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Dispersal and persistence of genetically modified oilseed rape around Japanese harbors

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Abstract
Background, aim, and scope The possibility of gene transfer from genetically modified oilseed rape (OSR) to its cultivated or wild relatives is of concern since its commercial cultivation, because of its potential weediness and impact on the environment. Introgression of modified genes can affect conservation of agricultural crops, because there are many cultivars and wild Brassicaceae that may cross with genetically modified OSR (*Brassica napus*) in Japan. Japan imports more than 2 million tons of OSR a year from Canada and other countries. Since volunteers of GM OSR were found around harbors in 2004, a lot of feral GM OSR was discovered in Japan. To consider the way how to keep domestic Brassicaceae from GM contamination, we surveyed and analyzed the dispersal and persistence of GM OSR around Japanese harbors. We present the cause and abundance of GM OSR in Japan by this paper.
Materials and methods Survey of the feral OSR was performed several times a year at different seasons when domestic OSR either grows or does not around port areas. Detection of herbicide tolerance in feral *B. napus* was done by test papers that cross react with the modified gene product. Two kinds of herbicide tolerance (glyphosate and glufosinate) were tested.
Results The feral *B. napus* were discovered around all 13 harbors that import rapeseeds from foreign countries. Genetically modified, herbicide-tolerant OSR were frequently found in the surveyed populations. Two kinds of herbicide-tolerant OSR (glyphosate- and glufosinate-tolerant) were discovered in a natural condition 40 km from port to an oil factory where 60,000 tons of OSR seed are processed a year. The cause of voluntary growth of OSR is seed spillage during transportation by trucks from harbors to oil factories and other processing facilities. Some of the feral OSR growing along the roadsides of transport paths exhibited perennial growth spilling their seeds around the places. Alteration of the generation of feral GM OSR was discovered for the first time in Japan as a result of this study. We studied the yearly change of feral OSR abundances focusing on Yokkaichi port over the 4 years

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since 2004. The rate of GM OSR increases year to year, and reaches nearly 90% in 2008.

Discussion The possibility and consequences of gene transfer from the genetically modified OSR to domestic species (*B. rapa* and *B. juncea*) were discussed in relation to impact on domestic agriculture and on environments. Evolutional meaning of the gene transfer was also discussed with respect to the gene construct of GM OSR. This study shows the importance of another pathway of modified gene transfer to non-GM relative species by seed transportation in addition to pollen transfer from commercial cultivation of genetically modified OSR.

Conclusions and recommendations We identified unintended dispersal and persistence of GM OSR around Japanese harbors that import OSR from Canada and other countries. Both glyphosate- and glufosinate-tolerant feral *B. napus* were discovered. The cause of volunteer OSR is spillage of the seeds during transportation by truck to oil factory. The feral GM OSR sometimes showed perennial growth in Japanese phonological conditions which are not observed for domestic Brassicaceae. In addition, we confirmed an alteration of generations by feral GM OSR in Japan. The possibility of cross pollination and GM gene introgression to domestic varieties can occur in these environments. To improve the situation, each responsible organization, company, administration, or government should establish measures how to stop the dispersal and persistence of GM OSR in nature. Also, the GM plant developers are responsible for revising this situation.

Keywords Dispersal and persistence · GM OSR · Introgression · Oilseed rape · Transportation

1 Background, aim, and scope

The possibility of contamination and gene transfer from genetically modified oilseed rape (GM OSR) to usually cultivated non-GM OSR has been discussed since the commercial cultivation of GM OSR in 1996 (Chevre et al. 1997; Brown et al. 2005; Mikkelson et al. 1996; Timmons et al. 1996; Treu and Emberlin 2000). Escape from commercial cultivation was of concern because of the potential of increased weediness and sustainability in nature (Brown et al. 2005). The acquisition of multiple, stacked modified genes by OSR was also reported (Hall et al. 2000). Introgression of genetically modified gene constructs from *Brassica napus* to other *Brassica* species by pollination is another issue in relation to conservation of agricultural crops (Treu and Emberlin 2000).

