Research Paper

Long-term monitoring of feral genetically modified herbicide-tolerant *Brassica napus* populations around unloading Japanese ports

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Genetically modified, herbicide-tolerant (GMHT) *Brassica napus* plants originating from seed spill have recently been found along roadsides leading from Japanese ports that unload oilseed rape. Such introductions have potential biodiversity effects (as defined by the Cartagena Protocol): these include replacement of native elements in the biota through competitive suppression or hybridization. We conducted surveys in the period 2006–2011 to assess such threats. We examined shifts in the population distribution and occurrence of GMHT plants in 1,029 volunteer introduced assemblages of *B. napus*, 1,169 of *B. juncea*, and 184 of *B. rapa* around 12 ports. GMHT *B. napus* was found around 10 of 12 ports, but its proportion in the populations varied greatly by year and location. Over the survey period, the distributions of a pure non-GMHT population around Tobata and a pure GMHT population around Hakata increased significantly. However, there was no common trend of population expansion or contraction around the 12 ports. Furthermore, we found no herbicide tolerant *B. juncea* and *B. rapa* plants derived from crosses with GMHT *B. napus*. Therefore, GMHT *B. napus* is not invading native vegetation surrounding its populations and not likely to cross with congeners in Japanese environment.

Key Words: *Brassica*, genetically modified, ferality, invasiveness, herbicide tolerance, seed spillage, persistence.

Introduction

Japan is dependent on imports of foreign raw materials such as oilseed. About 2.5×10^6 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. juncea* 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
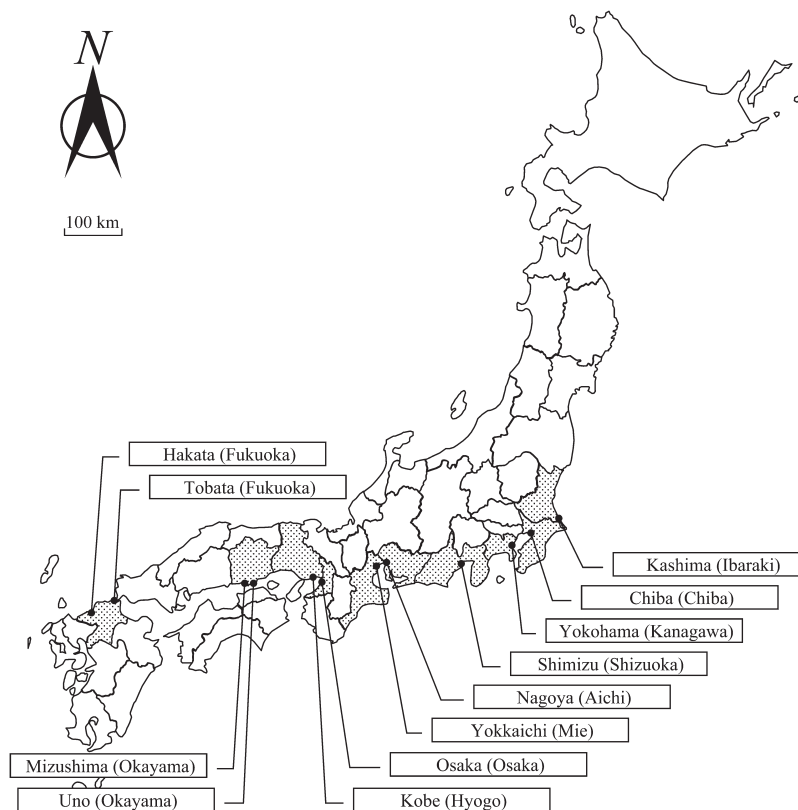

Fig. 1. Twelve ports in Japan that import oilseed rape; we surveyed *Brassica napus* (plants originating from spilled seeds), *B. juncea*, and *B. rapa* populations around these unloading ports.

a maximum of eight plants per population, we collected four or more fresh leaves per individual, and held them at –20°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.

