Japan is dependent on imports of foreign raw materials such as oilseed. About $2.5 \times 10^6$ t of *Brassica napus* seeds were imported in 2014 (MOF 2015) mostly from Canada, which supplies 97% of total Japanese imports (MOF 2015). The Canadian product is 98% genetically modified (GM) (ISAAA 2013): thus, most of the *B. napus* seeds imported by Japan come from GM cultivars. *B. napus* populations have been found recently along roadsides leading from Japanese ports that unload the seeds and around processing plants. The alien populations are thought to have originated from seed spilled during transportation (Aono *et al.* 2006, 2011, Kawata *et al.* 2009, Mizuguti *et al.* 2011, Nishizawa *et al.* 2009, Saji *et al.* 2005).

The safety of GM herbicide-tolerant (HT) *B. napus* is evaluated prior to importation into Japan. Based on the existing knowledge, there is a very low possibility of these cultivars spreading into the surrounding flora and replacing existing elements of the vegetation. The threat of native species ousting by aliens is recognized as a biodiversity effect under the Cartagena Protocol (J-BCH 2012, MAFF 2012, MHLW 2012). The distribution of Japanese *B. napus* populations derived from seed spillage has been surveyed over time periods of 3–48 mo; however, continuous monitoring over 48 mo has been limited to the region surrounding Yokkaichi (Aono *et al.* 2011). There are no data for evaluating increases and decreases in the extent of Japanese *B. napus* populations derived from seed spillage. Thus, we initiated a 6 yr continuous monitoring program (2006–2011) of populations (including those comprising GMHT *B. napus*) located around 12 Japanese importation facilities.

*B. napus* plants derived from seed spillage may hybridize with volunteer wild relatives in Japan, such as *B. juncea* and...
B. rapa (Bing et al. 1991, FitzJohn et al. 2007, Jørgensen et al. 1998). B. juncea and B. rapa are not native Japanese species (Mizushima and Tsunoda 1969, Takematsu and Ichizen 1993). Even if they were to hybridize with B. napus, the products of these crosses would not be considered as biodiversity effects under the Cartagena Protocol (Yogo 2005). B. juncea populations occur along riversides in various regions of Japan (Shimizu et al. 2001). Were they to cross with B. napus, there is a risk that the hybrids would have elevated fitness levels and invade the surrounding vegetation. However, the average crossing rate of B. juncea and B. napus is only 1.62% even when they are planted together (Tsuda et al. 2012). Furthermore, GM B. napus distributions are limited to port environs and roads leading from them (Aono et al. 2006, 2011, Kawata et al. 2009, Mizuguti et al. 2011, MAFF 2012, Saji et al. 2005). Consequently, the possibility of natural hybridization between B. napus and other related species in the wild seems extremely remote. Spontaneous hybridizations of GM B. napus and B. rapa have not been reported yet in the world. Although GM B. napus and B. rapa have spontaneously hybridized in Canada (Warwick et al. 2008, Yoshimura et al. 2006) and Japan (Aono et al. 2011), there has been no long-term monitoring of such events to date. Accordingly, we undertook our survey to determine whether the volunteer populations examined included HT plants.

### Materials and Methods

#### Sampling B. napus, B. juncea and B. rapa

During the April–May periods of the years 2006–2011, we examined the distributions of B. napus, B. juncea and B. rapa populations within 5 km radii of the 12 unloading ports. We distinguished the species morphologically at each sampling site. B. juncea is distinguished from the other two species by the deep clefts at the leaf bases and the absence of leaf clasps on the stem. The upper leaves of B. napus are sessile, partially clasp the stem and have waxy surfaces. The upper leaves of B. rapa are also sessile and fully clasp the stem.

We defined population units as follows. Even when there was only one plant, it was considered as an independent population. When a plant was >10 m from another plant, the two were considered as members of different population. Assemblages in obviously different growing conditions, such as the presence of soil (i) in a planted zone, or (ii) in accumulations in cracked road surfaces, were considered to be different populations, regardless of the distance between them.

