
RESEARCH PAPERS

Fixed-route monitoring and a comparative study of the occurrence of herbicide-resistant oilseed rape (*Brassica napus* L.) along a Japanese roadside

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ABSTRACT. Previously, we conducted a roadside survey to reveal the occurrence of genetically modified (GM) oilseed rape along a Japanese roadside (Route 51). In this study, we performed successive and thorough fixed-route monitoring in 5 sections along another road (Route 23). Oilseed rape plants were detected on both sides of the road in each section between autumn 2009 and winter 2013, which included 3 flowering seasons. In four sections, more plants were found on the side of the road leading from the Yokkaichi port than on the opposite side. In the fifth section, the presence of clogged drains on the roadside, where juvenile plants concentrated, caused the opposite distribution: oilseed rape predominantly occurred along the inbound lanes (leading to the Yokkaichi port) in 2010 and 2012. Unlike in our previous survey, glyphosate- or glufosinate-resistant oilseed rape plants were abundant (>75% of analyzed plants over 3 years). Moreover, a few individuals bearing both herbicide resistance traits were also detected in some sections. The spillage of imported seeds may explain the occurrence of oilseed rape on the roadside. The abundance of herbicide-resistant

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Received 10 November 2015; Revised 25 December 2015; Accepted 30 December 2015.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kgmc.

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oilseed rape plants may reflect the extent of contamination with GM oilseed rape seed within imports.

KEYWORDS. environmental concern, fixed-route monitoring, genetically modified crop, GM plants, herbicide resistance, oilseed rape, *Brassica napus*, roadside monitoring, seed spillage, seed transport

INTRODUCTION

Brassicaceous plants are among the most popular plants; in Japan, they blossom in spring, and large feral populations of *Brassica rapa*, *B. napus*, and *B. juncea* often appear on riverbanks, in river beds, and in fallow fields. In addition to being a component of feral plant populations, brassicaceous plants have been cultivated as crops since ancient times and are used as leaf or root vegetables, livestock feeds, and for oil production (reviewed in Nishizawa et al., 2010). However, despite their use as leaf vegetables, they are rarely used for oil production in Japan since the latter half of the 1970s (Nishizawa et al., 2010). On the other hand, spontaneous occurrence of oilseed rape plants (*B. napus* L.), some of them genetically modified (GM) to be herbicide-resistant, has been observed in recent years in areas around ports and along primary national roads (Saji et al., 2005; Aono et al., 2006, 2011; Nishizawa et al., 2009). To the best of our knowledge, GM oilseed rape has not been cultivated commercially in Japan. An extremely small number of non-GM oilseed rape plants have been cultivated in recent years for oil and feed production, and domestic demand is almost completely satisfied by import. Imported oilseed rape seed is transported from abroad mainly by sea (with a small fraction transported by airfreight), followed by land transportation, mainly by motor vehicles.

Recently, an escape of GM oilseed rape and environmental concerns it caused were also reported in Switzerland, where neither cultivation nor import of GM oilseed rape is allowed until the end of 2017 (Hecht et al., 2014; Schoenenberger and D'Andrea, 2012; Schulze et al.,

2014); feral oilseed rape including glyphosate-resistant plants occurred along railways and around port areas (Hecht et al., 2014; Schoenenberger and D'Andrea, 2012; Schulze et al., 2014), and glufosinate-resistant plants occurred around port areas (Schulze et al., 2014). The occurrence of feral oilseed rape plants in ruderal areas relevant to seed transportation or handling (road verges, railway tracks, and around port areas) has been also reported in countries other than Japan and Switzerland (Crawley and Brown, 1995, 2004; Elling et al., 2009; Heenan et al., 2004; Knispel and McLachlan, 2010; Peltzer et al., 2008; Pessel et al., 2001; Pivard et al., 2008a, 2008b; von der Lippe and Kowarik, 2007a, 2007b; Yoshimura et al., 2006). Although most of these populations seem to be temporal, the seed input from various sources including spillage is sufficient for their persistence (reviewed in Devos et al., 2012). Thus, in the cases like in Japan where GM oilseed rape is not cultivated commercially, an escape into the natural environment, especially because of the spillage of imported seed during land transportation, is the focal point of the assessment of the environmental risk of GM oilseed rape.

In our previous study, (Nishizawa et al., 2009) over a 3 y period (2005 to 2007), we monitored the roadside of Route 51, a major route of transportation of imported oilseed rape seed discharged at the port of Kashima. We have reported that: (i) oilseed rape plants occurred mainly along the lane leading away from the port, (ii) oilseed rape plants appeared each year on the roadside in substantially different numbers, (iii) a few herbicide-resistant oilseed rape plants were detected over the 3 years, but their proportion was quite low, and (iv) human activity such as road improvement work sharply decreased the occurrence of oilseed rape plants. Taking into account the results

of our field surveys and the status of oilseed rape cultivation in Japan, we assumed that the origin of feral populations of oilseed rape plants (which include some GM individuals) growing in these artificial environments was the spillage of imported oilseed rape seed (Nishizawa et al., 2009). However, it was not clear whether the results for Route 51 would be indicative of the general situation with the roadside plants (including GM individuals) in Japan.

Spontaneous populations of feral oilseed rape plants in the ruderal environment are dynamic (Crawley and Brown, 1995, 2004; Elling et al., 2009; Nishizawa et al., 2009; Peltzer et al., 2008). Various road structures (such as chinks between curbstones and asphalt pavement, drains, and gutters) allow oilseed rape plants to prosper (Nishizawa et al., 2009). Thus, the establishment of a spontaneous population from spillage of imported seed would be affected by regional conditions (such as landforms, sharp road bends, and the state of the road surface), the form of transportation, the distance from the port to oil-processing facilities, and the traffic volume of grain trucks. According to recent trade statistics of the Ministry of Finance of Japan, import of oilseed rape seed into Japan passes through more than 10 ports and airports, and the amount of seed discharged differs substantially between these points of entry (reviewed in Nishizawa et al., 2010; e.g., see table 3 in this reference). Therefore, it was very important to expand monitoring to another area for a comparison with the data obtained for Route 51, because this information would serve as a reference for further elucidation of the environmental impact of escaped GM oilseed rape plants growing under natural conditions in Japan.

To generate data for comparison with those for Route 51, we monitored the roadside of a national road leading to the port of Yokkaichi, Route 23, which is presumably a major transportation route of imported oilseed rape seed. In the area around this port and on the roadside of Route 23, we have reported the occurrence of a large number of GM oilseed rape plants bearing a single herbicide resistance trait (Aono et al., 2006,

2011). Of note, we also detected oilseed rape resistant to both glyphosate and glufosinate in each of the continuous years from 2005 to 2008 (Aono et al., 2011). No plants resistant to both herbicides were detected along Route 51 during the 3-year monitoring period (Nishizawa et al., 2009). However, the intra-annual and year-to-year changes in oilseed rape occurrence in the roadside habitats in the Yokkaichi area remained unknown, because our studies in this area were based on intensive collection of specimens to determine the frequency of GM individuals in each year, rather than on regular long-term investigation.

