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Relationship between hybridization frequency of *Brassica juncea* × *B. napus* and distance from pollen source (*B. napus*) to recipient (*B. juncea*) under field conditions in Japan

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Several imported transgenic canola (*Brassica napus*) seeds have been spilled and have grown along roadsides around import ports. *B. juncea*, a relative of *B. napus* with which it has high interspecific crossability, is widely distributed throughout Japan. There is public concern about the harmful impacts of feral *B. napus* plants on biodiversity, but spontaneous hybridization between spilled *B. napus* and weedy *B. juncea* populations is hardly revealed. We evaluated the relationship between the hybridization frequency of *B. juncea* × *B. napus* and their planting distance in field experiments using the mutagenic herbicide-tolerant *B. napus* cv. Bn0861 as a pollen source for hybrid screening. The recipient *B. juncea* cv. Kikarashina was planted in an experimental field with Bn0861 planted in the center. No hybrids were detected under natural flowering conditions in 2009. However, the flowering period was artificially kept overlapping in 2010, leading to a hybridization frequency of 1.62% in the mixed planting area. The hybridization frequency decreased drastically with distance from the pollen source, and was lower under field conditions than estimated from the high crossability, implying that spontaneous hybridization between spilled *B. napus* and weedy *B. juncea* is unlikely in the natural environment.

Key Words: *Brassica napus*, *B. juncea*, spontaneous hybridization, distance, mutagenic herbicide-tolerant canola.

Introduction

The *Brassica* genus consists of 3 diploid and 3 amphidiploid species (U 1935). Interspecific crossability among these species based on cross-combination has been reported in numerous studies (Morinaga 1934, Nishi 1964, Olsson 1960, Roy 1980, Scheffler and Dale 1994). *B. rapa* (AA, 2n = 20) and *B. juncea* (AABB, 2n = 36) have especially high crossability with *B. napus* (AACC, 2n = 38) (Bing *et al*. 1996, Jørgensen *et al*. 1998, Scheffler and Dale 1994). *B. rapa* is the first-ranked cross-compatible recipient of *B. napus*, and *B. juncea* the second (Scheffler and Dale 1994). In fact, hybrids of *B. rapa* × *B. napus* and *B. juncea* × *B. napus* are easily produced (Bing *et al*. 1996, Jørgensen *et al*. 1998, Scheffler and Dale 1994).

Japan imported approximately 2.3 million tons of canola seeds from several countries in 2010 (Ministry of Finance Japan 2011), comprising mainly a mixture of non-transgenic and transgenic canola. The imported transgenic canola met all required safety assessments (J-BCH 2011, MAFF 2010, MHLW 2011). Several countries have reported feral transgenic canola plants derived from seeds spilled along roadsides between the import ports and oilseed processing factories (Claessen *et al*. 2005, Kawata *et al*. 2009, Nishizawa *et al*. 2009, Yoshimura *et al*. 2006). As a matter of fact, a putative hybrid between weedy *B. rapa* and spilled transgenic *B. napus* has also been reported in Japan (Aono *et al*. 2011, J-BCH 2010). There is public concern about the harmful impacts of such hybrids on biodiversity. In particular, *B. juncea* is considered a likely recipient of *B. napus* in Japan because it is widely distributed throughout Japan as a naturalized plant (Shimizu 2003, Shimizu *et al*. 2003). Although hybrids between weedy *B. juncea* and transgenic *B. napus* have not been reported in Japan (J-BCH 2010, MAFF 2010), there is some potential for their occurrence due to the high interspecific crossability between *B. juncea* and *B. napus*. However, compared with the relatively extensive research on hybridization and introgression between *B. napus* and *B. rapa*, there have been few reports on hybridization and introgression between *B. napus* and *B. juncea* (Lei *et al*. 2011). Obtaining an understanding of the spontaneous hybridization frequency between *B. juncea* and *B. napus* is a key step in evaluating gene flow and introgression.

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The spontaneous hybridization frequencies of *B. juncea* × *B. napus* under mixed planting conditions have been reported in several studies (Bing *et al*. 1991, 1996, Huiming *et al*. 2007, Jørgensen *et al*. 1998). Bing *et al*. (1991, 1996) reported a hybridization frequency of 3.29%, and Huiming *et al*. (2007) reported a frequency ranging from 0.109%–0.951%. Jørgensen *et al*. (1998) reported a frequency ranging from 0.3%–2.3%. All these experiments only assessed gene flow from a transgenic canola cultivation field to a weedy *B. juncea* population within the fields and/or in adjacent areas, and there has been no study of spontaneous hybridization between a small pollen source as spilled *B. napus* and a weedy *B. juncea* population. We therefore planned to assess the relationship between spontaneous hybridization frequency and distance from *B. napus* to *B. juncea*.