In the ports of Kashima, Chiba, Yokohama, Shimizu, Nagoya, Yokkaichi, Osaka, Kobe, Mizushima, Uno, Hakata and Tobata (Fig. 1), we found a total of 1,029 populations of B. napus, 1,169 of B. juncea, and 184 of B. rapa. From

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Fig. 1. Twelve ports in Japan that import oilseed rape; we surveyed *Brassica napus* (plants originating from spilled seeds), B. juncea, and B. rapa populations around these unloading ports.
a maximum of eight plants per population, we collected four or more fresh leaves per individual, and held them at \(-20^\circ C\) before proceeding with biochemical analyses. We checked for the existence of transgenes as detailed below. When at least one GMHT plant was found, we considered the assemblage from which it had been taken to be a GMHT population.

**Immunological analyses**

Following the procedures of Aono et al. (2006), we used the TraitChek RUR and TraitChek LL Test Kit (Strategic Diagnostics Inc., Newark, DE, USA) test kits to detect GMHT *B. napus*. We checked for the presence of glyphosate-tolerant protein (CP4 EPSPS) and glufosinate-tolerant protein (PAT) in tissue extracts from ground fresh leaf samples.

**DNA analyses**

We used cetyletrimethylammonium bromide to extract DNA from leaves in which CP4 EPSPS and PAT had been found. Genes were detected by PCR using the two primers for the cp4-epsps gene that encodes EPSPS and the bar gene that encodes PAT. Amplifications were carried out in a 25 μL total reaction volumes using a GeneAmp9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The final concentrations of PCR components were as follows: PCR buffer II × 1 (Applied Biosystems); MgCl₂, 1.5 mmol/L; genomic DNA, 25 ng; primers, 0.5 μmol/L; dNTPs, 200 μmol/L; AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA), 0.625 units/reaction. Recombinant DNA segments were detected using the following PCR stepcycle program: preincubation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s followed by a final extension at 72°C for 7 min. The PCR-amplified products were then subjected to electrophoresis on 3%-agarose gels. DNA bands of appropriate sizes were recovered from the gels and their nucleotide sequences were determined (using a DNA sequencer) to confirm that these products corresponded to fragments of the respective genes.

**Statistical analysis**

R version 2.15.3 software (R Core Team 2013) was used for the generalized linear regression analyses of the GMHT *B. napus* population growth trend at each port. Since the dependent variable comprised count data, and population occurrences were rare at some ports, we considered a Poisson distribution to be appropriate.

**Results**

**Distribution of *B. napus* populations; trends in growth and contraction**

Many *B. napus* plants, including GMHT individuals were distributed around the 12 unloading ports and along roadsides. Over 6 yr, we found a total of 1,029 populations around the ports of Kashima, Yokkaichi and Hakata. Thus, there were >200 populations per port over the survey period (>35 populations in each year). Around Chiba, we found a total of 122 populations over 6 yr (>20 populations in each year). However, around each of the ports of Yokohama, Shimizu, Nagoya, Osaka, Kobe, Mizushima, Uno and Tobata, we found <100 over 6 yr, and no populations were observed in some years.

We found GMHT *B. napus* individuals in 414 populations (ca. 40%) of 1,029 around the 12 ports (Table 1). The proportion of populations containing GM individuals was high (>60%) in Chiba, Yokkaichi and Hakata. In other ports, the proportion was <30%, and zero in the ports of Osaka and Tobata (Table 1). Our generalized linear model regression

