cv. Ooi).

2.3 Harmful impact on other plants (experiments in a closed and a semi-closed greenhouse)

The possibility of harmful influences of transgenic melon on the environment was examined. The following compounds were compared between transgenic and non-transgenic plants: (1) phenolic acids, generally considered as allelochemical substances produced in the plant body and secreted from the root; (2) volatile compounds, released from the plant into atmosphere. To estimate the potential impacts on other crops, cabbage plants were grown on the soil that cultivated transgenic or non-transgenic melon plants and on the soil mixed with dry powder prepared from these respective melon plants. The germination ratio, root length and fresh weight of cabbage plants were then measured for investigating the effect.

Specific phenolic acids and volatile compounds were not detected from the transgenic melon plants. There were no differences in germination ratio, root length and fresh weight of cabbage cultivated on the soil. These results suggested that transgenic melon plants do not produce any specific compounds influencing on the environment and other plants.

2.4 Overwintering (experiments in an isolated field)

To compare the weediness of transgenic and non-transgenic melon plants, melon plants and seeds in fruits were place on and buried under the ground. Since the greenhouse was not equipped with any heating facility, transgenic and non-transgenic plants in greenhouse without heating facility withered and died at low temperature by the end of December. The fruits obtained by artificial pollination were either placed on or buried under the ground in the isolated field. The seeds germinated from fruits put on the ground following decomposition of the fruits. However, these seedlings were withered and dead by the low temperature condition before they could bear any fruit. Although germinated seedlings were not observed from fruits buried under the ground during autumn, seedlings emerged from these fruits in the following spring. This observation suggested that if seeds in melon fruits are buried under the ground they could overwinter unlike the whole plants. However, the characteristic for overwintering of transgenic and non-transgenic melon plants was not different, and weediness by overwintering was not changed in transgenic melon.

2.5 Influence on soil microflora

The influence of transgenic melon cultivation on the soil microflora was investigated in a semi-closed green-

Table 2 Comparison of pollen dispersal between transgenic and non-transgenic melon plants

	Distance from donor (m)	Number of progenies with NPT-II gene	Number of progenies exhibiting resistance to *Fusarium* wilt
No. 1	5	5/100[a)]	12/195[b)]
No. 2	10	3/100	7/192
No. 3	15	0/100	2/198
No. 4	15	0/100	0/194
No. 4	25	0/100	0/194
No. 5	25	0/100	0/194
No. 6	25	0/100	0/194
No. 8	30	0/100	0/194
No. 9	40	0/100	0/194
Cont 1		0/30	0/197
Cont 2		12/30	0/196
Cont 3		0/32	30/30

[a)] Number of progenies with NPT-II gene/total number of progenies examined.
[b)] Number of progenies exhibiting resistance to *Fusarium* wilt/total number of progenies examined.
Cont 1: progenies of recipient melon plants by self pollination.
Cont 2: progenies of transgenic melon (M5) plants by self pollination.
Cont 3: progenies of transgenic melon (cv. Ooi) plants by self pollination.

house and isolated field. In a semi-closed greenhouse, plants were cultivated in pots filled with unsterilized soil. After cultivation, the number of microbes, bacteria, actinomycetes and fungi, in the soil were counted. The numbers of actinomycetes and fungi in the soil cultivated with transgenic melon were slightly higher than in soil cultivated with non-transgenic melon plants. But the difference is not significant. Subsequently, the influence on soil microflora was examined in an isolated field. The soil in which melons were planted was sampled before planting (May 7), at flowering (July 8) and after the harvest (September 3). The numbers of fungi in the soil cultivated with transgenic melon were slightly larger than that of non-transgenic melons. Since the differences were not significant, we concluded that the effects of transgenic melons on microflora are the same as those of non-transgenic melons.

2.6 Residual Agrobacterium as vector

To prevent genetically modified *Agrobacterium* from spreading to environment, *Agrobacterium* used as vector was detected from plant body, surface and rhizosphere of transgenic melon plant. No *Agrobacterium* was detected from any part of the transgenic melon plants.

3. Conclusion

There was no difference between transgenic melon plants and non-transgenic melon plants within these experiments except in the increase in CMV resistance. Therefore, we concluded that the influence of transgenic melons on environment was the same as that of non-transgenic melons.

The transgenic melon (M-5) was given official approval for cultivation in ordinary field from Minister of Ministry of Agriculture, Forestry and Fisheries in 1996. This CMV resistant melon was cultivated according to ordinary planting pattern in 1997 and no difference of the general traits for cultivation in transgenic melon was observed in ordinary cultivation.