Immunochemical 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 cetyltrimethylammonium 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); MgCl$_2$, 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 prod-

ucts 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

Table 1. Numbers of *Brassica napus* populations containing genetically modified, herbicide-tolerant (GMHT) plants surveyed over 6 yr around 12 Japanese ports

	Ports	Number of populations								Number of populations with GMHT individuals								GM ratio[a] (%)
		Year						Total	Average	Year						Total	Average	
		2006	2007	2008	2009	2010	2011			2006	2007	2008	2009	2010	2011			
1	Kashima	38	38	21	31	41	43	212	35.3	1	0	0	0	1	0	2	0.3	1
2	Chiba	15	19	27	13	18	30	122	20.3	14	12	20	8	12	22	88	14.7	72
3	Yokohama	4	19	19	9	1	5	57	9.5	0	8	4	0	1	1	14	2.3	25
4	Shimizu	3	0	2	3	7	0	15	2.5	0	0	0	1	3	0	4	0.7	27
5	Nagoya	10	14	13	9	22	14	82	13.7	1	1	2	2	3	3	12	2.0	15
6	Yokkaichi	40	43	23	38	36	30	210	35.0	25	17	17	26	24	27	136	22.7	65
7	Osaka	0	0	1	1	4	1	7	1.2	0	0	0	0	0	0	0	0.0	0
8	Kobe	4	13	6	10	9	4	46	7.7	2	5	0	3	0	2	12	2.0	26
9	Mizushima	5	2	1	3	2	2	15	2.5	2	0	0	0	0	0	2	0.3	13
10	Uno	0	1	1	3	0	1	6	1.0	0	0	0	0	0	1	1	0.2	17
11	Hakata	25	35	46	42	41	43	232	38.7	7	19	37	27	27	26	143	23.8	62
12	Tobata	0	1	0	5	3	16	25	4.2	0	0	0	0	0	0	0	0.0	0
	Total	144	185	160	167	184	189	1029		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		5.8	