| Ports     | Number of populations | Number of populations with GMHT individuals | GM ratioa (%) |
|-----------|-----------------------|--------------------------------------------|---------------|
|           | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | Total | Average  | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | Total | Average |
| Kashima   | 38   | 38   | 21   | 31   | 41   | 43   | 212   | 35.3     | 1     | 0     | 0    | 1    | 0    | 1    | 2     | 0.3     |
| Chiba     | 15   | 19   | 27   | 13   | 18   | 30   | 122   | 20.3     | 14    | 12    | 20   | 8    | 12   | 22   | 88    | 14.7    |
| Yokohama  | 4    | 19   | 19   | 9    | 17   | 5    | 57    | 9.5      | 0     | 8     | 4    | 0    | 1    | 1    | 14    | 2.3     |
| Shimizu   | 3    | 0    | 2    | 3    | 7    | 0    | 15    | 2.5      | 0     | 0     | 0    | 1    | 3    | 0    | 4     | 0.7     |
| Nagoya    | 10   | 14   | 13   | 22   | 14   | 8    | 82    | 13.7     | 1     | 1     | 2    | 3    | 3    | 12   | 12    | 2.0     |
| Yokkaichi | 40   | 43   | 23   | 38   | 36   | 30   | 210   | 35.0     | 25    | 17    | 17   | 26   | 24   | 27   | 136   | 22.7    |
| Osaka     | 0    | 0    | 1    | 1    | 4    | 1    | 7     | 1.2      | 0     | 0     | 0    | 0    | 0    | 0    | 0     | 0.0     |
| Kobe      | 4    | 13   | 6    | 10   | 9    | 4    | 46    | 7.7      | 2     | 5     | 3    | 0    | 2    | 12   | 2     | 2.0     |
| Mizushima | 5    | 2    | 1    | 3    | 2    | 2    | 15    | 2.5      | 2     | 0     | 0    | 0    | 0    | 0    | 2     | 0.3     |
| Uno       | 0    | 1    | 1    | 3    | 0    | 1    | 6     | 1.0      | 0     | 0     | 0    | 0    | 0    | 1    | 1     | 0.2     |
| Hakata    | 25   | 35   | 46   | 42   | 41   | 43   | 232   | 38.7     | 7     | 19    | 37   | 27   | 27   | 26   | 143   | 23.8    |
| Tobata    | 0    | 1    | 0    | 5    | 3    | 16   | 25    | 4.2      | 0     | 0     | 0    | 0    | 0    | 0    | 0     | 0.0     |
| Total     | 144  | 185  | 160  | 167  | 184  | 189  | 1029  | 14.3     | 52    | 62    | 80   | 67   | 71   | 82   | 414   | 40      |
| Average   | 12.0 | 15.4 | 13.3 | 13.9 | 15.3 | 15.8 | 14.3  | 4.3      | 5.2   | 6.7   | 5.6  | 5.9  | 6.8  | 6.8  | 5.8   | 0.0     |

* Proportion of GMHT populations: (total number of populations containing GMHT *B. napus* / total number of populations) × 100.
Fig. 2. Time series analyses of the numbers of *Brassica napus* populations around 12 unloading Japanese ports. Temporal trends are represented by fitted regression lines (with 95% confidence envelopes).
Fig. 3. Time series analyses of the numbers of *Brassica napus* populations containing genetically modified, herbicide-tolerant (GMHT) individuals around 12 unloading Japanese ports. Temporal trends are represented by fitted regression lines (with 95% confidence envelopes). Regression fits are not provided for Tobata, Uno, Mizushima and Osaka because GMHT individuals were either absent or found only once.
Glyphosate-tolerant protein (CP4 EPSPS) and glufosinate-tolerant protein (PAT) were not detected in any port or year. We did not detect glyphosate-tolerant protein (CP4 EPSPS) or glufosinate-tolerant protein (PAT) in any of the populations examined. Thus hybrid progeny of GMHT B. napus × B. juncea or GMHT B. napus × B. rapa crosses were absent from our samples.

### Discussion

**Relationship between B. napus import volume and the number of populations around each port**

The total import volumes of B. napus were stable (212.7–235.3 × 10^5 t/yr) during the period 2006–2011 (Table 3). During these years, Tobata imported B. napus in only 2006. We found B. napus populations more frequently around Hakata, Kashima and Yokkaichi (in descending rank order) than other ports, but the import volumes of these three ports ranked only 8th, 6th and 9th among the 12, respectively. Kobe imported more than any of the other ports (>400 × 10^5 t), and the volume unloaded there increased over 6 yr. Nevertheless, we found <50 populations in total around Kobe (7th in rank order among 12 ports). Thus, there was no relationship between the import volume of B. napus and the numbers of populations growing around ports. The establishment of the plant populations depended on the transport method from tanker to domestic facility, the presence or absence of soil around the ports and weed management protocols. As expected, there were few populations around Uno and Osaka, which imported <25 × 10^5 t. However, there were populations around Tobata, even though there were no imports in the period 2007–2011. Thus, there may be alternative B. napus habitats around Tobata, or the habitat requirements of B. napus populations around the 12 ports around Japan were not rejected for all ports except Tobata. Therefore, the populations showed no increase or decrease over 6 yr around all ports other than Tobata, where the temporal trend was positive. However, a single outlier value for 2011 was responsible for this positive trend in Tobata; since we found no correlated shifts in favorable environmental condition associated with the high value in 2011, we consider this data point to be incidental. Furthermore, the Tobata populations did not contain GMHT plants.