The relationship between spontaneous hybridization frequency and distance from the pollen source to the recipient in *B. rapa* × *B. napus* and *B. napus* × *B. napus* has been reported previously (Bing *et al*. 1996, Halfhill *et al*. 2004, Scheffler *et al*. 1993). In these reports, hybridization frequencies decreased as the distance from the pollen source increased (Halfhill *et al.* 2004, Scheffler *et al*. 1993). Since the hybridization frequency of *B. juncea* × *B. napus* is low even under mixed planting conditions (Bing *et al*. 1991, 1996, Huiming *et al*. 2007, Jørgensen *et al*. 1998), the hybridization frequency at plots distant from the pollen source is likely to be extremely low. Thus, efficient and reliable methods of selecting hybrids from a lot of progeny seeds are essential.

Herbicide tolerance is useful for mass screening, and herbicide-tolerant transgenic canola has been utilized for mass screening in hybridization experiments with herbicide-sensitive plants (Bing *et al*. 1991, 1996, Downey 1999, Scheffler *et al*. 1993). However, cultivation of herbicide-tolerant transgenic *B. napus* is difficult in Japan, because guidelines issued by the Ministry of Agriculture, Forestry and Fisheries (MAFF 2004) demand that transgenic canola must be cultivated more than 600 m away from other cultivated *Brassica* crops. Therefore, we used a single-gene homozygous mutant herbicide (imazamox)-tolerant *B. napus* cv. Bn0861. Since the heterozygous F_1 hybrids of *B. juncea* × *B. napus* cv. Bn0861 are more sensitive to herbicides than homozygous Bn0861, we evaluated the screening conditions for hybrid plants using the herbicide before attempting to detect spontaneous hybridization.

Here we report on the relationship between the spontaneous hybridization frequency of *B. juncea* × *B. napus* and distance from the pollen source to the recipient under field conditions. Furthermore, we designed the field conditions to investigate the effect of overlapping flowering between the pollen donor and recipient plants on hybridization frequency by controlling the flowering of the pollen donor. Data from these experiments were used to estimate the spontaneous hybridization between *B. napus* and *B. juncea* in the natural environment.

Materials and Methods

Plant materials

Herbicide (imazamox)-tolerant canola cv. Bn0861 (*B. napus*, provided by BASF Plant Science Company GmbH) was used as the pollen source. The leaf mustard cultivar *B. juncea* Coss. cv. Kikarashina (Takii & Co., Ltd. Kyoto, Japan) was the recipient. Kikarashina × Bn0861 hybrids were produced by artificial pollination to determine the most appropriate screening conditions with the herbicide "Beyond 1AS" (BAS 720 01 H*, 120 g/l of imazamox, BASF Plant Science Company GmbH).

Screening of hybrids

Kikarashina, Bn0861 and interspecific hybrid plants produced by artificial pollination were grown in 9-cm-diameter plastic pots in a glass greenhouse programmed with day/night temperatures of 25°C/22°C.

We evaluated the responses of seedlings at the 2–3 (Table 1) and 5–6 leaf stages as well as that of the mature plant before flowering, to different imazamox concentrations (1 μM, 3 μM, 5 μM, 7.5 μM, 10 μM and 30 μM). All herbicide solutions contained 0.25% of the spreading agent "Induce" (BASF Plant Science Company GmbH). Herbicide was applied once a day for 2 weeks, and then herbicide sensitivity was evaluated at 2 weeks after the end of herbicide application. Herbicide tolerance/sensitivity was evaluated as follows: tolerance (++) indicates the agent had no influence on the seedling; weak tolerance (+) indicated stunted growth without dying; and sensitivity (−) indicated dead seedlings (Table 1).