[a] Proportion of GMHT populations: (total number of populations containing GMHT *B. napus* / total number of populations) × 100.
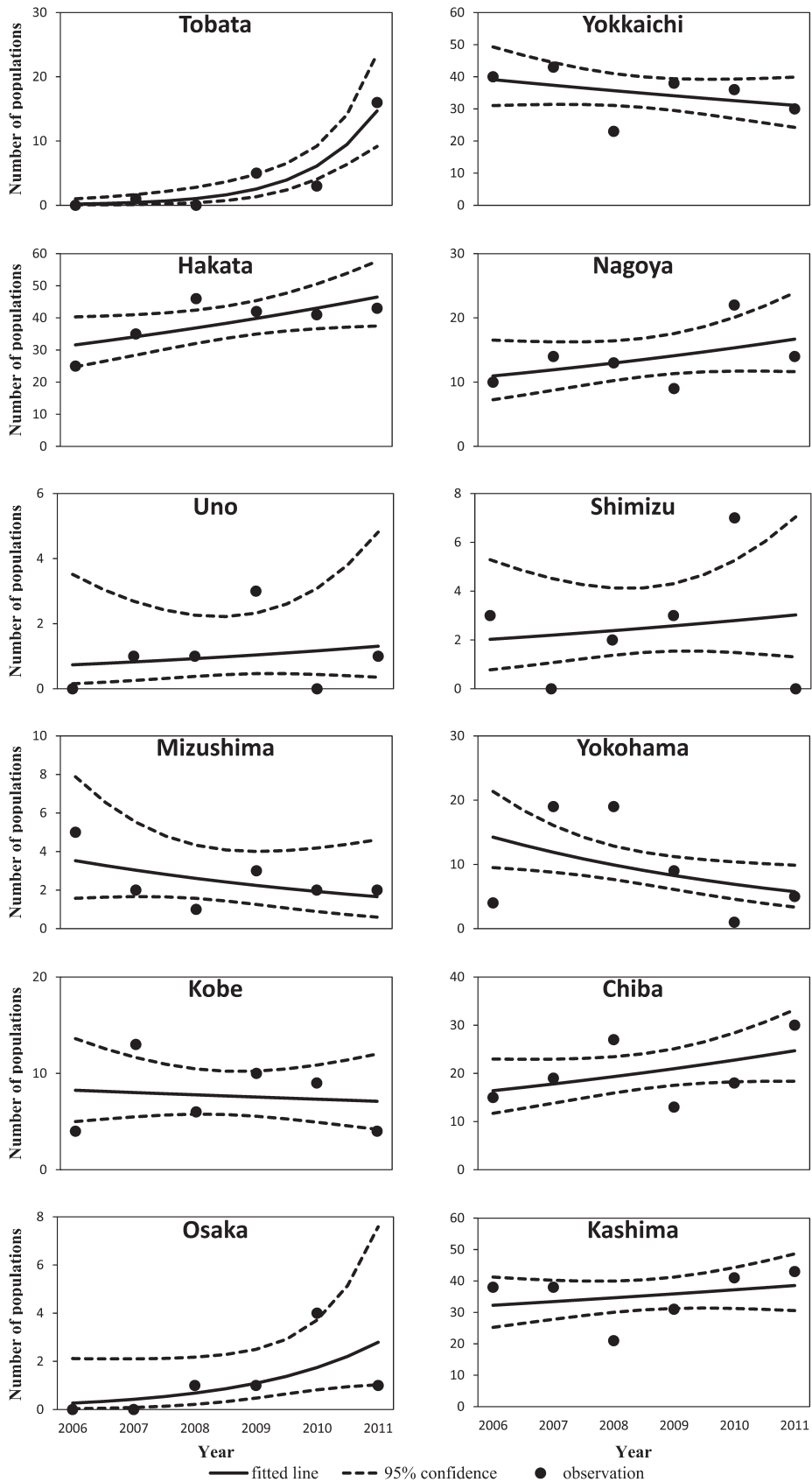
Fig. 2. Time series analyses of the numbers of *Brassica napus* populations around 12 unloading Japanese ports. Temporal trends are represented by fitted regression lines (with 95% confidence envelopes).
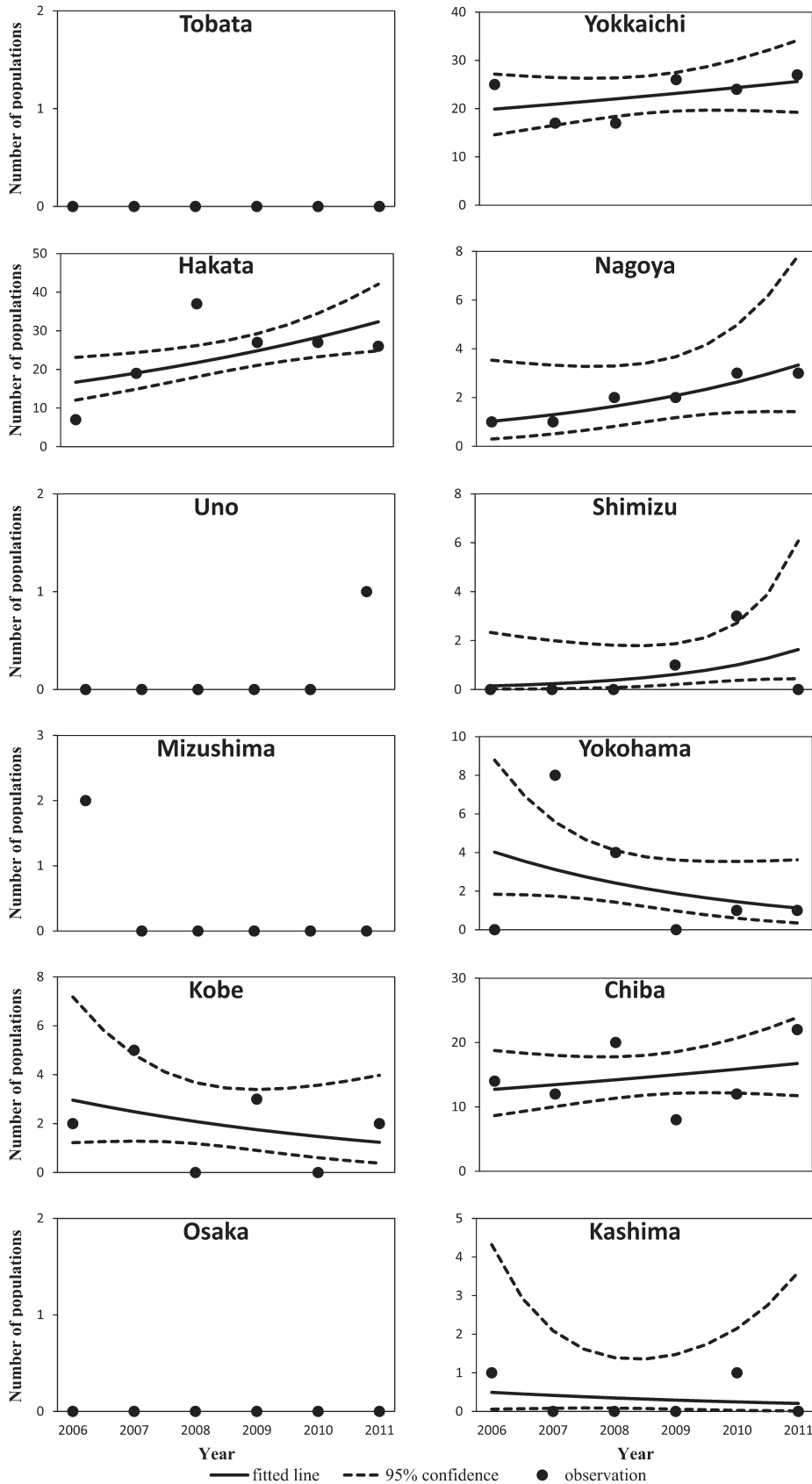
Fig. 3. Time series analyses of the numbers of *Brassica napus* populations containing genetically modified, herbicide-tolerant (GMHT) individuals around 12 unloading Japanese ports. Temporal trends are represented by fitted regression lines (with 95% confidence envelopes). Regression fits are not provided for Tobata, Uno, Mizushima and Osaka because GMHT individuals were either absent or found only once.