### Crossing between GMHT B. napus and congeners

We found 1,169 B. juncea populations around in the 12 ports over 6 yr (Table 2). These populations were found in all ports and years other than Chiba in 2011. Over 6 yr we found >100 in Nagoya, Osaka, Kobe, Mizushima, Uno and Tobata. Tobata had the largest number (197); Kashima, Chiba, Yokkaichi and Hakata had <30 over the same time period.

The population distribution of B. rapa was smaller than that of B. juncea. Only 184 B. napus populations were found in the 12 ports over 6 yr (Table 2). We found no populations around Yokkaichi and Osaka over the same time period. Populations (96; >50% of the total) were found in every year around Tobata.

### Table 2. Numbers of Brassica juncea and B. rapa populations surveyed over 6 yr around 12 Japanese ports

| Ports   | B. juncea | B. rapa |
|---------|-----------|---------|
|         | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | Total | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | Total |
| 1 Kashima | 6   | 6   | 7   | 4   | 1   | 1   | 25 | 3 | 1 | 2 | 0 | 3 | 0 | 9 |
| 2 Chiba   | 5   | 1   | 3   | 2   | 1   | 0   | 12 | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| 3 Yokohama | 14  | 26  | 6   | 12  | 10  | 10  | 78 | 0 | 0 | 0 | 3 | 2 | 1 | 6 |
| 4 Shimizu | 10  | 13  | 15  | 15  | 21  | 24  | 98 | 2 | 0 | 1 | 0 | 1 | 0 | 4 |
| 5 Nagoya  | 41  | 8   | 15  | 16  | 18  | 26  | 124 | 0 | 0 | 0 | 5 | 4 | 12 |
| 6 Yokkaichi | 6   | 2   | 1   | 3   | 3   | 11  | 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 Osaka   | 12  | 13  | 17  | 19  | 19  | 21  | 101 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 Kobe    | 30  | 18  | 26  | 16  | 24  | 24  | 132 | 0 | 1 | 7 | 9 | 4 | 6 | 27 |
| 9 Mizushima | 32  | 31  | 33  | 23  | 26  | 31  | 176 | 0 | 0 | 1 | 2 | 2 | 0 | 5 |
| 10 Uno    | 22  | 29  | 28  | 37  | 25  | 31  | 172 | 0 | 0 | 1 | 1 | 1 | 0 | 3 |
| 11 Hakata | 11  | 6   | 2   | 3   | 3   | 3   | 28 | 0 | 1 | 3 | 2 | 0 | 2 | 20 |
| 12 Tobata | 36  | 32  | 31  | 28  | 32  | 38  | 197 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total     | 225 | 185 | 184 | 180 | 175 | 220 | 1169 | 35 | 33 | 35 | 25 | 38 | 18 | 184 |

Glyphosate-tolerant protein (CP4 EPSPS) and glufosinate-tolerant protein (PAT) were not detected in any port or year.
Long-term monitoring of feral GMHT Brassica napus around Japanese ports

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Table 3. Total Brassica napus import volumes and proportion arriving at 12 Japanese ports from Canada over 6 yr

| Ports | B. napus import volume (k ton) | Import volume from Canada (%) |
|------|-------------------------------|-------------------------------|
|      | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 |
| 1    | Kashima | 199  | 183  | 166  | 164  | 195  | 180  | 95   | 96   | 100  | 100  | 100  |
| 2    | Chiba   | 367  | 365  | 352  | 351  | 346  | 322  | 94   | 100  | 100  | 100  | 100  |
| 3    | Yokohama| 348  | 363  | 354  | 377  | 349  | 368  | 95   | 99   | 99   | 90   | 99   |
| 4    | Shimizu | 157  | 201  | 196  | 190  | 220  | 216  | 100  | 100  | 100  | 100  | 100  |
| 5    | Nagoya  | 287  | 253  | 226  | 278  | 260  | 318  | 92   | 88   | 95   | 96   | 95   |
| 6    | Yokkaichi| 145  | 116  | 103  | 93   | 95   | 131  | 52   | 82   | 100  | 92   | 92   |
| 7    | Osaka   | 6    | 2    | 5    | 6    | 5    | 7    | 0    | 0    | 0    | 0    | 0    |
| 8    | Kobe    | 430  | 426  | 433  | 466  | 500  | 494  | 75   | 89   | 95   | 83   | 91   |
| 9    | Mizushima | 188  | 160  | 159  | 152  | 190  | 160  | 93   | 83   | 100  | 63   | 91   |
| 10   | Uno     | 25   | 23   | 23   | 15   | 16   | 19   | 94   | 95   | 100  | 100  | 100  |
| 11   | Hakata  | 114  | 126  | 110  | 118  | 130  | 139  | 88   | 89   | 88   | 90   | 95   |
| 12   | Tobata  | 3    | 0    | 0    | 0    | 0    | 0    | 49   | –    | –    | –    | –    |
| Total|        | 2270 | 2218 | 2127 | 2211 | 2307 | 2353 | 87   | 93   | 97   | 91   | 96   |