In the present study, we performed periodic fixed-route census to monitor the occurrence of spontaneous oilseed rape plants in 5 sections along Route 23 and compared these data with those we previously reported for Route 51 (Nishizawa et al., 2009).

RESULTS AND DISCUSSION

Distribution of Oilseed Rape Plants on the Roadside along Route 23

A total of 65 field censuses were conducted in 5 monitored sections along Route 23 (Fig. 1). Through the whole duration of this study, which included 3 flowering seasons, oilseed rape plants were detected on both sides of the road in all sections (Figs. 2 and 3). Annual phenological changes were similar among plants detected in different sections and similar to those of feral individuals growing in riverbanks, riverbeds, and fallow fields in Japan. The occurrence peaked in spring (from late March to early May), and a few or almost no plants were found in summer (from early July to late September) (Figs. 2 and 3). Annual changes in the frequency of oilseed rape plants in the *Shiroko*, *Toyotsuueno*, and *Kumozu* sections showed a distinct kurtosis distribution, whereas those in the *Shiohama* and *Suzuka* sections showed gradual changes (Figs. 2 and 3).

The occurrence of oilseed rape plants was predominantly observed along the outbound lanes, which lead from the Yokkaichi port to

FIGURE 1. Roadside sections along Route 23 used to detect the occurrence of oilseed rape (*Brassica napus*) from October 21, 2009 to January 15, 2013. The monitored area is located on the west coast of Ise Bay south of the Yokkaichi port. Periodic censuses throughout the year and specimen collection for DNA analysis during the flowering season were conducted on both sides of the road at 5 locations indicated by black squares.

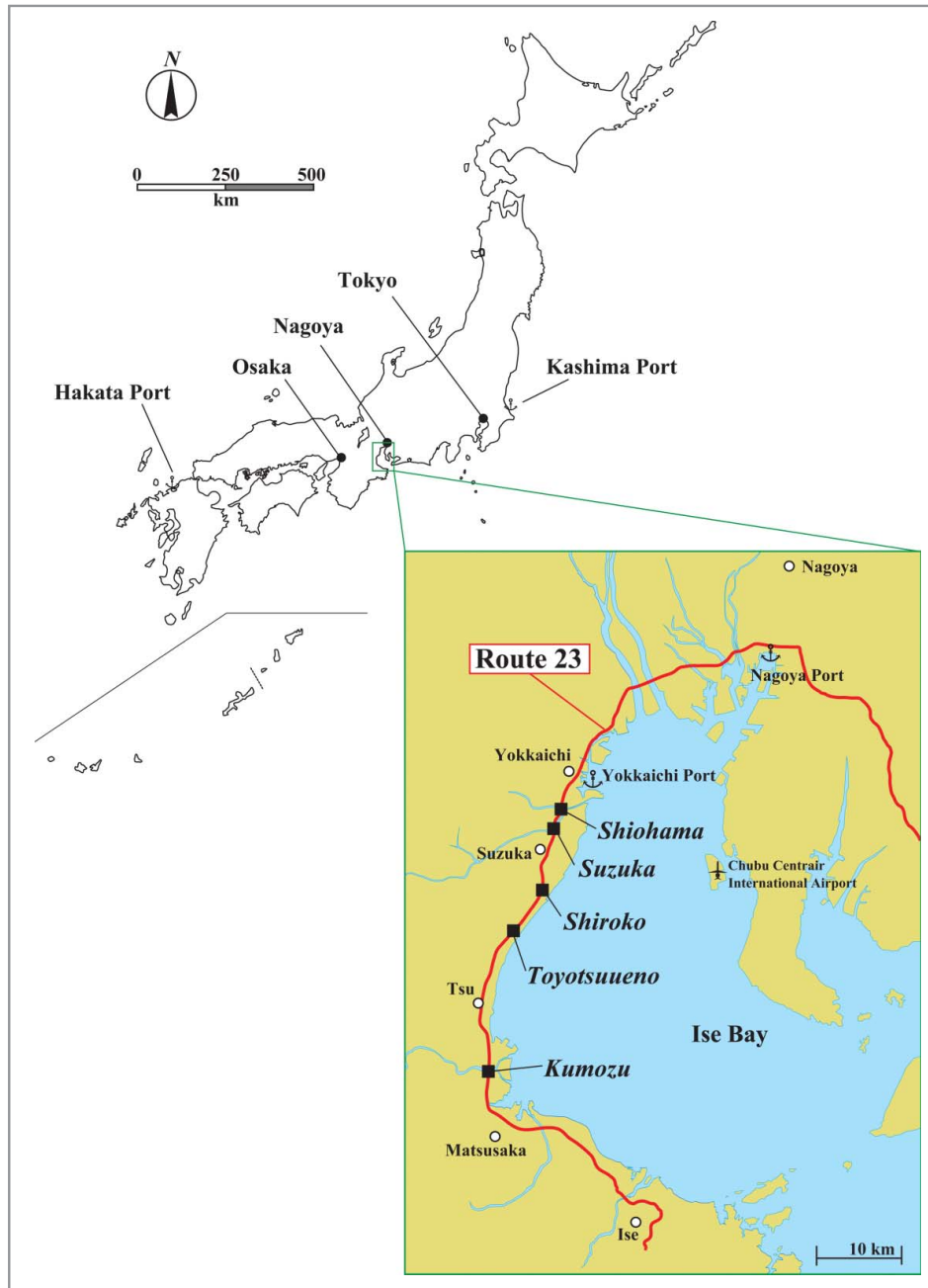


FIGURE 2. The numbers of oilseed rape plants observed on both sides of the road within 4 monitored sections, *Shiohama*, *Suzuka*, *Shiroko*, and *Toyotsuueno*, from October 21, 2009 to January 15, 2013. The dates of specimen collection for DNA analysis are denoted by asterisks. Each survey continued for 2 days; the date of each census is the first day of the survey.

