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Original Paper

Molecular based evidence for a lack of gene-flow between *Rosa×hybrida* and wild *Rosa* species in Japan

Noriko Nakamura[1], Ursula Tems[2], Masako Fukuchi-Mizutani[1], Steve Chandler[2], Yoshiie Matsuda[3], Shunsuke Takeuchi[4], Shogo Matsumoto[5], Yoshikazu Tanaka[1,*]

[1] Institute for Plant Science, Suntory Holdings Ltd., Mishima, Osaka 618-8503, Japan; [2] Florigene Pty Ltd., Bundoora, Victoria 3083, Australia; [3] NISSHOKU Corporation, Tsuyama, Okayama 708-8652, Japan; [4] Keisei Rose Nurseries, Inc., Yachiyo, Chiba 276-0046, Japan; [5] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan

* E-mail: Yoshikazu_Tanaka@suntory.co.jp Tel: +81-75-962-8807 Fax: +81-75-962-3791

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Abstract An important part of the assessment of the potential environmental impact from the introduction of a genetically modified (GM) plant is an evaluation of the potential for gene flow from the GM plant to related wild species. This information is needed as part of the risk-assessment process, in the context of whether gene flow to wild species is possible. One method for evaluating gene flow is to use molecular techniques to identify genes in wild species populations that may have originated from a cultivated species. An advantage of this method is that a phenotypic marker or trait is not required to measure gene flow. In the present study we analyzed the seedlings of seeds from three wild native *Rosa* species (*R. multiflora* Thunb., *R. luciae* Rochebr. et Franch. ex Crép. and *R. rugosa* Thunb.) selected from several locations across Japan where the wild rose was growing in close proximity to cultivated rose plants (*Rosa×hybrida)*. To determine whether gene flow from cultivated rose had occurred, young leaves of 1,296 seedlings from the wild *Rosa* plants were analyzed by PCR for the presence of the *KSN* locus. This locus originated from a sport of *R. chinensis* Jacq. var. *spontanea* (Rehd. et Wils.) Yu et Ku and is involved in the recurrent flowering phenotype observed for cultivated rose hybrids, but is absent in Japanese species roses. The *KSN* locus was absent in all seedlings sampled, indicating no gene flow to wild *Rosa* species from the cultivated rose had occurred, and providing evidence that the probability of gene flow from cultivated to wild *Rosa* species in Japan is low or non-existent.

Key words: Gene flow, genetically modified, risk assessment, *Rosa×hybrida*, rose.

Roses are the most beloved of garden flowering plants and are also the most commercially important plant species for cut-flowers. *Rosa×hybrida* accounts for about 25% of the cut flower market and is the most widely sold cut-flower species in the world. Approximately 330 million stems of cultivated rose (*Rosa × hybrida*) were produced in Japan in 2009, with roses having the third highest production value of all cut flower types following chrysanthemum and lily, according to Statistics from the Agriculture, Forestry and Fisheries issued by Ministry of Agriculture, Forestry and Fishery. Roses are widely grown in most regions of Japan for landscaping purposes. It is estimated that between 400 and 500 garden cultivars are currently marketed in Japan. While considerable efforts to make new rose cultivars have been made for hundreds of years using hybridization breeding, more recently, genetic engineering has been adopted to confer novel characters to *Rosa×hybrida.* Colour modified roses expressing a pansy flavonoid 3′, 5′-hydroxylase gene have been generated (Katsumoto et al. 2007) and one transgenic variety is now commercially produced and sold in Japan.

Gene flow is a valid concern during the evaluation for the introduction of cultivated species into areas where wild relatives are present, and there are examples where genetically modified (GM) plant species have escaped cultivation, or where phenotypic traits have been transferred from crop species to crop species, or from crop species to a wild or weedy related species (De Marchis et al. 2003; Kawata et al. 2009; Knispel and McLachlan 2010; Rieger et al. 2002). Assessment of probability of gene flow from a GM plant is therefore crucial before release into the environment can be considered, especially when there are native related species of the GM plant present. Molecular techniques are useful to detect gene flow in cases where there is no obvious phenotype to monitor (Chandler and Dunwell 2008). For example, molecular microsatellite markers

Abbreviations: GM, genetically modified; GAPDH, glyceraldehyde 3-phosphate dehydrogenase
This article can be found at http://www.jspcmb.jp/

Table 1. Distribution and flowering period of common native *Rosa* species found in Japan