Field design

The evaluations of spontaneous hybridization were performed in an experimental field of the National Institute of Agrobiological Sciences located at Tsukuba, Ibaraki, Japan, in 2009 and 2010. The total field area was 18 a (40 m × 45 m). Bn0861 plants were grown in the center of the experimental field (5 m × 5 m) (Fig. 1). A total of 15,300 Kikarashina seeds were directly sown throughout the field in

Table 1. Examination of screening condition on 2–3 leaf stage seedling by imazamox

Concentration of imazamox (μM)	Kikarashina	Interspecific hybrid	Bn0861
0 (water)	++[a]	++	++
1.0	+[b]	++	++
3.0	+	++	++
5.0	−[c]	++	++
7.5	−	+	++
10.0	−	+	++
30.0	−	−	++

[a] Tolerance: The agent had no influence on the seedling.
[b] Weak tolerance: Stunted growth without dying.
[c] Sensitivity: Dead seedlings.

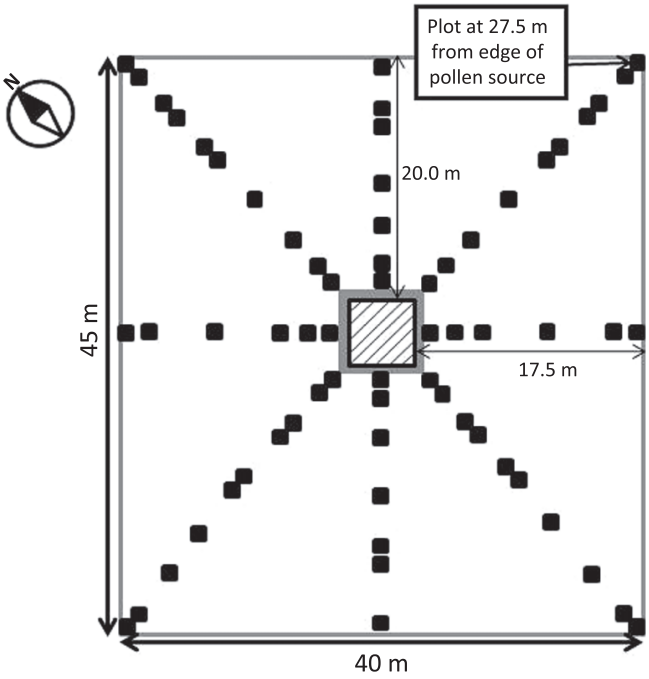

Fig. 1. Experimental field design. The recipient Kikarashina seeds were sown in an experimental field (40 m × 45 m) in 68 rows that were 45 m long with 60 cm spacing between rows and 20 cm spacing between plants. The pollen source area (5 m × 5 m) was located in the center of field and is depicted as the diagonal zone. Two hundred and twenty five seedlings of Bn0861 were transplanted into the field in 2009 and the same number of flowering pots was continuously supplied in place in 2010 to keep flowering. To create mixed planting plots, 25 recipient plants were cultivated in the same location as the pollen source plants. Adjacent planting areas (gray shadow) are areas within 1 m of the edge of the pollen source. Other plots (black areas) were located 1 m, 3 m, 5 m, 10 m, 15 m, 17.5 m, 20 m, 21.5 m, 25 m and 27.5 m from the edge of the pollen source area in the experimental field.

2009 and 2010. The cultivation design was as follows: 68 rows 45 m long with 60 cm spacing between rows and 20 cm spacing between plants. Kikarashina seeds were sown on April 16, 2009 and March 31, 2010. We also planted 25 pots of Kikarashina within the pollen source area in 2010 to represent a mixed planting plot. Bn0861 seedlings at 5–6 leaf stage were directly transplanted at a density of 9 plants/m^2 in the center of field on April 24, 2009. This field experiment in 2009 was performed under natural flowering conditions. In contrast, efflorescent plants of Bn0861 planted in pots were continuously supplied and kept flowering to ensure that the flowering period overlapped completely from May 12 to July 3, 2010. Two hundred and twenty five seedlings of Bn0861 were transplanted into the field in 2009 and the same number of flowering pots was continuously supplied in 2010.

Counting of the number of flowering flower of Kikarashina during the experiments was initiated with the start of Kikarashina flowering and was performed every seventh day. The number of flowering flower of Bn0861 was counted at the same time. The dates at which the flowering periods of Kikarashina and Bn0861 ended were also noted.