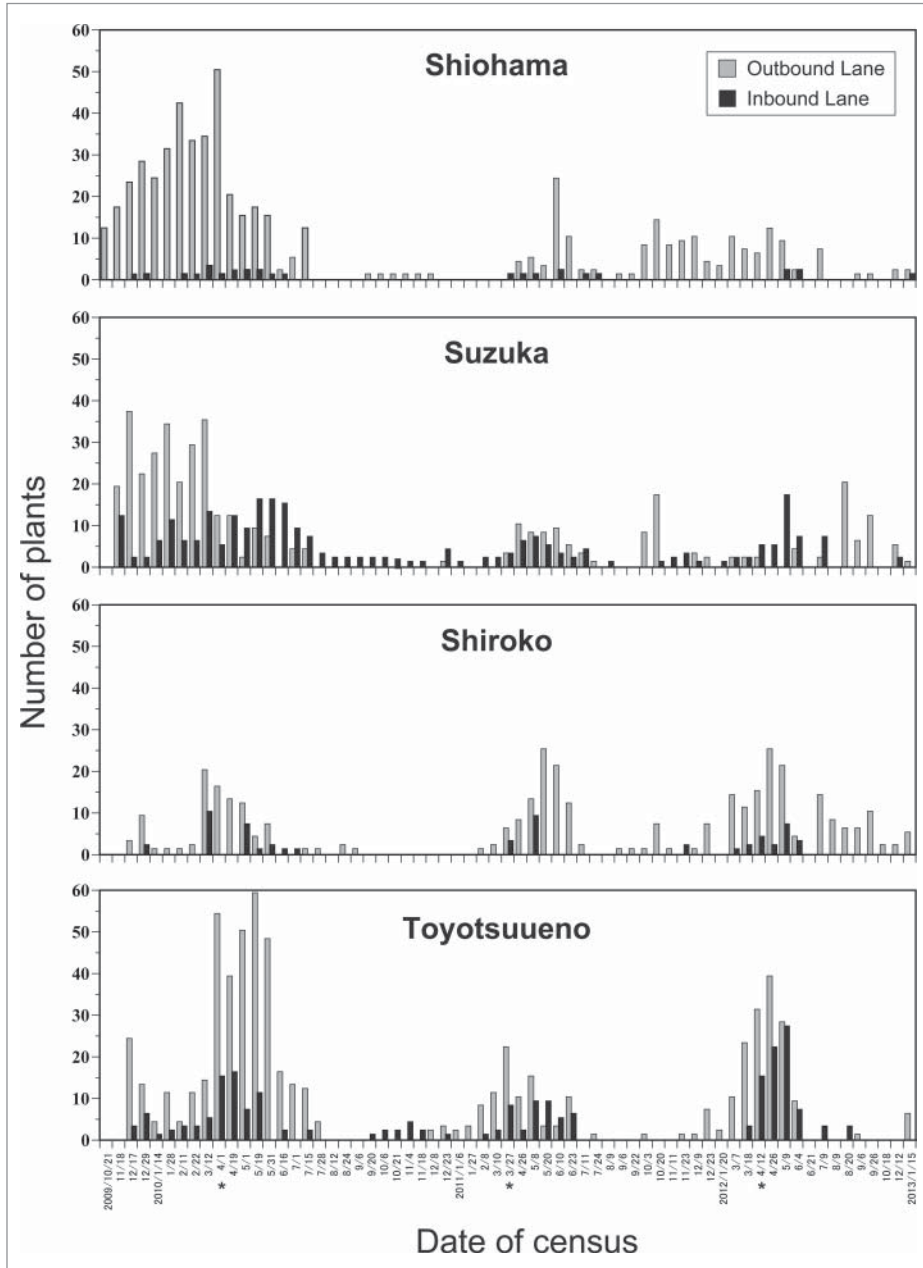
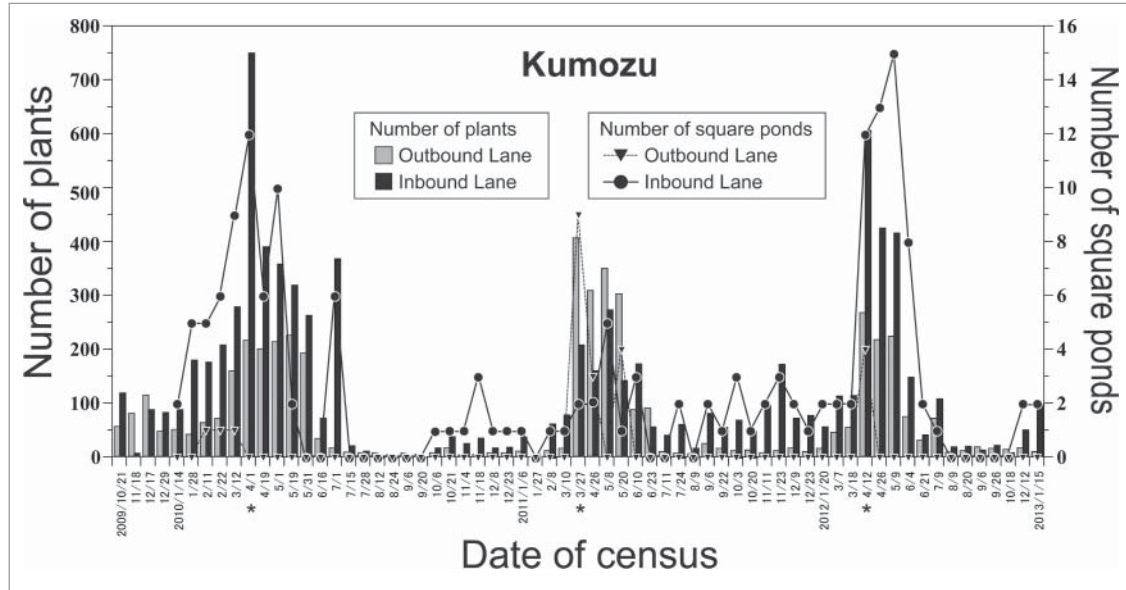


FIGURE 3. The numbers of oilseed rape plants observed and the number of densely populated square ponds where juvenile plants concentrated on both sides of the road in the *Kumozu* section from October 21, 2009 to January 15, 2013. The dates of specimen collection for DNA analysis are denoted by asterisks. Each survey continued for 2 days; the date of each census is first day of the survey.



Ise City (Fig. 2). Similar asymmetry was also observed along Route 51: oilseed rape distribution was limited to the roadside of lanes leading from the port of Kashima (Nishizawa et al., 2009). On the other hand, the distribution in the *Kumozu* section was quite unique, especially the asymmetrical pattern in 2010 and 2012, which was opposite to those in the other sections and that along Route 51 (Fig. 3); we discuss this phenomenon in the following subsection.

Spontaneous occurrence of oilseed rape plants along transportation routes (roads or railway verges) has been reported in many other countries (e.g., Bailleul et al., 2012; Crawley and Brown, 1995, 2004; Elling et al., 2009; Hecht et al., 2014; Knispel and McLachlan, 2010; Nishizawa et al., 2009; Pivard et al., 2008a, 2008b; Reuter et al., 2008; Schoenenberger and D'Andrea, 2012; Schulze et al., 2014; von der Lippe and Kowarik, 2007a, 2007b; Yoshimura et al., 2006); many studies reported that plant distribution along roads reflected the direction of oilseed rape seed transport (Crawley and

Brown, 1995, 2004; Hecht et al., 2014; Nishizawa et al., 2009; Pivard et al., 2008a, 2008b; von der Lippe and Kowarik, 2007a, 2007b, 2008). As in those studies, we considered seed spillage from vehicles as a major source of roadside plant populations along Route 51, since there were no potential seed sources around the census route. Oilseed rape distributions along Route 23 (other than in *Kumozu*) also corresponded well to the situations reported in various countries (Crawley and Brown, 1995, 2004; Hecht et al., 2014; von der Lippe and Kowarik, 2007a). We also found no evidence of potential seed sources or large-scale cultivation of oilseed rape (e.g., for biofuel production) around Route 23. Thus, the spillage of imported seeds is likely to lead to the occurrence of oilseed rape plants along Route 23.