In Japan, there are many cultivated varieties of *Brassica rapa* used as food resources. *Brassica juncea* is also used as food and occurs as a wild plant in Japan. Both are known to hybridize with *B. napus*. *B. napus* has not been grown before it was imported from foreign countries. Japan imports about 2 million tons of OSR a year as food oil sources and feed for livestock. Since volunteers of GM OSR was reported first in 2005 (Saji et al. 2005), volunteers or feral growth of genetically modified OSR has occurred in Japan through losses during transport of the seeds by trucks from the harbor to other places like oil factories. To consider how to keep domestic and wild relative species free from introgression of modified genes, we surveyed and analyzed the abundance and rate of genetic modification in volunteer OSR around the Japanese harbors since 2004. The paper presents the available information on a relevant process of unintended dispersal of genetically modified plants into environments where they have not been cultivated. To analyze the ongoing process is of high relevance for biosafety assessment, also with respect to other countries. This report represents the issue of discussion concerning biodiversity conservation in scheduled COP10 international meeting in Nagoya, Japan in 2010.

2 Materials and methods

Survey of the feral OSR was performed several times a year at different seasons when domestic OSR either grows or does not. Feral OSR (*B. napus*) was more easily discovered when domestic OSR (*B. rapa* and *B. juncea*) finished their growth season, because feral *B. napus* grows all year round. The test samples were collected from port areas and the roadsides leading to an oil factory 40 km from the Yokkaichi harbor. Every plant was tested when growing singly, and several samples were collected when they were growing as large populations. The discrimination of Brassicaceae was performed by their morphological characteristics. The samples were analyzed whether they contained the protein that confers herbicide tolerance by an immunochromatographic method called lateral flow test. The sensitivity of the test is 0.1% of the target protein content in the sample. This method detects the protein of herbicide tolerance as an antigen. Test strips of glyphosate tolerance detects CP4EPSPS protein originating from *Agrobacterium* sp. strain CP4, and that of glufonsinate-tolerance detect PAT protein from *Streptomyces hygroscopicus* or *S. viridochromogenes* in GM OSR. Small pieces of leaf (~0.5 cm^2) were extracted in 1 ml of water by crushing in a test tube. A test strip was inserted into the extract. After about 10 min, a red signal line is developed on the strip if the sample contained the target protein. Two types of herbicide tolerance (glyphosate and glufosinate) were tested. The test kit of Agri-

Screen from NEOGEN Corporation (Lansing, MI, USA) and from Strategic Diagnostics Inc. (Newark, DE, USA) were used. In the beginning of the survey, the lateral flow tests and the results of PCR analysis for the corresponding DNA were compared. The results showed good coincidence (data not shown).

3 Results

A lot of feral OSR plants (*B. napus*) were discovered around Yokkaichi harbor in July 2004 when we first surveyed the area. They grew under conveyor belts from ship to silos and around silos. Also, feral OSRs were found in the streets from the harbor to the inland. Some of OSR bloomed and others were just germinating or producing seeds, although domestic *B. rapa* and *B. juncea* had already finished the growth season in this hot summer (July) in Japan. The feral OSRs were also observed outside of the Yokkaichi port area, along roads to an oil factory 40 km distant from the harbor. The frequency of feral OSR was high in the outbound road to the oil factory and low in the road inbound to Yokkaichi harbor. From the situation, the cause of feral OSR was concluded to be spilling of the seeds from the trucks during transportation. Almost all of the imported 60,000 tons of OSR seeds in Yokkaichi harbor were processed in the oil factory. Some of the OSR developed to large populations and others grew as single, isolated individuals. Flowering, seeding, and germination were observed all year round, which could not be observed for domestic varieties of *B. rapa*, of which the flowering season is only in spring. The season independent growth of *B. napus* may increase the chance of hybridization with domestic cultivars of related species and wild species, e.g., *B. juncea*. In a few cases, large plant individuals of feral OSR were observed which lived for several years, showing a perennial growth.

The transgenic feral OSRs produce and scatter seeds making offspring themselves, so that feral transgenic populations persist every time year round in the Yokkaichi port area and along the roads to the oil factory.

From a national scale survey by a citizens group, feral OSR has been observed around all 13 harbors in Japan that import OSR seeds from foreign countries, although the extent was very different from port to port (Fig. 1).The newly constructed oil factories are just neighboring the silos where the seeds are unloaded from ships. Thus, a chance of spilling seeds may be lower. With an increase of transportation distance, the frequencies of feral OSR populations become higher.