Sampling of progeny seeds

Unsynchronized flowers of *B. juncea* with *B. napus* were eliminated prior to harvest in 2009. The sampling plots are illustrated in Fig. 1. The sampling plots comprised 77 sites which included adjacently planting plot (<1 m from the pollen source) and 1 m, 3 m, 5 m, 10 m, 15 m, 17.5 m, 20 m, 21.5 m, 25 m and 27.5 m from the pollen source in eight directions (north, south, east, west, northeast, northwest, southeast and southwest). Plots in the northeast and southwest directions were up to 20 m from the pollen source and those in the northwest and southeast directions were up to 17.5 m from the pollen source. Each sampling plot had a 1-m radius.

Screening of hybrid in progeny

Harvested recipient plants were dried in the shade and placed in a drying machine at 32°C for 12 h. All harvested seeds were collected, counted and stored at 4°C. All of the obtained seeds were tested in 2009. Tested seeds in 2010 were randomly selected and we attempted to test more than 2,000 seedlings from each sampling plot in 2010. Seeds were germinated in petri dishes with filter paper moistened with water at 25°C for 48 h. They were then transplanted into a combined nursery tray (hole size: 40 mm × 40 mm × H50 mm) and grown in a glass greenhouse with programmed day/night temperatures of 25°C/22°C. The number of progeny seedlings was counted prior to herbicide treatment.

For the screening experiments, all progeny plants that reached the 2–3 leaf stage were treated with 5 μM imazamox once a day for 2 weeks. Kikarashina, Bn0861 and interspecific hybrids were always used as control plants. The plants were allowed to grow for 15 days after herbicide application and the number of herbicide-tolerant seedlings was counted.

Confirmation of hybridity by morphological characteristics and simple sequence repeats (SSR) markers

First, the hybridity of tolerant plants was confirmed by observing their morphological characteristics, namely flower organ size, leaf margin shape, leaf rugosity, leaf fairness and leaf waxiness (Tsuda *et al*. 2011).

Second, SSR markers were investigated. Genomic DNA was extracted from young leaves with an ISOPLANT II kit (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. To confirm the reliability of the screening conditions, leaves of several herbicide-sensitive progeny were randomly sampled in addition to those of the herbicide-tolerant progeny before imazamox application. Five *B. napus* C genome-specific SSR markers were used for identification of interspecific hybrids (Na12-E02B, Na10-D03A, CB10288, MR129 and CB10544B). These markers were used to construct the *B. napus* genetic map by

Piquemal *et al*. (2005). PCR reactions were performed according procedure described by Piquemal *et al*. (2005) with a few modifications. The reaction mix used was as follows: 0.5 U/μl Taq DNA polymerase (Gene Taq: Nippon Gene. Co., Ltd.); 1× PCR Buffer for Gene Taq; 0.2 mM dNTP; 0.25 mM forward primer; 0.25 mM reverse primer and 2 ng/μl template DNA. The PCR reaction was performed using a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturing step at 94°C for 4 min, followed by 15 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C; 23 cycles of 30 s at 94°C, 30 s at 53°C, 1 min at 72°C and a final extension step at 72°C for 5 min. The PCR products were electrophoresed on 5% acrylamide gels and visualized by ethidium bromide staining.

Results

Hybrid screening with herbicide

For determination of the most appropriate hybrid screening condition, the imazamox tolerance of each hybrid plant was examined. Six different concentrations of imazamox were sprayed on seedlings at the 2–3 and 5–6 leaf stages as well as on mature plants. Bn0861 was used as a positive control and tolerated in all test conditions. However, the tolerance of Kikarashina and interspecific hybrids varied according to their growth stage. Kikarashina and interspecific hybrids at the 2–3 leaf stage were sensitive to >1 μM and >7.5 μM imazamox, respectively. Reliable hybrid selection was possible with the application of 5 μM imazamox at the 2–3 leaf stage (Table 1). The growth of Kikarashina and interspecific hybrids was stunted by the application of 3 μM and 10 μM imazamox, respectively, at the 5–6 leaf stage and by the application of 5 μM and 30 μM imazamox at the mature plant stage. Reliable hybrids were obtained with 7.5 and 10 μM imazamox at the 5–6 leaf and mature plant stages, respectively (data not shown). The 2–3 leaf stage was chosen for further experiments as it allowed early selection and less labor intensive. Consequently, the treatment of 5 μM imazamox on 2–3 leaf stage seedlings was used as screening conditions (Table 1 and Fig. 2A, 2B).