Data are from Trade Statistics of Japan, MOF (Ministry of Finance Japan); http://www.customs.go.jp/toukei/info/index.htm (2015).

populations may have originated from sources other than oilseed imports.

Invasive potential of B. napus populations including GMHT plants

B. napus is generally thought to be an opportunistic species (Warwick et al. 1999). It may dominate in habitats disturbed by humans, but has little ability to establish in undisturbed habitats due to competitive exclusion by native elements in the flora. Therefore, it is not considered to be an invasive species (Crawley et al. 1993, 2001, Damgaard and Kjaer 2009, Hails et al. 2006, Warwick et al. 1999). Non-GM and GM B. napus population frequencies increased significantly over 6 yr around only Tobata (Fig. 2) and Hakata (Fig. 3), respectively. We observed no common trends of increase/decrease across the ports. B. napus including GMHT plants is therefore considered to be neither expanding nor invasive species in Japanese environment. Feral B. napus spreads in areas with high oilseed rape cultivation frequencies (Squire et al. 2011), along busy roads (Crawley and Brown 1995, 2004, Knispel and McLachlan 2009), and near seed handling storage and processing facilities (Peltzer et al. 2006). More than 87% of B. napus were 85%, 88%, 86%, 93%, 94%, and 96% in the years 2006, 2007, 2008, 2009, 2010 and 2011, respectively. We found 414 populations of glyphosate- or glufosinate-tolerant GM B. napus, i.e., 40% of the total population number. More than 87% of B. napus imported since 2006 came from Canada (Table 3), where the proportions of GMHT B. napus were 85%, 88%, 86%, 93%, 94%, and 96% in the years 2006, 2007, 2008, 2009, 2010 and 2011, respectively (ISAAA 2013). Therefore, the GM ratio is estimated more than 41.7% (85% × 49% at Tobata in 2006) at least. The maximum portion of persistent populations in a single location is low; most disappear rapidly (Charters et al. 1999, Crawley and Brown 1995, 2004, Elling et al. 2009, Knispel and McLachlan 2009, Nishizawa et al. 2009, Peltzer et al. 2008, Squire et al. 2011). However, periodic disturbance disturbed by anthropogenic effects, such as weeding and herbicide application, or by natural forces, such as flooding, promote feral population persistence over protracted periods (Claessen et al. 2005, Garnier et al. 2006). Planted zones near Kashima ports are weeded several times a year (Mizuguti et al. 2011). Therefore, repeated seed inputs via transportation vectors and artificial disturbances, such as weeding may be main factors enabling the persistence of B. napus populations near Japanese ports.

We found 414 populations of glyphosate- or glufosinate-tolerant GM B. napus, i.e., 40% of the total population number. More than 87% of B. napus imported since 2006 came from Canada (Table 3), where the proportions of GMHT B. napus were 85%, 88%, 86%, 93%, 94%, and 96% in the years 2006, 2007, 2008, 2009, 2010 and 2011, respectively (ISAAA 2013). Therefore, the GM ratio is estimated more than 41.7% (85% × 49% at Tobata in 2006) at least. The GM ratios in Kashima, Yokohama, Shimizu, Nagoya Mizushima and Uno (Table 1) were obviously lower than...
expected values by multiplying the proportions of GMHT B. napus cultivation in Canada and import volume from Canada (Table 3). Aono et al. (2011) reported similar findings. The reasons for the mismatch between GM proportions in the Canadian sources and introduced Japanese populations are unknown, but may include recruitment from seeds that persisted in the soil from a time period when the GM proportion in the imports was lower. Alternatively, some of the populations may have originated from sources other than oilseed imports.