The origin of oilseed rape plants growing in the inbound lane would result from the spilled seed in the outbound lane. The median strip, in which the width is less than 1 m, is installed along the center of the road in all monitored

sections. However, we assume that the central structure would not act as a barrier to bouncing of seed, because an intensive turbulence caused by frequent traffic continually occurs. In the 3 sections, *Shiohama*, *Suzuka*, and *Kumozu*, which included part of the road with a steel bridge across rivers, there was a strong wind blowing from Ise Bay. Thus, the weather conditions throughout the year would also contribute to second dispersal of the spilled seed.

We have frequently seen grain trucks carrying poultry feeds in the outbound lane, although there is no quantitative traffic data for grain trucks. Poultry farming is popular in and to the south of Matsusaka City in Mie Prefecture. On the other hand, seed spillage from the grain trucks returning to the port would also be the potential to become the origin of oilseed rape plants on the inbound lane. Thus, investigation of the frequency of grain trucks and containment of imported seed would be necessary in future studies.

Unique Oilseed Rape Distribution in the Kumozu Section

The occurrence of oilseed rape plants in the *Kumozu* section differed greatly from that in the other 4 sections (Fig. 3). Similar to the other sections, plant presence in spring 2011 was predominantly observed in the outbound lanes, and high kurtosis in spring that similar to the *Shiroko* and *Toyotsuueno* sections was observed in all years (Fig. 3). However, the occurrence of a large number of oilseed rape plants predominantly in the inbound lanes in 2010 and 2012 is worthy of special mention, even if we take into account that *Kumozu* was the longest (1,300 m) among the 5 sections. In each flowering season, the maximum number of oilseed rape plants detected in *Kumozu* was one order of magnitude greater than that in each of the other 4 sections (Figs. 2 and 3).

A suitable space for germination of spilled seeds and for survival of juvenile plants, such as accumulated mud and sand at the bottom of a gutter, can cause a massive occurrence of spontaneous oilseed rape plants (Nishizawa et al., 2009). Unlike the other 4 sections, accumulation of mud in the drains in *Kumozu* has

been remarkable, especially along the inbound lane, and extremely large numbers of seedlings and juveniles were detected there (Figs. 3 and 4). We found that square ponds connected to the drains were densely populated by oilseed rape, and considered square ponds with more than 10 juvenile plants per pond to be a dense population (Fig. 4D and E). In each census, we counted the number of densely populated square ponds together with that of oilseed rape plants. Higher numbers of densely populated square ponds was associated with higher numbers of spontaneous oilseed rape plants (Fig. 3). This relationship provides a convincing explanation for the presence of oilseed rape plants predominantly in the inbound lane in 2010 and 2012, because during the flowering seasons in these 2 years, densely populated drain bottoms were predominantly observed along the inbound lane (Fig. 3). For example, along the inbound lane, 746 individuals were observed on April 1, 2010 and 602 individuals on April 12, 2012; on each date, 12 densely populated square ponds were observed. Along the outbound lane, 403 individuals were observed on March 27, 2011; 9 densely populated square ponds were observed (Fig. 3).

As mentioned above, if seed spillage during transportation is a source of oilseed rape plants in roadside habitats, their distribution would depend on the direction of traffic. However, the roadside of the inbound lane in the *Kumozu* section had multiple clogged drains, so that the effect of drains would mask the pattern formed by seed spillage. There were almost no clogged drains in the other 4 sections, and the majority of oilseed rape plants were detected in chinks between asphalt pavement and curbstones (Fig. 4F-H). These chinks were small and sufficient only for limited numbers of oilseed rape plants, which probably explains why much fewer plants were found in the 4 other sections than in *Kumozu*.

Human-Mediated Disturbance and Plant Distribution

On the roadside, the exposure of oilseed rape plants to severe environmental conditions, especially to human-induced disturbance,

FIGURE 4. Typical habitats of oilseed rape plants in road structures and on the roadside along Route 23. (A) A joining section of the Kumozu-ohashi bridge. The steel frame of the bridge is at the road surface level. (B) A close-up view of a joining section of the Kumozu-ohashi bridge with accumulated mud and sand. (C) Oilseed rape seedlings growing in the mud accumulated at the bottom of the joint. (D) Seedlings and juvenile plants growing in the mud accumulated in a drain. (E) Densely populated square pond connected to the drain. (F) An adult plant flowering in a chink between asphalt pavement and curbstones. (G) An adult plant just before flowering in a crack in a curbstone. (H) An adult plant just before flowering in a crack in a rubber seal that coated a chink between curbstones to prevent weed growth.



would affect the dynamics of their populations. In our previous investigation, we found that artificial structures on the roadside and human activities strongly affect the abundance of spontaneous oilseed rape plant populations along Route 51: the number of aggregated plants growing in the mud at the bottom of the gutters was dramatically decreased when the mud disappeared (to 2% of the previous year's level), and road cleaning using Unimog vehicles removed juveniles that grew on the side strip along the road (Nishizawa et al., 2009).

Hand weeding along Route 23 seemed to have affected the dynamics of oilseed rape populations on the roadside. According to an interview of a citizens' group, they performed weeding along Route 23 on June 13–20, 2010. This civil activity was conducted south of the boundary between Yokkaichi and Suzuka Cities, which lies on the Suzuka River, and thus did not affect the *Shiohama* and *Suzuka* sections. In the remaining 3 sections, *Shiroko*, *Toyotsuueno*, and *Kumozu*, the number of plants detected on June 16, 2010 considerably decreased in comparison with that on May 31 (Figs. 2 and 3). This change was clearly observed in the *Kumozu* section: 189 (outbound lane) and 259 (inbound lane) plants were found on May 31 but only 30 and 68 on June 16, respectively (Fig. 3). The number of oilseed rape plants in the inbound lane dramatically increased to 364 individuals again on July 1 but decreased to 17 on July 15, with the increase and decrease in the number of densely populated square ponds (Fig. 3). The dramatic decrease in the number of oilseed rape plants from May 31 to June 16, 2010 in the *Kumozu* section seems to have been caused by weeding rather than by drain cleaning, because densely populated square ponds were not observed on May 31 and June 16 (Fig. 3). Thus, hand weeding appears to dramatically decrease the number of oilseed rape plants but its effect may be lasting for only a limited period of time; not permanent.

On August 12, 2010, we saw a sign of drain cleaning initiated by the road administrator in the *Kumozu* section; accumulated mud at the bottom of square ponds was also to be cleaned.