Spontaneous hybridization frequency

The Bn0861 and Kikarashina flowering periods overlapped for 19 days (May 15–June 3) in 2009 and 34 days (May 28–June 30) in 2010. The total length of the flowering periods was as follows. In 2009, Kikarashina flowered from April 24–June 24 and Bn0861 from May 15–June 3; in 2010, Kikarashina flowered from May 28–June 30. The days with the greatest number of Kikarashina and Bn0861 flowers were May 22 and May 29, respectively, in 2009. In total, 178 and 352 recipient plants were harvested in 2009 and 2010, respectively (Tables 2, 3). In 2009, 5,535 progeny seeds were obtained. The number of harvested seeds was very low in 2009 compared with that in 2010 (Tables 2, 3), because we eliminated Kikarashina flowers that were not synchronized with Bn0861 flowers. No hybrid plants were detected from any of the progeny in 2009 (Table 2).

The number of hybrids detected and the hybridization frequency in 2010 are shown in Table 3. Thirty-six hybrid plants from a total of 2,227 progeny (1.62%) were detected in the mixed planting areas and 15 hybrids from 4,906 progeny (0.306%) were detected in adjacent planting areas. One hybrid plant was detected at distances of 1 m, 5 m, 10 m and 17.5 m from the pollen source; the corresponding hybridization frequencies were 0.050% (1 plant from 2,005 plants at 1 m), 0.037% (1 plant from 2,710 plants at 5 m), 0.040% (1 plant from 2,528 plants at 10 m) and 0.034% (1 plant from 2,905 plants at 17.5 m). No hybrid plants were detected at distances of 3 m, 15 m and >20 m from the pollen source (Table 3).

Confirmation of selected hybrids based on morphological characteristics and SSR markers

The hybridity of the herbicide-tolerant progeny (Fig. 2C) was confirmed by assessment of their morphological characteristics. All herbicide-tolerant plants had intermediate characteristics between *B. juncea* and *B. napus* in terms of flower organ size, leaf margin shape, leaf rugosity, leaf fairness and leaf waxiness (data not shown).

Subsequently, the hybridity of the herbicide-tolerant plants was also examined by SSR analysis to confirm that the chromosomal region was derived from *B. napus*. Five SSR markers were detected in all herbicide-tolerant plants, and these herbicide-tolerant plants were confirmed as hybrids (Fig. 3). In a control experiment, no SSR markers were detected from randomly sampled herbicide-sensitive progeny (Fig. 3). Because the hybridity of all the herbicide-tolerant plants was confirmed, we can conclude that the herbicide screening conditions used in this study are practical and reliable.

Discussion

We examined the spontaneous hybridization frequency of *B. juncea* × *B. napus* in field conditions over two years. It was apparently difficult to detect hybrid plants from an enormous number of progeny seeds because the spontaneous hybridization frequency of *B. juncea* × *B. napus* was considered very low in previous reports (Beckie *et al*. 2003, Downey 1999, Halfhill *et al*. 2004, Scheffler *et al*. 1993). Therefore, we established a mass screening method using herbicide (imazamox)-tolerant *B. napus* cv. Bn0861 to ensure efficient detection of hybrid plants (Table 1 and Fig. 2).

Bn0861 has a homozygous herbicide-tolerant gene due to a single base pair mutation in the acetohydroxyacid synthase (AHAS) gene (personal communication, BASF Plant Science Company GmbH). When mutagenic herbicide-tolerant plants, such as Bn0861, are used, screening conditions for detecting interspecific hybrids must be adjusted. Mutagenic herbicide-tolerant Bn0861 with its mutagenic AHAS gene has the same herbicide tolerance as plants with a mutated acetolactate synthase (ALS) gene, because AHAS and ALS are