B. napus is a poor competitor and is not regarded as an environmentally hazardous colonizing species (Beckie et al. 2001, Dignam 2001). The persistence and invasiveness of GMHT and non-GM B. napus should remain similar as long as glyphosate and glufosinate herbicides are not used. Simard et al. (2005) examined volunteer B. napus with single or multiple HT traits in a greenhouse study and found that their fitness values differed very little or not at all from those of non-GMHT plants when herbicide was not applied. Furthermore, there is no evidence that dormancies of glyphosate- and glufosinate-tolerant seeds are different from those of non-GM seeds (Hails et al. 1997, Lutman et al. 2005, 2008, Messean et al. 2007, Sweet et al. 2004).

Based on the previous studies (Beckie et al. 2004, Hails et al. 1997, Lutman et al. 2005, 2008, Messean et al. 2007, Simard et al. 2005, Sweet et al. 2004), it is reasonable to conclude that persistence and the ability to recruit are similar between GMHT and non-GM B. napus and that GMHT B. napus would quickly disappear without environmental disturbance. Populations around ports that are maintained by newly spilled seeds, are highly unlikely to invade surrounding vegetation. Although GMHT B. napus has been grown and marketed in Canada since 1996, its distribution around Vancouver in 2005 was largely restricted to sites along railroad tracks and roads used for seed transport; GM B. napus has not invaded the surrounding flora (Yoshimura et al. 2006).

Frequency of detecting glyphosate- and glufosinate-tolerant B. napus

In Canada, gene flow through pollen has induced unintended HT trait stacking in native (Beckie et al. 2003, Hall et al. 2000) and feral (Knispel et al. 2008) plants. Unintended glyphosate-and-glufosinate-tolerances in B. napus plants have recently been reported in the USA (Schafer et al. 2011) and in Japan (Aono et al. 2006). There are two possible cases for such multiple HT trait stacking: (i) hybridization in the exporting country, where the two types of GMHT B. napus are grown in adjacent fields, and hybrid seeds exported to recipient locations; (ii) co-occurrence and hybridization of glyphosate-tolerant and glufosinate-tolerant B. napus plants in feral populations, leading to seed production. Yoshimura et al. (2006) examined 381 feral B. napus plants in western Canada, where GMHT B. napus is widely grown, and in the port of Vancouver, from which the seeds are exported, but no stacked individuals were found. We analyzed 1,029 populations (1,904 plants), but none were tolerant of both herbicides. Thus, the frequency of stacked plant was lower than the detection resolution of this study.

Frequency of crossing between GMHT B. napus and B. juncea

Previous studies showed that B. napus produces most seeds through self-crossing, but readily crosses with B. juncea (Bing et al. 1991, FitzJohn et al. 2007, Jørgensen et al. 1998). Since B. juncea grows widely as a weed in Japan (Takematsu and Ichizen 1993), it is very likely to receive pollen from B. napus. No transgenes were found in feral B. juncea seeds sampled from 57 locations and 58 locations by Saji et al. (2005) and Aono et al. (2006), respectively; the collection sites were located near several ports, along roadsides, and riversides in Japan. In this six-year study, we examined 1,169 B. juncea populations (3,965 plants) but found no transgenes in our study either.

Hybridization probabilities are related to flowering synchrony between B. napus and B. juncea, the distance between the two species, and the frequency and activity of the pollinators. According to Matsuo and Ito (2001), the flowering periods of the species overlap (early April to mid-May in the Kanto region, and late March to late April in the Kinki region). Consequently, hybridization may be prevented by physical isolation. When both species are cultivated together in large fields, the crossing rates was 0.1-3.29% (Bing et al. 1991, 1996, Huiming et al. 2007, Jørgensen et al. 1998); when they grew together on unintended terrain the crossing rate was 1.62% (Tsuda et al. 2012). The largest number of B. juncea populations we encountered were located in Nagoya (in 2006), but only 41 populations occurred in a 5 km radius. Under circumstances in which the two species rarely grow adjacent to one another, hybridization between B. napus and B. juncea is unlikely. Bees and bumblebees are the most important long-distance pollinators of rape plants (OECD 1997), but they are rarer in port environments than in cultivation fields. The pollination rates in the volunteer populations we examined were therefore likely to have been low.