Table 1 shows the surveyed results for 4 years around Yokaichi harbor since 2004. Contamination rates of GM OSR have increased considerably over these 4 years (Fig. 2). Nearly 90% of feral OSR in this area was herbicide tolerant in 2007. There are two kinds of herbicide tolerance for glyphosate and glufonsinate. Rate of the two traits has changed since 2004. In the beginning, the glyphosate-tolerant population was dominant, but the glufosinate-tolerant population recently became the majority to be found. We could not as yet trace the reason why the change occurred. The source through spilled imported seeds might have changed or maybe the results of artificial selection pressure due to spraying of complementary herbicides.

We observed sympatric co-existence of herbicide-tolerant OSR (*B. napus*) and wild OSR (*B. juncea*) in the area. There are big populations of *B. juncea* on riverbanks in the south of Yokkaichi port area. Feral OSRs grow together with the feral population of *B. juncea*.

4 Discussion

Japan imports over 2 million tons of OSR seeds a year, most of which are from Canada, where herbicide-tolerant OSR was approved as food in 1994 and for commercial cultivation in 1995. In 2007, more than 90% of OSR from Canada was genetically modified. Canadian farmers prefer herbicide-tolerant traits because of easy weed management. Reflecting the cultivating situation in Canada, the feral OSR around Japanese harbors contains a considerable rate of herbicide tolerance as described in the Section 3.

The possibility of hybridization between oilseed rape and its wild relatives have been discussed by many authors since development of genetically modified crops because of the concern about the potential escape of genetically modified gene and its introgression into cultivated or wild relatives (Chevre et al. 1997; Brown et al. 2005; Mikkelson et al. 1996; Timmons et al. 1996; Treu and Emberlin 2000). Extensive experimentation was carried out for pollination by herbicide-tolerant canola (*B. napus*) with its wild relatives in the field condition by J. Brown et al. before commercial cultivation of GM canola (Brown et al. 2005). They suggested that seed transportation will make volunteer weed, pollen movement will be affected by wind direction, hybrids will be made between GM canola and its wild relatives in field conditions, and bridge crosses between the hybrid and its relatives could play a major role in the movement of herbicide-resistant genes into the natural weed population. A large-scale field experiment was performed to quantify gene flow from herbicide-tolerant canola by pollination to non-genetically modified canola in Australia in 2002 (Rieger et al. 2002). The study showed the pollen from GM canola can move more than 2 km from the cultivation field, although the frequency was less than 0.2%. The gene flow from the feral GM OSR to the