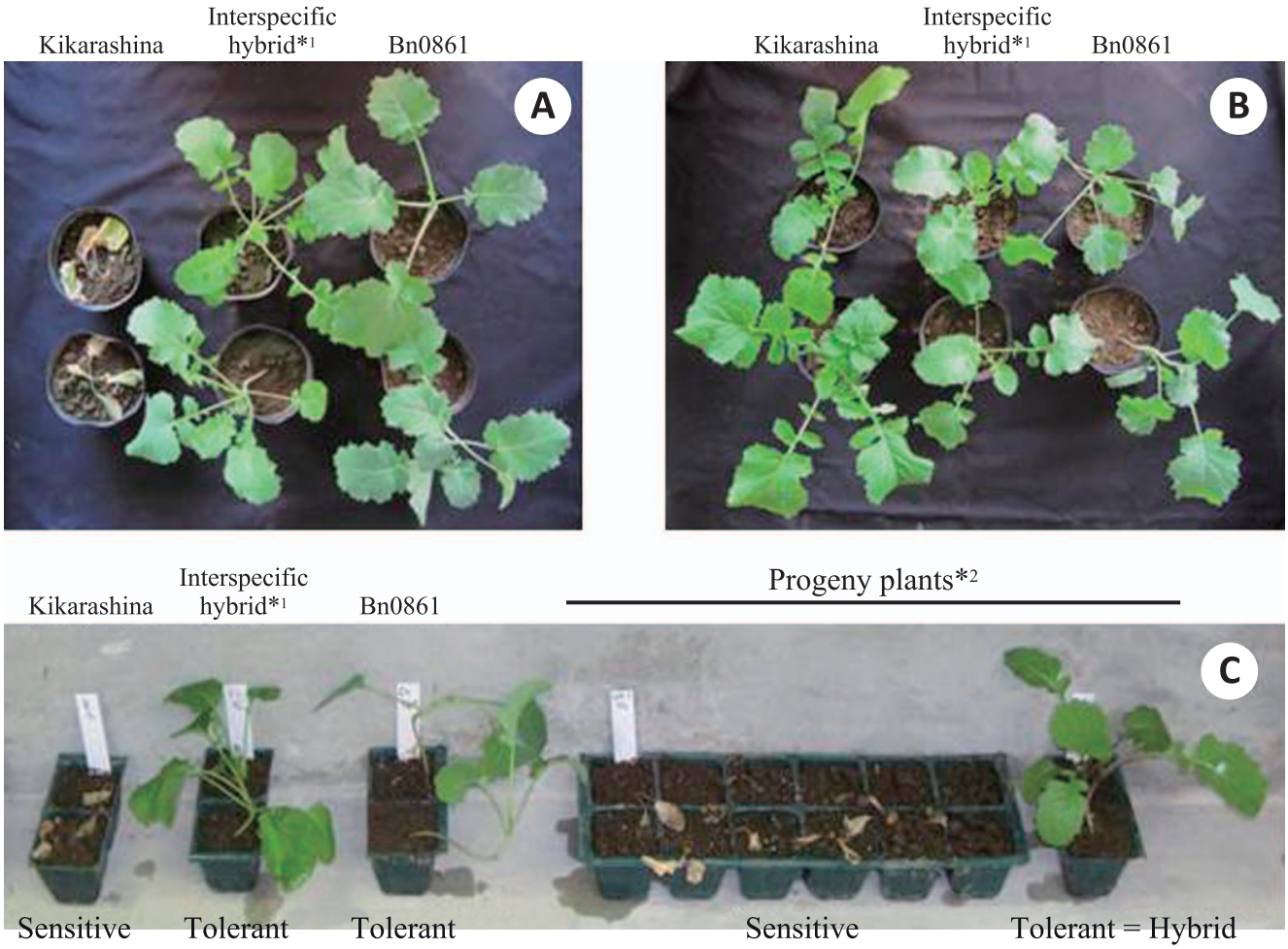

Fig. 2. Examination of screening conditions for herbicide-tolerant progeny. Kikarashina, Bn0861 and interspecific hybrid seedlings at the 2–3 leaf stage were used. A) After 15 days of spraying with 5 μM imazamox solution, all Kikarashina plants died but interspecific hybrid and Bn0861 plants survived. B) After 15 days of spraying with 0 μM imazamox solution (water with spreading agent), all tested plants survived. C) Response of sample progeny to 5 μM imazamox treatment. All Kikarashina and 12 progeny plants died but interspecific hybrid, Bn0861 and 2 progeny plants survived. *[1]Interspecific hybrids were produced by artificial pollination of Kikarashina × Bn0861. *[2]Progeny plants were obtained from adjacent planting areas.

the same enzyme and are distinguished by their different substrates (Shimizu *et al*. 2002). Endo *et al*. (2007) speculated that chimeric ALS complexes containing both bispyribac-tolerant and bispyribac-sensitive subunits may be inhibited by bispyribac. In fact, our F_1 plants showed more sensitivity than the homozygous Bn0861 (Table 1).