Even when the two species hybridize, the pollen fertility of the hybrid is as low (0–28%) (Frello et al. 1995), and fecundity is less than that of the parents. Since fruiting plants are rare along roadsides due to insect damage and abiotic stresses, including water shortages (Charters et al. 1999), the frequencies of hybrids or their progeny were likely very low, and probably undetectable at the resolution of our sampling program.

Frequency of crossing between GMHT B. napus and B. rapa

Since B. rapa and B. napus both have A genomes, their crossing compatibility is high (Norris et al. 2004); some natural crossings have been reported. Reported crossing rates of 0.4–1.5% (Scott and Wilkinson 1998), 0.2% (Wilkinson et al. 2000), 1.1–17.5% (Simard et al. 2006), and 1.93% (Wilkinson et al. 2000) have been reported. In
all of these cases, the plants of the two species were growing close together.

Saji et al. (2005) did not find transgenes in volunteer feral B. rapa seeds collected from two Kobe port locations. Aono et al. (2006) made collections in 17 locations around ports, along roadsides, and along riversides, but did not find transgenes. We also found no hybrid offspring of B. rapa and B. napus in 184 feral B. rapa populations (485 plants).

Factors influencing the probability of hybrid crosses between B. rapa and B. napus are similar to those influencing the frequency of hybridization between B. juncea and B. napus (synchronization of flowering, the distance between the two species, and the frequency and activity of pollinators). Observations of herbarium specimens and field studies by Matsuo and Ito (2001) indicated that the flowering periods of B. rapa and B. napus overlap (early April to mid-May in the Kanto region, and late March to late April in the Kinki region). B. rapa occurred less frequently than B. juncea in our study; a maximum of 22 populations (around Tobata in 2007) occurred within 5 km of unloading sites. Thus, physical isolation is the primary mechanism inhibiting hybridization. Furthermore, although the hybrid morphology is similar to that of the B. rapa seed parent, pollen fertility is low (55% according to Warwick 2003; 0–28% according to Frello et al. 1995) because the hybrids are triploid. Even when hybridization occurs, fecundity is low. The frequency of hybrids is very low, but they have been found in riverbeds in Yokkaichi (Aono et al. 2011) and in Vancouver, from which B. napus is exported (Yoshimura et al. 2006). When the natural habitat of B. rapa is near B. napus transportation routes, hybridization can occur. The fitness of B. rapa × B. napus hybrids is lower than those of the parent populations, even though transgenes are present (Warwick 2007). Recent observations in Canada have shown, however, that offspring of GMHT B. napus × B. rapa hybrids are able to persist for 6 yr without herbicide application, despite the fitness cost of hybridization (Warwick et al. 2008). Thus, the fitness of B. rapa × B. napus hybrid plants may be higher than previously thought. This topic should be further researched in previous studies.

Conclusion

The analyses of data from our six-year survey demonstrated that the distributions of GMHT B. napus population showed no increase or decrease significantly around 12 unloading ports while B. napus including GMHT plants import volume were stable. Therefore, GMHT B. napus is not invasive and would not eliminate elements of the native flora in Japanese environment.

We also found no hybrid in 1,169 populations of B. juncea or 184 populations of B. rapa around the 12 ports. Thus, GMHT B. napus is not likely to cross with congeners in the Japanese environment. GMHT B. napus is therefore not considered to be a plant that impacts biodiversity in Japan according to the criteria of the Cartagena Protocol.

Most of the GM crops approved in Japan have a HT trait. Development of traits that increase tolerance of environmental stressors, such as insect tolerance and drought tolerance, is to be expected in the future (Warwick et al. 2009, Yoshimura and Matsuo 2012). Therefore, the importance of continued long-term monitoring is increasing as baseline data of feral GM B. napus.

Acknowledgments

We thank Ms. Yukiko Yamada, Mr. Kenji Asakura, Mr. Masahide Fujikawa, Ms. Ayako Yoshio, Mr. Fumio Kato and Mr. Noriya Shioda of the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan for valuable discussions and advice for this survey. We also thank Dr. Satoshi Furui and Dr. Kazumi Kitta for transferring the materials for detecting genetically modified organisms. We appreciate Agriculture, Forestry and Fisheries research council, MAFF of Japan for technical supports of DNA analyses. This project was funded by the MAFF of Japan.

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