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

	Ports	*B. juncea*							*B. rapa*						
		Year						Total	Year						Total
		2006	2007	2008	2009	2010	2011		2006	2007	2008	2009	2010	2011	
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	0	1	0	1	4
5	Nagoya	41	8	15	16	18	26	124	0	0	0	3	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	18	16	24	132	1	7	9	4	6	0	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	13	3	2	0	2	0	20
12	Tobata	36	32	31	28	32	38	197	16	22	20	10	16	12	96
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.

analyses of temporal trends in the populations (both GM and non-GM) around each port are depicted in **Fig. 2**. A positive temporal trend was detected for the ports of Tobata, Hakata, Uno, Osaka, Nagoya, Shimizu, Chiba and Kashima. We found negative trends for the ports of Mizushima, Kobe, Yokkaichi and Yokohama. The fitted slopes were never steep and the confidence envelopes were broad. We tested null hypotheses postulating that the gradient of each slope was zero (with 95% confidence intervals); the hypotheses 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.

Generalized linear model regression analyses of temporal trends in populations containing GMHT individuals are depicted in **Fig. 3**. Four populations (Tobata, Uno, Mizushima and Osaka) are excluded from these trend analyses because GMHT plants were either absent or occurred in only a single year. A significant positive trend was detected only in the fit for the GMHT population in Hakata; the slope gradient was gradual in this case. Overall, GMHT populations appeared not to be increasing in frequency over time.

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.

We did not detect glyphosate-tolerant protein (CP4 EPSPS) or glufosinate-tolerant protein (PAT) in any of the *B. juncea* and *B. rapa* 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

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 (%)					
		Year						Year					
		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	100
2	Chiba	367	365	352	351	346	322	94	100	100	100	100	100
3	Yokohama	348	363	354	377	349	368	95	99	99	90	99	99
4	Shimizu	157	201	196	190	220	216	100	100	100	100	100	100
5	Nagoya	287	253	226	278	260	318	92	88	95	96	95	97
6	Yokkaichi	145	116	103	93	95	131	52	82	100	92	92	99
7	Osaka	6	2	5	6	5	7	0	0	0	0	0	0
8	Kobe	430	426	433	466	500	494	75	89	95	83	91	94
9	Mizushima	188	160	159	152	190	160	93	83	100	63	91	92
10	Uno	25	23	23	15	16	19	94	95	100	100	100	100
11	Hakata	114	126	110	118	130	139	88	89	88	90	95	96
12	Tobata	3	0	0	0	0	0	49	–	–	–	–	–
Total		2270	2218	2127	2211	2307	2353	87	93	97	91	96	97

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.* 2008, Yoshimura *et al.* 2006). Pivard *et al.* (2008) reported that feral populations depended not only input via spillage of new seeds, but also on seed supply from soil seed banks and reproduction of feral populations. The maximum duration of secondary dormancy in *B. napus* seeds is 5 yr under field conditions, but may extend to >10 yr (Beckie and Warwick 2010, Begg *et al.* 2006, D'Hertefeldt *et al.* 2008, Gruber *et al.* 2008, Gulden *et al.* 2003, Jørgensen *et al.* 2007, Lutman *et al.* 2003, 2005, 2008, Messean *et al.* 2007, Simard *et al.* 2002). However, <5 viable seeds/m^2 have been found in roadside seed banks in western Canada, where feral *B. napus* populations occur. Thus, dormant seeds are exceedingly rare (Knispel *et al.* 2008) and repetitive seed inputs from crop fields and transportation spillage

were likely the main supply sources that enabled population persistence of these feral *B. napus* populations (which occurred near fields and along transportation routes).

A 10 yr survey in the UK found the populations of *B. napus* in quadrats distributed along roadsides persisted for only 1–4 yr (Crawley and Brown 2004). Based on a continuous survey of spilled *B. napus* around Kashima port, Mizuguti *et al.* (2011) found that population persistence in the short-term depended on repeated spilled seed input; only a few populations in specific clear areas were able to persist for a number of years through self-crossing.

Thus *B. napus* populations appear to alternate. The proportion 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 export 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 *et al.* 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 future 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*.

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