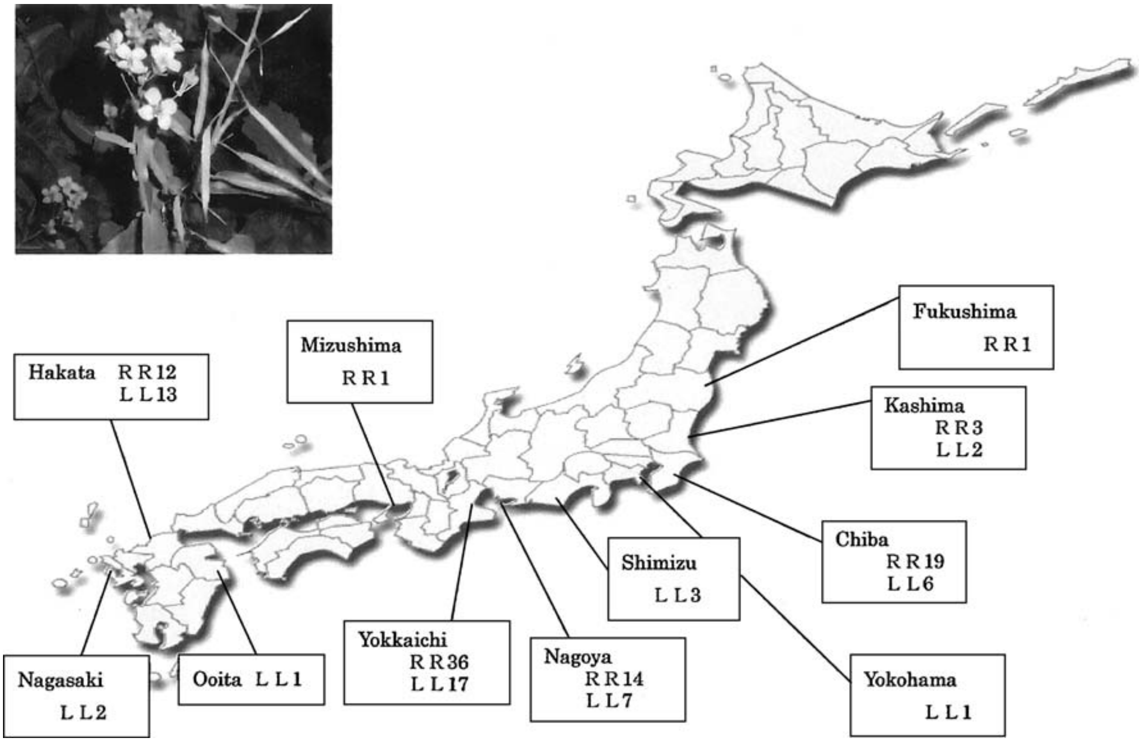

Fig. 1 Map of genetically modified OSR around port areas in Japan. Survey by No-GMO Campaign Japan (2006)

domestic varieties is inherent, because hybridization between these species via pollen has been also documented (Scheffler and Dale 1994).

In the national scale, assessment of hybridization between *B. napus* and *B. rapa*, Wilkinson et al. (2003) identified 47 hybrids among 3,230 plants from eight sympatric populations in the UK, and estimated 32,000 hybrids will form annually in waterside *B. rapa* populations across the land. Hansen et al. (2001, 2003) demonstrated extensive introgression between *B. napus* and *B. rapa* in the natural population and persistence of the introgressed gene in the following generations. Transfer of modified genes from *B. napus* to *B. rapa* was genetically analyzed in detail under Japanese conditions by Lu et al. (2002). Persistence and expression ability of the introgressed genes was tested using green fluorescent protein (GFP) gene between genetically modified *B. napus* with insect resistant gene (Bt) and GFP gene and *B. rapa* (Halfhill et al. 2003). The authors made several hybrid generations of the plants and confirmed that the F_1 hybrid generations contained 95–97% of the *B. napus*-specific GFP marker gene. Although successive backcross generation showed reduction of the markers to 15–29%, formation of homozygous individuals within hybrid populations increase the level of transgene expression as generations progress.

Considering these backgrounds and the circumstances in the Japanese port area, the chance of modified gene introgression into its wild relative species and cultivated crops may happen because there are fields of cultivated OSR (*B. rapa*) for food and large populations of wild relatives *B. juncea* alongside of the truck road to the oil factory, although we have not yet found the hybrids between them. The Yokkaichi region is known as a suitable place for OSR cultivation due to its climatic environment.

Table 1 Results of survey around Yokkaichi harbor for 2004–2007

Date	Sample no.	RR		LL		Total	
		+	%	+	%	+	%
040721	12	3	25.0	0	0.0	3	25.0
040823	17	6	35.3	3	17.6	9	52.9
050217	20	2	10.0	1	5.0	3	15.0
050317	21	12	57.1	0	0.0	12	57.1
050407	7	3	42.9	0	0.0	3	42.9
050412	10	6	60.0	1	10.0	7	70.0
050528	9	0	0.0	4	44.4	4	44.4
050717	6	5	83.3	0	0.0	5	83.3
051003	14	4	28.6	1	7.1	5	35.7
060209	41	19	46.3	4	9.8	23	56.1
060705	16	9	56.3	7	43.8	16	100.0
060705	15	9	60.0	4	26.7	13	86.7
061009	21	10	47.6	8	38.1	18	85.7
061125	45	17	37.8	22	48.9	39	86.7
070307	28	12	42.9	14	50.0	26	92.9
070505	15	6	40.0	8	53.3	14	93.3
071103	25	6	24.0	14	56.0	20	80.0