Between July 15 and September 20, 2010, we found no densely populated square ponds (Fig. 3). To control oilseed rape plants on the roadside in the *Kumozu* section, periodic sweeping of road verges would be useful and mud removal from drains would also be required.

Road maintenance work by the road administration also affected the dynamics of oilseed rape populations along Route 23. Crawley and Brown (Crawley and Brown, 1995, 2004) have stated that soil disturbance because of roadwork was significantly associated with a decrease in oilseed rape density. Similarly, we have reported that pavement renewal with exfoliation of asphalt or sand replacement decreased the occurrence of oilseed rape plants along Route 51 (Nishizawa et al., 2009). By August 9, 2012, road improvement work, which included the replacement of asphalt pavement, had been completed on the *Kumozu*-ohashi bridge (*Kumozu* section). Road and drain cleaning seemed to have been conducted before the road work because the densely populated square ponds rapidly disappeared from June to July 2012 (Fig. 3). Thus, the small number of oilseed rape plants between August 9 and October 18, 2012 may have been caused by road works.

Another factor that might affect the dynamics of oilseed rape populations on the roadside was a chemical substance used by the Ministry of Land, Infrastructure, Transport and Tourism to melt snow in winter. We found signs of road cleaning and deposits of this substance on the roadside in 3 sections, *Shiohama*, *Suzuka*, and *Kumozu*, in the census of December 8, 2010. Dispersion of a snow-thawing agent in these sections would frequently be done in winter because of the presence of steel-frame bridges. However, we do not know how this disturbance during winter affects the occurrence of oilseed rape plants at this time. It would be interesting to examine the survival of juvenile plants upon application of snow-thawing agents such as those containing chloride ion or ethylene glycol.

Unlike the other 3 sections, the inter-annual distribution of oilseed rape plants in the *Shiohama* and *Suzuka* sections showed a

moderate change in plant number without high kurtosis, except in the *Shiohama* section in 2010. It is likely that the absence of hand weeding affected the inter-annual distribution of oilseed rape plants in these sections since 2011; however, we have no clear evidence about the relationship between weeding and the inter-annual distribution of oilseed rape plants. Further investigations of the effect of human-induced disturbance (such as weeding, road work, and road maintenance during winter) on the presence of oilseed rape plants along the roads in Japan would be helpful for understanding the dynamics of these spontaneous populations.

Comparison of the Occurrence of Herbicide-Resistant Oilseed Rape on the Roadside of Route 23 and Route 51

Although herbicide-resistant GM oilseed rape plants were detected in every section along Route 23 during the 3-year monitoring period, the frequency of their detection differed greatly among sections (Table 1). Herbicide-resistant plants on the roadside of Route 23 showed some characteristics that differed from those found along Route 51 (Nishizawa et al., 2009): (i) the proportion of herbicide-resistant plants was quite large, (ii) the proportion of glufosinate-resistant plants was larger than that of glyphosate-resistant plants, and (iii) double herbicide-resistant plants (those bearing both glyphosate and glufosinate resistance traits) were detected at specific sections over the 3 seasons.

The length of the monitored part of Route 51 (ca. 20 km) was much greater than the total length of the 5 monitored sections along Route 23 (ca. 3.4 km), and thousands of plants were found along Route 51 (2,162 in 2005, 4,066 in 2006, although only 278 in 2007; Nishizawa et al., 2009). However, the number of herbicide-resistant oilseed rape plants detected along Route 51 was low (26 glyphosate-resistant plants in 2005, 8 in 2006, and 5 in 2007; 9 glufosinate-resistant plants in 2005 and none in 2006 or 2007; Nishizawa et al., 2009). The proportion of herbicide-resistant plants ranged

from 0.2% to 1.8% (Nishizawa et al., 2009). In contrast, a much higher proportion of herbicide-resistant plants was consistently detected along Route 23 (77.0% in 2010, 75.2% in 2011, and 77.7% in 2012; Table 1). Although the number of oilseed rape plants growing along Route 23 differed considerably among sections, over 60% of the analyzed plants in each section (with one exception) were herbicide-resistant every year (Table 1). The much larger proportion of herbicide-resistant individuals along Route 23 than along Route 51 is not unexpected if we consider the increases in the quantity of oilseed rape seed imported by Japan and the cultivation of GM oilseed rape in oilseed-producing countries since the Route 51 study.

Studies conducted in many countries where GM oilseed rape is widely grown have shown that feral GM oilseed rape plants occur widely along the margins of arable fields and along transportation routes, and that the proportion of feral GM individuals roughly corresponds to the proportion of GM oilseed rape grown or in transit (Knispel et al., 2008; Yoshimura et al., 2006). Schulze et al. (2014) showed that the occurrence of GM oilseed rape in the port area where unloading and transport of seed cargo was underway was much greater than in area with no current transport or loading of seed cargo in Switzerland, where cultivation of GM oilseed rape and import of its seed were not approved.

According to the trade statistics of the Ministry of Finance of Japan, the amount of imported oilseed rape seed has remained over 2 million tons per year during the past decade, and tended to increase over the last several years, with over 2.4 million tons in 2012 and later (Table 2; for the data before 2009, see Nishizawa et al., 2010). For the last 10 years, Canada has been the biggest supplier of oilseed rape seed to Japan (over 90% of import), followed by Australia (less than 10%; Table 2; for the data before 2009, see Nishizawa et al., 2010). Production of GM oilseed rape in Canada was approved in 1995, and over 90% of oilseed rape produced in the country until 2013 was herbicide-resistant (Smyth, 2014). Therefore, we have estimated that about 80% of oilseed rape seed imported into Japan might bear a

Table 1. Total numbers of oilseed rape plants, numbers of samples collected and analyzed, and numbers of herbicide-resistant individuals detected in the 5 monitored sections on both sides of Route 23 from 2010 to 2012