We selected 5 μM imazamox treatment of 2–3 leaf stage seedlings for detection of hybrids. Previous reports using transgenic herbicide-tolerant canola have not systematically investigated screening conditions for detecting heterozygous progeny (Bing *et al*. 1991, 1996, Downey 1999, Scheffler *et al*. 1993) because the bar and cp4-epsps genes of the heterozygous progeny exhibit stable herbicide tolerance. In the current study, the hybridity of all herbicide-tolerant progeny selected by 5 μM imazamox treatment was confirmed by morphological characteristics and SSR marker analysis. Interspecific hybrids could be screened using mutagenic herbicide-tolerant *B. napus* instead of transgenic *B. napus*. Our results revealed a hybridization frequency of 1.62% in mixed planting areas, supporting previous results under mixed cultivated conditions (Bing *et al*. 1991, 1996, Huiming *et al*. 2007, Jørgensen *et al*. 1998). Therefore, we believe that this screening method is useful for detecting hybrid plants in plots distant from the pollen source.

Flowering of *B. juncea* and *B. napus* is observed during spring in Japan (Takematsu and Ichizen 1993). If flowering periods overlapped, spontaneous hybridization frequency may be increased. In contrast, it has generally been reported that either a drastic reduction or no spontaneous hybridization occurs when the flowering periods do not overlap. Spontaneous hybridization was not detected when the heading date differed by >13 days in rice (Endo *et al*. 2009), and spontaneous hybridization frequencies in maize declined from 14.2% to 0.05% when the number of days with asynchronous flowering increased from 1 to 20 (Montserrat *et al*. 2008).

In the *Brassica* genus, Jørgensen and Andersen (1994)

Table 2. Number of hybrid plants between *B. napus* and *B. juncea* at each distance plot under field condition in 2009

Sampling plot		No. of sampling recipient plants	No. of obtained seeds	No. of tested seedlings	No. of hybrids
Mixed Setting		NT[a]	NT	NT	NT
Adjacently planting		12	110	110	0
Distance from pollen source (m)	1.0	30	792	792	0
	3.0	30	823	823	0
	5.0	37	906	906	0
	10.0	20	1,934	1,934	0
	15.0	15	328	328	0
	17.5	4	42	42	0
	20.0	8	321	321	0
	21.5	2	147	147	0
	25.0	6	46	46	0
	27.5	14	86	86	0
	total	178	5,535	5,535	0

[a] NT: Not tested.

reported that the spontaneous hybridization frequency was 60% when flowering of *B. rapa* and *B. napus* was synchronized, but the frequency was decreased to 13 or 22% when *B. rapa* flowers opened 1 week earlier than *B. napus* flowers. Landbo *et al*. (1996) also noted that hybridization frequency may be influenced by overlapping of the flowering period. Bing *et al*. (1991, 1996) obtained 9.34 and 4.02 hybrid plants per artificially pollinated flower in *B. rapa* × *B. napus* and *B. juncea* × *B. napus*, respectively; however, the spontaneous hybridization frequencies were low, as indicated by the 0.99% result for *B. juncea* × *B. napus* compared with 3.29% for *B. rapa* × *B. napus*. Bing *et al*. (1991, 1996) speculated that the reason for the low spontaneous hybridization frequency of *B. rapa* × *B. napus* was that flowering of *B. rapa* occurred approximately 2 weeks earlier than that of *B. napus*.

The flowering of Bn0861 was delayed by 20 days relative to that of Kikarashina in our experiment in 2009 and the day with the maximum number of flowers in *B. napus* was 1 week later than that for *B. juncea*. Moreover, the overlapping period in 2009 was 20 days, which was approximately half the period in 2010. We speculate that no hybrids were detected because the shorter period of synchronized flowering affected spontaneous hybridization in 2009.