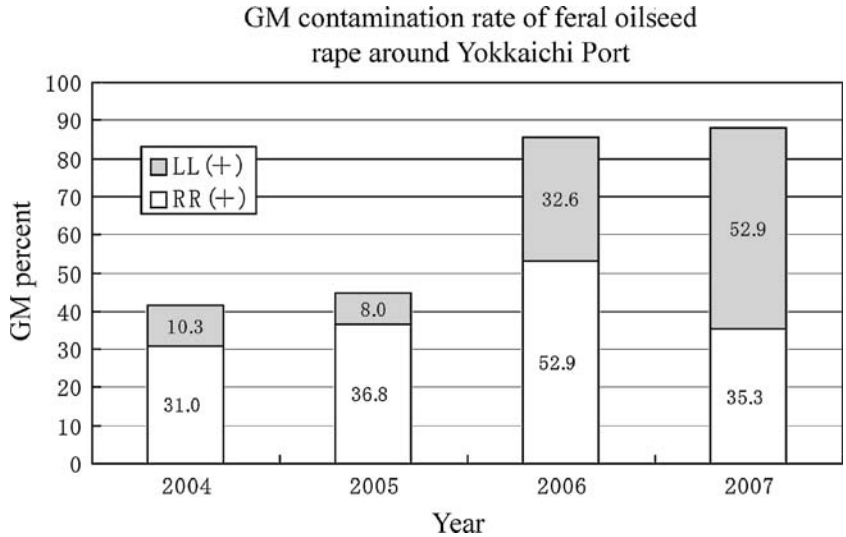

Fig. 2 GM contamination rate of feral oilseed rape around Yokkaichi port

As mentioned in Section 3, we have found many volunteers of GM OSR around the harbors and roadside to the oil factory. These had resulted from spilling of OSR seeds during truck transportation. Hodkinson and Thompson (1997) discussed the role of man in plant dispersal in 1997. Zwaenepoel et al. (2006) also studied the problem in 2006. They noticed seed movement with topsoil by motor vehicles in the case of small seed plants like OSR for their dispersal. Garnier et al. (2008) did an experiment on feral OSR seed dispersal along roadverges to evaluate the influence of genetically modified OSR to the ecosystem. They deposited OSR seeds on the roadverges and checked their anthropogenic secondary dispersal. They reported the dispersal was only limited to small distances from the roadverges (d_{max}= 21.5 m). From the experiment, they concluded that the impact of GM OSR seed dispersal is unlikely to be due to the spread of the GM feral OSR. However, our observation shows the dispersal of GM OSR was caused not only by seed movement by vehicles but by spilling from the truck containers. In addition to primary spilling and dispersion by trucks, wind and birds may contribute to the dispersal. Pollen dispersal by wind and hybridization between relative species would make more long-distance effects on the environment and crop fields. These cases of GM OSR volunteers are already known in Canada, where the commercial cultivation of GM OSR has been performed most in the world since 1996 (Beckie et al. 2006). The report describes the fact of GM OSR volunteer appeared 2 years after commercial cultivation (1998), and the volunteers showed two to three herbicide-resistant properties in a plant body from pollination between different GM individuals. These multi-resistant plants are known as stacked GM plants. The report also describes that one GM volunteer was detected in a place of 550 m from pollen source in the field. Gene flow from herbicide-tolerant canola (*B. napus*) to *B. rapa* and *B. juncea* was also observed in eastern Canada.

There is a Japanese report that a feral OSR with stacked, herbicide-tolerant genes was discovered in 2005 and 2006 around the road from port area to the oil factories (Saji et al. 2007). This double herbicide-resistant OSR was considered to be a hybrid between glyphosate-tolerant and glufosinate-tolerant *B. napus*, because it was found together with single herbicide-tolerant OSRs in the same population. The stacked gene OSR was found at two port areas in Japan. Hybrids were also detected with both *B. rapa* pollinated by *B. napus* and *B. napus* pollinated by *B. rapa* in feral populations in Japan, although the authors did not describe whether the hybrids were genetically modified or not (Matsuo et al. 2005).

Table 2 Domestic Brassicaceae for foods and feral species that can be hybridized with genetically modified *Brassica napus* in Japan

Species name	Variety	Chromosome (2n) (genome type)
Brassica napus	GM oilseed rape (canola)	38 (AACC)
Brassica napus	var. oleifera	38 (AACC)
Brassica rapa	var. campestrisvar. rapavar. pekinensisvar. chinensisvar. farinosavar. japonicavar. Parachinensis	20 (AA)
Brassica carinata		34 (BBCC)
Brassica juncea	var. oleiferavar. cernusvar. napiformisvar. integrifolisvar. rugosavar. Tsasai	36 (AABB)

Namai (personal communication)

There are many domestic OSR varieties for foods in Japan that could be a target of gene introgression from GM OSR (Table 2; Namai, personal communication). Most of these are consumed as vegetables and salads. Then Japanese farmers and consumers are concerned about the potential contamination of GM OSR to domestic OSR species and are afraid of unintended gene transfer from GM OSR to their products. Considering farmer and consumer anxiety, some local administrations decree independent regulation rules for cultivation of genetically modified crops, although the Japanese government approved cultivation of most GM crops that are imported from foreign countries. No farmers as yet cultivate GM crops in Japan. However, some institutes and laboratories are cultivating GM crops in their test fields under controlled environments. In small fields in Japan (mean field size is 1.8 ha), hybridization may easily occur by pollination between GM and non-GM crops, especially in maize and OSR when commercial cultivation of GM crops begins. This study shows another route of gene transfer due to spilled seeds during transportation from the import harbors.