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Review

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Ecological Risks of Growing Genetically Modified Crops

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Genetic modification can become a major achievement to plant breeding. However, genetic modification differs from traditional breeding in that totally new traits—for example from unrelated organisms—can be added to plants at a high rate, and that these traits are usually introduced many at a time as precisely designed stacks of genes with their own regulating sequences. These differences demand that plants developed by genetic modification are risk assessed. The possible risks are that transgenic phenotypes with altered fitness could change in abundance in the ecosystem, with unwanted effects on other species and on ecosystem integrity or that the ecosystems are affected indirectly by the transgenic plants. The risk analysis should provide information about the following: (1) the possibility of transfer of the transgene by spontaneous crosses between crop and weedy or wild relatives, (2) fitness of the genetically modified crop as well as fitness of the crop relatives that received the transgene by introgression and (3) other types of transgene provoked interactions between the recipient plant and the environment. As an example of a risk analysis data are presented from the model genus *Brassica*.

1. Introduction

The risk of transgene transfer from oilseed rape (*Brassica napus*, 2n=38, genomic constitution AACC) to the weedy relative *Brassica rapa* (2n=20, genomes AA) has been studied. *B. rapa* is one of the parental species of oilseed rape. *B. rapa* is a common annual weed in agricultural fields worldwide in the temperate zone. Particulary in oilseed rape fields with a potential of introgression of oilseed rape genes from the crop to the weed. Outside the field *B. rapa* is ephemeral as seeds will only germinate when the soil is turned. Frequency of gene transfer as well as fitness analysis of offspring plants from crosses have been studied.

2. Results and Discussion

2.1 Frequencies of spontaneous hybridisation and backcrossing between oilseed rape and B. rapa

Our results indicate that spontaneous hybrids between oilseed rape and weedy *B. rapa* are easily obtained. The hybrids also backcrossed spontaneously to the weedy species in the field. The results have been reported [1–6] and they are summarized in **Table 1**.

B. rapa and interspecific transgenic hybrids were sown together in field experiments to assess the extent of backcrossing. Seed set per pod on interspecific hybrids was low (approximately 2.5) compared to seed set on the parental species (typically 16 to 23). The reciprocal cross *B. rapa* × hybrid did not seem to take place, as judged from analysis of seeds harvested on *B. rapa*. A large number of transgenic plants developed from seeds harvested on the interspecific hybrids, and individuals with a *B. rapa*-like morphology were selected for further analysis. Most of the selected plants were clearly backcross plants and a few were almost identical to *B. rapa* (chromosome number 2n=20, high pollen fertility) and had a high seed set in crosses with genuine *B. rapa* (**Fig. 1**) [6].

2.2 Analysis of marker transfer in controlled crosses and backcrosses between B. napus and B. rapa

Hybrids obtained from crosses between different maternal *B. napus* cultivars/transgenic lines and *B. rapa* individuals from cultivars or a wild Danish population were backcrossed (as females) to *B. rapa* individuals from the same population. The backcross, (*B. napus* cv. Drakkar × *B. rapa* cv. Indus) × *B. rapa* cv. Indus, has been analysed with RAPD markers to characterise the transfer of genetic material from the interspecific hybrid to the first backcross generation. We used 33 markers, specific to the *B. napus* parent of the cross. The vast majority of the markers were localized to the C-genome of the crop. All of the markers were transferred to the first backcross generation and most of them in a ratio that were not significantly different from 50% [7].

Another backcross progeny of the type (*B. napus* cv. Drakkar × *B. rapa* (weedy Danish population Bc 25)) × *B. rapa* (weedy Danish population Bc 25) was characterised with respect to both molecular markers, chromosome number and pollen fertility. Also in this cross all the markers—most of them specific to the C-genome—were found in the backcross plants. Plants with 20 chromosomes and a high pollen fertility, both characters of the weedy *B. rapa* were identified among the backcross plants.

2.3 Fitness of F_1, BC_1, F_2 and BC_3 plants from crosses between populations of B. rapa and varieties of B. napus (B. rapa as recurrent parent)

Plants from each of three weedy *B. rapa* populations and three *B. napus* varieties have been intercrossed, and

Table 1 Gene transfer from oilseed rape to *Brassica rapa* in spontaneous crosses

Hybridization, F_1	
female, *B. rapa*:	0–69% hybrids
female, *B. napus*:	0–9% hybrids
Backcrossing, BC_1	
female, F_1:	0–77% backcross plants (~1% *B. rapa*-like)
female, *B. rapa*	low

the fruit and seed set estimated. The offspring plants were grown in the field, and monitored for survival and reproduction. Combining these fitness components, hybrids were intermediate to *B. rapa* and *B. napus* (**Fig. 2**) [8].

Hybrids, *B. rapa* and *B. napus* plants, originating from crossings between two populations and two varieties in the first generation, were intercrossed to obtain F_2, backcross (on both *B. rapa* and hybrids), and pure *B. rapa* and *B. napus* seeds. The same fitness components as described above were estimated in the field for these offspring plants. In average, offspring from backcrossings and F_2 matings had a reduced fitness relative to offspring from intraspecific matings (pure species) [9].