In contrast, some hybrids were detected in 2010 because the maximum period of flowering synchrony was maintained by artificial supply of efflorescent Bn0861. These results showed that spontaneous hybridization frequency is affected by the synchrony of flowering between *B. juncea* × *B. napus*, as in the previous reports on *B. rapa* × *B. napus* (Bing *et al*. 1996, Jørgensen and Andersen 1994, Landbo *et al*. 1996). The spontaneous hybridization under mixed planting conditions was 1.62% in our results, and this is similar to 0.109–3.29% under mixed planting conditions in previous reports (Bing *et al*. 1991, 1996, Huiming *et al*. 2007, Jørgensen *et al*. 1998). Regarding the relationship between distance and spontaneous hybridization, the hybridization frequency decreased markedly with distance from the pollen source. Although a decrease in spontaneous hybridization frequency was also reported in *B. rapa* × *B. napus* and *B. napus* × *B. napus* (Bing *et al*. 1996, Halfhill *et al*. 2004, Scheffler *et al*. 1993), these spontaneous hybridization frequencies gradually decreased with the by distance compared to that of *B. juncea* × *B. napus*. No hybrids were detected at located plots of >20 m from the pollen source (Table 3). Moreover, our results also show that the spontaneous hybridization frequency of *B. juncea* × *B. napus* under field conditions is extremely low, despite the high interspecific crossability of *B. juncea* with *B. napus* (Hauser *et al.* 1998, Scheffler and Dale 1994, Tsuda *et al*. 2011) and the entire synchrony of flowering between *B. juncea* and *B. napus*. These results are due to the high self-compatibility of *B. juncea* (Ohsawa and Namai 1987, Rakow and Woods

Table 3. Hybridization frequencies between *B. napus* and *B. juncea* at each plot under field condition in 2010

Sampling plot		No. of sampling recipient plants	No. of obtained seeds	No. of tested seedlings	No. of hybrids	Hybridization frequency (%)[a]
Mixed setting		19	45,243	2,227	36	1.62
Adjacently planting		54	317,752	4,906	15	0.306
Distance from pollen source (m)	1.0	22	135,275	2,005	1	0.0499
	3.0	27	156,240	2,846	0	0.0000
	5.0	31	159,605	2,710	1	0.0369
	10.0	31	164,108	2,528	1	0.0396
	15.0	32	181,982	2,303	0	0.0000
	17.5	35	157,208	2,905	1	0.0344
	20.0	42	130,607	3,034	0	0.0000
	21.5	24	147,513	2,646	0	0.0000
	25.0	19	102,348	2,133	0	0.0000
	27.5	16	20,064	2,050	0	0.0000
	total	352	1,717,945	32,293	55	—

[a] Hybridization frequency (%) = No. of hybrids / No. of tested seedlings × 100.

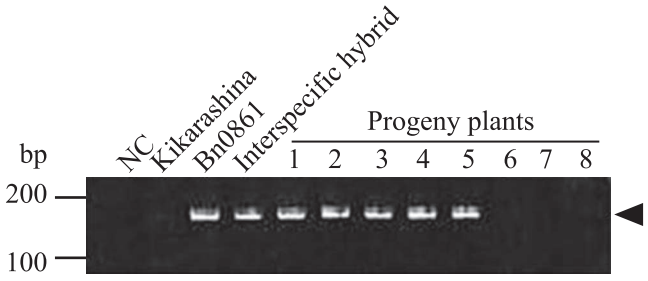

Fig. 3. Confirmation of hybrid plants using *B. napus* C genome-specific SSR markers. The bands indicate that the plant has a *B. napus* C genome-specific region. (Forward primer: ATGATTTGCCTTGAA ATGCC; Reverse primer: GATGAAACAATAACCTGAGACACA). 1–5: Herbicide-tolerant plant progeny. The band was detected in all herbicide-tolerant plants. 6–8: Herbicide-sensitive plant progeny. The band was not detected in sensitive plants. NC: Negative control, amplified by PCR without DNA sample. Kikarashina: *Brassica juncea* cv. Kikarashina. Bn0861: Herbicide-tolerant *Brassica napus* cv. Bn0861. Interspecific hybrid: Interspecific hybrids obtained by artificial pollination of Kikarashina × Bn0861. Arrowhead indicates the specific band derived from *B. napus* C genome-specific SSR markers: NA10-D03 (150–162 bp).

1987). In fact, despite a putative hybrid between *B. rapa* and transgenic *B. napus* being confirmed, hybrids between weedy *B. juncea* and transgenic *B. napus* have not been reported in Japan (J-BCH 2010, MAFF 2010). Feral *B. napus* populations are generally small, and most feral transgenic *B. napus* seeds cannot grow into mature plants because of human disturbance (Mizuguti *et al*. 2011). Moreover, weedy *B. juncea* population is usually separated from feral *B. napus*. Spontaneous hybridization of *B. juncea* × *B. napus* will probably be not easy in Japan.

However, the possibility of introgression between wild *B. juncea* and *B. napus* remains. Therefore, we will continue further investigation for fitness and the transfer of genomic regions derived from *B. napus* in hybrid and their progenies will be essential for a further understanding of introgression from *B. napus* to *B. juncea*.

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