Section	Shiohama		Suzuka		Shiroko		Toyotsuueno		Kumozu		Total
	240		380		750		750		1,300		
Lanes: out, outbound; in, inbound	out	in	out	in	out	in	out	in	out	in	(out, in)
Collection on April 1 and 2, 2010											
Total number of individuals	50	1	12	5	16	0	54	15	213	746	1,112 (345, 767)
Number of sampled oilseed rape plants	50	1	12	5	16	0	51	10	199	408	752 (328, 424)
Number of samples analyzed for GM traits	44	1	5	4	15	0	35	10	158	188	460 (257, 203)
Number of herbicide-resistant plants	36	1	4	3	9	—*	24	8	125	144	354 (198, 156)
Proportion of herbicide-resistant plants (%)	81.8	100	80.0	75.0	60.0	—	68.6	80.0	79.1	76.6	77.0 (77.0, 76.8)
Glyphosate-resistant plants	16	0	3	0	6	—	10	6	43	52	136 (78, 58)
Proportion of glyphosate-resistant plants (%)	36.4	0	60.0	0	40.0	—	28.6	60.0	27.2	27.7	29.6 (30.4, 28.6)
Glufosinate-resistant plants	20	1	1	3	3	—	14	2	79	84	207 (117, 90)
Proportion of glufosinate-resistant plants (%)	45.5	100	20.0	75.0	20.0	—	40.0	20.0	50.0	44.7	45.0 (45.5, 44.3)
Double herbicide-resistant plants	0	0	0	0	0	—	0	0	3	8	11 (3, 8)
Proportion of double herbicide-resistant plants (%)	0	0	0	0	0	—	0	0	1.9	4.3	2.4 (1.2, 3.9)
Collection: March 27 and 28, 2011											
Total number of individuals	0	1	3	3	6	3	22	8	403	204	653 (434, 219)
Number of sampled oilseed rape plants	0	1	3	3	6	3	22	8	84	100	230 (115, 115)
Number of samples analyzed for GM traits	0	1	3	3	6	3	22	8	84	100	230 (115, 115)
Number of herbicide-resistant plants	—	1	2	3	5	2	14	8	58	80	173 (79, 94)
Proportion of herbicide-resistant plants (%)	—	100	66.7	100	83.3	66.7	63.6	100	69.0	80.0	75.2 (68.7, 81.7)
Glyphosate-resistant plants	—	0	1	1	2	1	8	5	27	38	83 (38, 45)
Proportion of glyphosate-resistant plants (%)	—	0	33.3	33.3	33.3	33.3	36.4	62.5	32.1	38.0	36.1 (33.0, 39.1)
Glufosinate-resistant plants	—	1	1	2	3	1	5	3	30	42	88 (39, 49)
Proportion of glufosinate-resistant plants (%)	—	100	33.3	66.7	50.0	33.3	22.7	37.5	35.7	42.0	38.3 (33.9, 42.6)
Double herbicide-resistant plants	—	0	0	0	0	0	1	0	1	0	2 (2, 0)
Proportion of double herbicide-resistant plants (%)	—	0	0	0	0	0	4.5	0	1.2	0	0.87 (0.87, 0)
Collection: April 12 and 13, 2012											
Total number of individuals	6	0	2	5	15	4	31	15	264	602	944 (318, 626)
Number of sampled oilseed rape plants	6	0	2	5	15	4	31	15	158	159	395 (212, 183)
Number of samples analyzed for GM traits	6	0	2	5	15	4	31	15	158	159	395 (212, 183)
Number of herbicide-resistant plants	6	—	2	4	14	3	18	14	122	124	307 (162, 145)
Proportion of herbicide-resistant plants (%)	100	—	100	80.0	93.3	75.0	58.1	93.3	77.2	78.0	77.7 (76.4, 79.2)
Glyphosate-resistant plants	1	—	0	1	5	0	9	6	47	47	116 (62, 54)
Proportion of glyphosate-resistant plants (%)	16.7	—	0	20.0	33.3	0	29.0	40.0	29.7	29.6	29.4 (29.2, 29.5)
Glufosinate-resistant plants	5	—	2	3	9	3	9	7	75	75	188 (100, 88)

(Continued on next page)

Table 1. Total numbers of oilseed rape plants, numbers of samples collected and analyzed, and numbers of herbicide-resistant individuals detected in the 5 monitored sections on both sides of Route 23 from 2010 to 2012 (*Continued*)

Section	Shiohama		Suzuka		Shiroko		Toyotsuueno		Kumozu		Total
	out	in	out	in	out	in	out	in	out	in	
Length of each section (m)	240		380		750		750		1,300		3,420
Lanes: out, outbound; in, inbound	out	in	out	in	out	in	out	in	out	in	(out, in)
Proportion of glufosinate-resistant plants (%)	83.3	—	100	60.0	60.0	75.0	29.0	46.7	47.5	47.2	47.6 (47.2, 48.1)
Double herbicide-resistant plants	0	—	0	0	0	0	0	1	0	2	3 (0, 3)
Proportion of double herbicide-resistant plants (%)	0	—	0	0	0	0	0	6.7	0	1.3	0.76 (0, 0.76)

[†]Hyphens mean that no oilseed rape plants were found at the roadside of lanes.

herbicide resistance trait. Over 10 Japanese sea ports are constantly involved in oilseed rape seed import, including the Yokkaichi port (Nishizawa et al., 2010). The amount of oilseed rape seed discharged at the Yokkaichi port is smaller than at other ports (about 5% or less during the past few years; Table 2). Over 90% of oilseed rape seed that passed through the Yokkaichi port was imported from Canada (except 85% in 2013; Table 2). Thus, a substantial proportion of oilseed rape seed spilled along Route 23 might originate from Canada. Throughout the monitoring period, the proportion of herbicide-resistant oilseed rape plants detected remained almost constant (>75%). Therefore, the occurrence of herbicide-resistant oilseed rape along Route 23 seems to reflect the extent of contamination with GM oilseed rape (mostly imported from Canada) resulting from spillage.

Another characteristic of oilseed rape plants found along Route 23 was that glufosinate resistance occurred at a similar or higher frequency than glyphosate resistance. The proportion of glyphosate-resistant plants found over the 5 sections was 29.6% in 2010, 36.1% in 2011, and 29.4% in 2012 (Table 1). The proportion of glufosinate-resistant plants was 45.0% in 2010, 38.3% in 2011, and 47.6% in 2012 (Table 1). In contrast, only a few oilseed rape plants bearing the glufosinate resistance trait were observed along Route 51 (Nishizawa et al., 2009). The occurrence of glufosinate-

resistant plants at high frequency was consistent with the results of our previous investigation conducted from 2005 to 2008 (Aono et al., 2011). The relative proportions of GM oilseed rape with resistance to different herbicides produced in Canada in 2010 were 47% glyphosate-resistant and 46% glufosinate-resistant (Canola Council of Canada, 2014). The proportion of glufosinate-resistant plants found along Route 23 seems to roughly correspond with the relative proportion of glufosinate-resistant plants in the product. This suggests that most spontaneous oilseed rape plants on the roadside originated from imported seed recently spilled during land transportation from the port area.

On the other hand, through the duration of this study, a small number of flowering (and occasionally fruiting) individuals were observed along Route 23 (Fig. 4F). We were not able to estimate the contribution of flowering individuals to the sustainment of roadside populations, because identification of each individual and follow-up investigation of them had not been performed. However, on the basis of the observations during each census, growing and continuous appearance of large matured individuals (such as the plant that was shown in figure 2C of Nishizawa et al., 2009) was not observed around the same point. Thus, we assume that seed supply from the matured plants would act minor contribution for the perpetuation of roadside populations. However, demographic

study of oilseed plants growing in the roadside habitat must be next task.