Of concern are also the effects on the natural environment, especially on *B. juncea* and *B. rapa* that is very common in riverbank areas in Japan. *B. rapa* originally came from Europe in old times and has been used for more than 1,000 years in Japan. *B. juncea* and *B. rapa* can hybridize with *B. napus*, and modified genes from GM OSR could thus be transferred as described above. Once a gene flow occurred to these feral OSRs, they can proliferate and disperse developing into stable populations. Although initial hybridization may be of low frequencies, the second or subsequent generations of the hybrids can transfer the modified gene to other OSR plants by back crosses and homozygous hybrid formation by cross-hybridization occurring between them as described by M.D. Halfhill et al. (2003). In addition to *B. rapa*, *B. napus*, *B. juncea*, and *Brassica oleracea* (cabbage), *Brassica nigra* and *Brassica carinata* are also important food sources. Genetically modified *B. napus* is originally a hybrid of *B. oleracea* and *B. rapa*. The evolutional significance and relations between these *Brassica* species were studied and reported in 1935 by Korean botanist Woo Jan-choon who studied in Japan. The theory is known as 'Triangle of U' that can explain how these Brassicaceae originated in nature. Even if the natural probability of hybridization between these different species is small, gene introgression between the species can occur.

To exclude these environmental influences, a mitigation strategy is proposed to introduce a dwarfing gene into cultivating OSR to reduce its competitive fitness in the natural condition (Gressel and Al-Ahmad 2004). However, Reuter et al. (2008) concluded that this strategy will instead increase feral GM OSR rather than reducing them, as demonstrated in their study involving a large-scale feral OSR in rural and urban areas in Germany.

As genetically modified plants have multiple genes from different organisms containing procaryotes and eucaryotes, to give new properties like herbicide tolerance or insecticide tolerance to the targeted plants, together with promoters or terminators, and for other purposes like signal peptides for modified protein transportation in the cell and as selection markers, modified gene introgression into natural species also has a possibility of affecting the ecosystem and evolution in future. To evaluate these effects, more extensive and detailed studies are required.

5 Conclusions

We could identify unintended dispersal of genetically modified OSR (*B. napus*) around all 13 harbors that import OSR for food oil in Japan. Both herbicide-tolerant (glyphosate and glufonsinate) OSRs were discovered. In some cases, persistence of the feral offspring of them was also confirmed that means alteration of generations of them in Japanese environment. A detailed survey was performed for Yokkaichi harbor and for around 4 years since 2004, where most feral GM OSR populations were found. The cause of the spontaneous growth of OSR is transportation and spillage of the seeds by trucks from import harbor to oil factory. The same situation was also found in another harbor area in Japan. The extent of dispersal is related to the distance between the sites of harbors and processing factories. This may be another way of contamination and dispersal of genetically modified OSR to relative species in crops (*B. rapa*) and in nature (*B. juncea*), in addition to well-known cross pollination by commercial cultivation of GM OSR. We suggest the similar situation may occur in other countries that import OSR. Also, the same may occur in transportation from cultivation fields to other places for processing and to export harbors. Possibilities of modified gene transfer to domestic varieties of foods and wild species are increasing by sympatric growth of GM OSR under natural conditions. More detailed surveys are required for evaluation of effects on foods and environments.

6 Recommendations and perspectives

Companies and governmental organizations associated for the import and processing of OSR are recommended to improve the circumstances described in Japanese port areas. Conveyer belt system in ports and truck containers are requested to improve by not spilling the seeds around the structures during transportation of the seeds. Port and roads administrators should reinforce the checking and management of environments and establish measures on how to stop the dispersal and persistence of GM plants around the port areas. The central government is also responsible to save environments and biodiversity from introgression of artificial gene construct to natural and cultivation plants. Domestic agriculture will be influenced by escape and introgression of modified genes from GM OSR to cultivation varieties and wild relatives. Finally, the responsibility of GM plant developers is an important subject. They have the patents for the GM plants to keep their monopoly, while they cannot control the unintended growth and persistence of their products in environments of foreign countries where GM crop is not cultivated.

All these problems and issues should be discussed extensively and be resolved scientifically and socially.

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