A BC_3 generation produced from backcrossing *B. rapa* like BC_1 plants for another two generations were quantified as to their seed production and pollen fertility. With respect to these two fitness parameters there were no differences between the BC_3 plants and genuine *B. rapa*. This BC_3 generation segregated in a 1 : 1 proportion transgenic (herbicide tolerant) : nontransgenic plants. There were no significant differences between transgenic and nontransgenic sister-plants in survivorship or number of seeds per plant, indicating that costs associated with the transgene are likely to be negligible [10].

2.4 Isolating mechanisms reducing gene flow between B. napus and B. rapa

Differences in seed germination pattern between oilseed rape, wild *B. rapa* and their hybrids were investigated on seeds from controlled crosses (to get well characterised seeds of the two species and their reciprocal hybrids) as well as seeds from a number of natural populations of weedy *B. rapa*. As expected, all *B. napus* seeds germinated at 20°C and ambient humidity, showing no sign of dormancy. *B. rapa* on the contrary, showed a very high degree of dormancy. In this species we had to apply temperature cycling, KNO_3 and sometime even scarification to get a high frequency of germination. As to the hybrids, both types (*napus* × *rapa*) and (*rapa* × *napus*) seeds showed almost no sign of dormancy [11]. Lack of dormancy could be disadvantageous, as the number of hybrids reaching the flowering state could be limited due to germination under unfavorable conditions e.g. with effective weed management thereby limiting interspecific geneflow (**Fig. 3**).

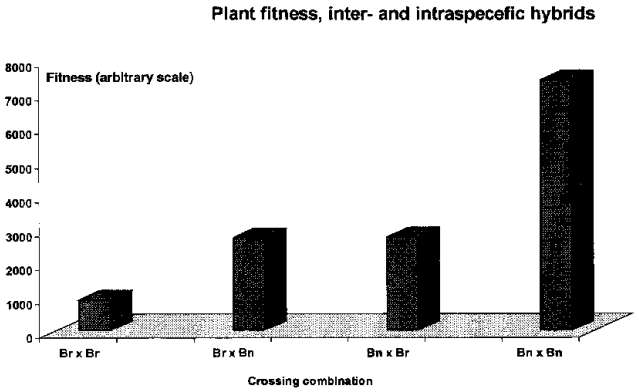

Fig. 2 Combined fitness estimate of F_1 hybrids and pure parental species (*B. rapa* and *B. napus*).

3. Conclusion

In conclusion we have found interspecific hybrid seeds in seed lots from both *B. rapa* and *B. napus*. Also adult hybrids and later generation introgression plants have been found to occur spontaneously in natural populations. The F_1, BC_1, F_2 and BC_3 generations are rather fertile and

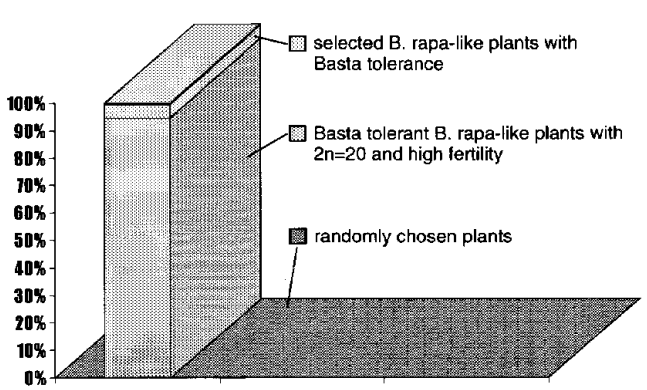

Fig. 1 The frequency of transgenic *Brassica rapa*-like plants in the first backcross generation between transgenic oilseed rape and *B. rapa* (*B. rapa* as recurrant parent).

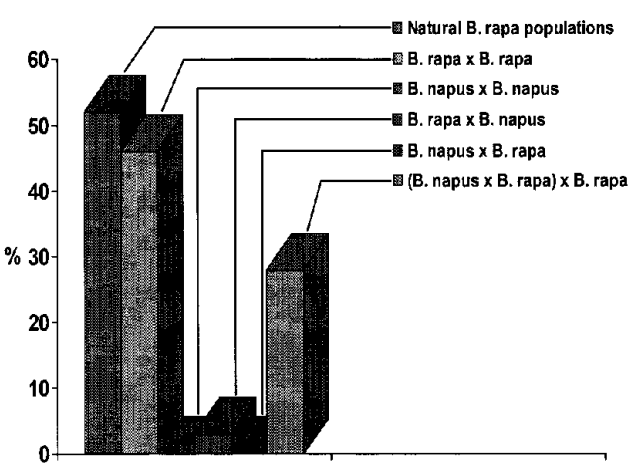

Fig. 3 Seed dormancy of *B. rapa*, *B. napus* and the interspecific hybrids.

fit and *B. rapa*-like plants were observed as early as the first backcross generation. Genomic positions providing "safe integration" of a transgene were not found, as all oilseed rape markers studied were transferred to the backcross plants from controlled crosses. Given these data it seems likely that transgenes will be incorporated into wild *B. rapa* populations. The consequences of this will depend on the transgene in question and the recipient ecosystem.

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