In our previous study along Route 51, we unexpectedly observed that the proportion of herbicide-resistant plants detected on the roadside was much smaller than the putative proportion of imported GM seed (Nishizawa et al., 2009). We assumed that an increase in the proportion of non-GM varieties, a change in herbicide resistance traits or cultivars, or in the country of origin might explain the difference. In the past several years, the amount of oilseed rape seed that passed through the Kashima port (>8% of total import) was about twice that through the Yokkaichi port, with almost all oilseed rape seed discharged at the Kashima port imported from Canada (Table 2). Thus, the distribution of herbicide-resistant oilseed rape along Route 23 suggests that the change of the route of oilseed transportation from the Kashima port may be a principal cause of the difference, although the proportion of GM varieties produced in Canada at the time of our previous monitoring (2005–2007) was somewhat different from the rate of today (>90%).

Occurrence of Double Herbicide-Resistant Individuals in Specific Sections

Notably, although the proportion of double herbicide-resistant plants was small, they were found in each year on both or only one side of the road in *Toyotsuueno*, *Kumozu*, or both sections (Table 1). So far, double herbicide-resistant oilseed rape plants have been detected in Japan only in the Yokkaichi, Mizushima, and Hakata areas, but the detection in Mizushima and Hakata was reported in only one year (Aono et al., 2006, 2011). Despite massive occurrence of spontaneous oilseed rape plants along Route 51 in 2005 and 2006, no double herbicide-resistant plants were detected (Nishizawa et al., 2009). On the other hand, double herbicide-resistant plants in the Yokkaichi area have been consistently detected since 2005 (Aono et al., 2006, 2011), and were also detected in the present study during 3 consecutive years. They were most abundant in *Kumozu* in 2010, when 11 plants (2.4% of all analyzed samples) were detected on both

sides of the road. In 2011, a total of 2 plants (0.87% of all samples) found in the outbound lanes in *Toyotsuueno* and *Kumozu* were double herbicide-resistant. In 2012, a total of 3 individuals (0.76% of all samples) found in the inbound lanes in *Toyotsuueno* and *Kumozu* were double herbicide-resistant. No plants resistant to both herbicides were detected in the other 3 sections, where smaller numbers of oilseed rape plants appeared. A continuous occurrence of double herbicide-resistant oilseed rape plants implies that their appearance is inevitable. We have suggested in a previous report that double herbicide-resistant oilseed rape plants are derived from a cross between 2 types of herbicide-resistant varieties; however, we could not determine whether the crossing events occur in the countries producing oilseed rape or within Japan (Aono et al., 2011). In this study which is intended to monitor the occurrence of plants and its fluctuation, the origin of double herbicide-resistant individuals still remains uncertain. Likewise, we have no definite explanation for the consistent detection of double herbicide-resistant oilseed rape plants around the port of Yokkaichi.

The occurrence of double herbicide-resistant plants in the *Kumozu* section fluctuated among the 3 years: most plants were observed in 2010 and their numbers decreased to less than one-third of the level of 2010 in the following 2 years (Table 1). The numbers of double herbicide-resistant plants were much lower than those of plants resistant to a single herbicide. If more oilseed rape plants occur in *Shiohama*, *Suzuka*, and *Shiroko*, where no massive presence and no double herbicide-resistant plants were observed, the number of double herbicide-resistant plants detected might also increase. Thus, further continuous monitoring along Route 23 and a comparative study including another area where double herbicide-resistant oilseed rape plants are present, such as Hakata, would be helpful to elucidate the impact of feral GM oilseed rape plants on Japan's environment.

Conclusion and Perspectives

Oilseed rape populations on the roadside of Route 23 differed from those on the roadside

Table 2. Statistics of oilseed rape seeds import into Japan from Canada, Australia (2 most active exporters), and other countries in 2009—2014

Year	2009	2010	2011	2012	2013	2014
Total amount of import [A] ^a	2,072,402	2,344,304	2,318,994	2,408,423	2,461,041	2,411,348
Canada total	1,957,239	2,145,095	2,260,447	2,331,636	2,309,279	2,243,361
Proportion of import from Canada (%)	94.4	91.5	97.5	96.8	93.8	93.0
Australia total	114,592	198,712	57,918	75,749	151,641	167,834
Proportion of import from Australia (%)	5.5	8.5	2.5	3.1	6.2	7.0
Other countries	571	497	629	1,038	121	153
Proportion of import from other countries (%)	0.028	0.021	0.027	0.043	0.005	0.006
Discharge at each port and from each country						
Port of Yokkaichi, total [B]	90,341	90,204	118,948	104,047	85,189	114,944
Proportion of discharge at Yokkaichi (%) [B/A]	4.4	3.8	5.1	4.3	3.5	4.8
From Canada [C]	84,119	81,500	117,984	104,047	72,612	109,811
Proportion of import from Canada (%) [C/B]	93.1	90.4	99.2	100.0	85.2	95.5
From Australia [D]	6,222	8,704	964	—	12,577	5,133
Proportion of import from Australia (%) [D/B]	6.9	9.6	0.8	—	14.8	4.5
Port of Kashima, total [E]	162,036	197,886	170,148	199,269	217,462	212,951
Proportion of discharge at Kashima (%) [E/A]	7.8	8.4	7.3	8.3	8.8	8.8
From Canada [F]	162,036	197,886	170,148	194,815	212,222	208,198
Proportion of import from Canada (%) [F/E]	100.0	100.0	100.0	97.8	97.6	97.8
From Australia [G]	—	—	—	4,454	5,240	4,753
Proportion of import from Australia (%) [G/E]	—	—	—	2.2	2.4	2.2
Number of exporter countries	11	13	11	10	7	7
Number of ports at which oilseed was discharged ^b	12	13 (1)	13	14 (1)	12	13 (1)

The source of these data is the trade statistics of the Ministry of Finance of Japan (http://www.customs.go.jp/toukei/info/index_e.htm).

^aThe letters in square brackets mean the guide to calculation.

^bThe numbers of airports are given in parentheses.

of Route 51, especially in the frequency of herbicide-resistant individuals and the proportion of plants resistant to different herbicides. Most crucial concern relevant to the unintended escape of GM oilseed rape into natural environment is the harmful rumor to the cultivation of Brassicaceous crops, because dissemination of accurate knowledge and the social acceptance regarding the application of them are not enough in Japan. We think that the temporal occurrence of GM oilseed plants along roadside itself does not bring an

immediate impact to the environment. As mentioned above, the management of roadside oilseed rape plants along R23 is mainly conducted by mowing, not the application of herbicides. However, weediness potential of oilseed rape (*B. napus*) is considered quite large, because this plant has been distributed throughout Japan after the introduction from abroad in the 19th century (reviewed in Nishizawa et al., 2010). The spread and establishment of feral population of oilseed plants bearing herbicide resistant traits, which might

increase the probability of transgene flow into the gene pool of Brassicaceous crops, threatens the commercial value of crops which are produced in Japan.

To design a plan to reduce the potential of transgene escape, it is necessary to consider the distribution of oilseed rape plants in each region. Phenological changes of oilseed rape plants growing along Route 23 corresponded well to those in feral populations. Simultaneous flowering of spontaneous and feral individuals may increase the potential of transgene transfer into the gene pool of feral plants. The number of individuals on the roadside was dramatically decreased after human activities (such as a weeding, cleaning of road structures, and road renewal works). Thus, periodic and feasible removal of individuals from the roadside and the accompanying structures, such as square ponds of drains, would control the risk of transgene escape after seed spillage.

Drain cleaning would remove juvenile plants from the bottom of square ponds of drains, and lead to the disappearance of densely populated sites. However, during this study, we became concerned about the potential dispersal of spilled seed by washing off the mud from the bottom of square ponds of drains. In our next paper (manuscript in preparation), we will address the details of the mechanism of carry-over of spilled seed into river areas where feral brassicaceous plants form large populations, and will discuss an appropriate method of drain cleaning and treatment of the residuum at the bottom of square ponds of drains.

MATERIALS AND METHODS

Monitored Sites

Route 23 is located in the Chubu District of Japan, mainly along the west coast of Ise Bay. It begins at Toyohashi City (Aichi Prefecture), goes through Yokkaichi City, and extends to Ise City (Mie Prefecture); its total length is 177 km (Fig. 1). This national road is one of the likely major routes of transportation of oilseed rape seed discharged at the Yokkaichi port, including to the urban area in southern Mie Prefecture.

The Yokkaichi port is a major port in Chubu District and is located in the northern part of Ise Bay, south-west from the Nagoya port.

To perform periodic monitoring based on thorough fixed-route surveys, we selected 5 sections of Route 23 as monitored areas (Fig. 1). Census was performed on both sides of the road: along the inbound lane, which leads to the Yokkaichi port (and also to Nagoya), and the outbound lane, which leads away from the Yokkaichi port and goes to Ise City. These five sections were selected because of massive occurrence of oilseed rape plants, including a herbicide-resistant individual, according to our previous research and a preliminary survey (Aono et al., 2006, 2011).

The first section, *Shiohama* (240 m long), was the northernmost of the 5 sections (34°55'49"N, 136°36'90"E). This section included part of the road with a steel bridge across the Utsube River (the Shiohama-ohashi bridge). The Utsube River is one of the tributaries of the Suzuka River, which is a first-class river in the northern part of Mie Prefecture.

The second section, *Suzuka* (380 m long), was located about 2 km south of the Shiohama-ohashi bridge (34°54'48"N, 136°36'31"E). This section included part of the road with a steel bridge across the Suzuka River (the Suzuka-ohashi bridge).

The third section, *Shiroko* (750 m long), was located about 8.3 km south of the Suzuka-ohashi bridge and about 300 m west of the Shiroko station of the Kintetsu Railway (34°50'37"N, 136°35'29"E). This section started from the "Shiroko Station Ent." intersection and continued to the "Gymnasium" intersection. In contrast to the 2 former sections, *Shiroko* did not include part of a steel bridge.

The fourth section, *Toyotsuueno* (750 m long), also did not include part of a steel bridge and was located about 6.5 km south of *Shiroko* and about 250 m west of the Toyotsuueno station of the Kintetsu Railway (34°47'34"N, 136°32'66"E). This section started from the "Nakabeppo" intersection and continued to the "Ueno S." intersection.

The fifth section, *Kumozu* (1,300 m long), consisted of 2 above-ground zones (a total length of 850 m) and a steel bridge zone

(450 m). This section was located approximately 9 km south of the center of Tsu City (Mie Prefecture), and was the southernmost of the 5 sections (34°38'81"N, 136°31'12"E). It included the Kumozu-ohashi bridge across the Kumozu River, which flows through the center of Mie Prefecture to Ise Bay. Around *Kumozu*, the Kumozu River flows at the boundary between Tsu City and Matsusaka City. The above-ground zones extended to the north and to the south of the bridge zone. The entire monitored zone started from the "Kumozu ohashi N." intersection and continued to the "Onoecho" intersection.

Periodic Roadside Surveys

Surveys were conducted from October 21, 2009 to January 15, 2013 and included 3 flowering seasons. We visited the 5 sections once every 2 or 3 weeks and performed a census of oilseed rape growing on the roadside a total of 65 times. Each survey continued for 2 days: 4 sections (except *Kumozu*) were surveyed on the first day, and *Kumozu* was surveyed on the second day. We traveled to a spot closest to each monitored section by public transport and performed a census on foot along the road.

A zone for census in each section was set at a distance of 1–2 m from the road edge, as we did previously while monitoring Route 51 (Nishizawa et al., 2009). We also surveyed various roadside structures: sidewalks, side strips, drains, gaps between asphalt pavement and curbstones, cracks in the pavement, gaps in steel frame joints, and flowerbeds. These structures were a habitat of oilseed rape revealed in our monitoring along Route 51 (Nishizawa et al., 2009). Most brassicaceous plants growing on the roadside of the monitored sections were *B. napus*, but we also found some *B. juncea*. Species identification in the field was conducted based on morphological traits (whitish leaf surface and the presence of amplexicaul leaves).

During the flowering season, we collected leaf specimens (a total area of approximately 5 cm² per plant) to test for herbicide resistance. Specimen collections were performed at the time of flowering of oilseed rape plants (April

1, 2010; March 27, 2011; and April 12, 2012). Leaf specimens were collected into paper envelopes in the field, brought to the laboratory, lyophilized and stored at room temperature.

Test for the Presence of Herbicide-Resistant Oilseed Rape

Detection of individuals carrying herbicide-resistant transgenes was performed by polymerase chain reaction (PCR). Lyophilized leaf specimens (<10 mg dry weight) were used to DNA analysis. Genomic DNA was extracted from a single leaf specimen with the Plant Genomic DNA Extraction Miniprep System (Viogene) according to the instruction manual. Amplification of the transgenes was conducted with the specific primers EPSPS7 and EPSPS8 for *cp4 epsps* and BAR7 and BAR8 for *bar*; *cp4 epsps* encodes CP4 EPSPS and *bar* encodes PAT (Aono et al., 2006). PCR was performed in a total volume of 15 μ L containing template DNA (~20 ng), 1 \times PCR buffer, 0.2 mM each dNTP, 0.2 μ M each primer, and 0.625 U of ExTaq DNA polymerase (TaKaRa Biotechnology Co.). The cycling profile was 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 60°C (*cp4 epsps*) or 55°C (*bar*) for 45 s, and 72°C for 60 s; and then 72°C for 1 min. Amplified PCR products were separated in 2.0% agarose gels. Samples that produced fragments of the expected size (320 bp with EPSPS7 and EPSPS8; 330 bp with BAR7 and BAR8; Aono et al., 2011) were considered as positive for the presence of transgenes.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ACKNOWLEDGMENTS

We thank Y. Otsuka, H. Watanabe, R. Nakazaki, and M. Nakajima for their technical support.

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