Species/hybrid	Distribution, character traits and usage	Flowering period
R. multiflora Thunb. (local name; Noibara)	Hokkaido to Kyushu and Korea Low climbing plant, used to create the cultivated spray rose 'Floribund'. Noibara is used as a rootstock in Japan	May and June
R. luciae Rochebr. et Franch. ex Crép. (local name; Terihanoibara)	Hokkaido, Shikoku, Kyushu, Okinawa, Korea, Taiwan and China Rambler rose, able to grow in a variety of habitats	Blooms relatively late, from June to July
R. rugosa Thunb. (Japanese rose) (local name; Hamanasu)	Temperate to arctic zones, including Hokkaido This species has been used to select a variety with good cold resistance (hybrid *rugosa*).	May to July Some evidence of longer flowering in some situations
R. davurica Pall. *var. alpestris* (Nakai) Kitag. (local name; Karafutoibara)	Hokkaido, Sakhalin, Korea	June to July
R. acicularis Lindl. (local name; Ohtakanebara)	Northeast China, Siberia, Northern Europe and America	June to July
R. onoei Makino var. *oligantha* (Franch. et Sav.) H. Ohba (local names; Ofujiibara, Azumaibara, Yamaterihanoibara)	Tokai and Kanto regions A climbing variety with hook-like thorns	May to June
R. hirtula (Regel) Nakai (local names; Sanshobara)	Fuji and Hakone regions	June

were used to evaluate population genetic structure for soybean (*Glycine max* L. Merrill) and to identify instances of introgression from cultivated to wild related species in Japan (Kuroda et al. 2006).

The centre of biodiversity for *Rosa* is Central and West Asia (Debener and Linde 2009), and there are native, wild, and introduced species of *Rosa* in Japan. Table 1 summarizes the distribution and flowering times of the most widely distributed wild *Rosa* species in Japan. Other wild *Rosa* species found in Japan are *R. nipponensis* Crep., *R. davurica* Pall. var. *alpestris* (Nakai) Kitag., *R. sambucina* Koidz. and *R. bracteata* J. C. Wendl.

Cultivated rose varieties are complex inter-specific hybrids derived from breeding with a handful of wild species (Gudin 2000); *R. canina* L., *R. multiflora* Thunb., *R. rugosa* Thunb., *R. phoenicea* Boiss, *R. luciae* Rochebr. et Franch. ex Crép., *R. gallica* L., *R. foetida* Hermann, *R. moschata* Hermann, *R. gigantea* Collet ex Crépin and *R. chinensis* Jacq. (Sakanishi 1989; Zlesak 2006). Three native *Rosa* species in Japan were used to create modern garden roses; *R. multiflora* Thunb., *R. luciae* Rochebr. et Franch. ex Crép., and *R. rugosa* Thunb. (Debener and Linde 2009; Gudin 2000; Hurst 1941; Wu et al. 2001). *Rosa rugosa* Thunb. is found predominantly along coastal regions in habitats including stabilized sand dunes and shorefront areas, with natural resistance to extreme environmental conditions (Bruun 2005; Kim 2005).

Modern hybrid tea and floribunda varieties of rose are tetraploid (Debener and Linde 2009; Zlesak 2006) and all exhibit a recurrent flowering phenotype. This phenotype is due to loss of a vernalization requirement for flowering and is controlled by a double recessive genotype (Zlesak 2006). Recurrent flowering was probably introduced from *R. chinensis* Jacq. (Debener and Linde 2009) which contains the recessive *evb* (ever blooming) allele at a single locus (Hess et al. 2007). Minor genes appear to be involved in the regulation of the recurrent blooming phenotype in cultivated *Rosa*×*hybrida* (Zlesak 2006) and one such element is a 9 kb transposon insertion in the recurrent flowering related *KSN* locus, which originated from *R. chinensis* Jacq. var. *spontanea* (Rehd. et Wils.) Yu et Ku (Iwata (2004) Protein and gene participating in perpetual blooming of angiosperm. International Patent Publication Number WO/2004/070036). The *KSN* locus is homologous to *Arabidopsis TERMINAL FLOWER1 (TFL1)*. The *tfl1-1* mutation in *Arabidopsis* causes early flowering and limits the development of the indeterminate inflorescence promoting terminal floral meristem formation (Melzer et al. 2008; Shannon and Ry Meeks-Wagner 1991). As a result of being homozygous for the *KSN* locus, cultivated *Rosa*×*hybrida* has a repeat flowering phenotype, while most wild *Rosa* species have a short, annual flowering period.

As part of an environmental impact assessment for the release of GM rose in Japan, the potential for gene flow from cultivated *Rosa*×*hybrida* to related wild *Rosa* species was assessed. Whether gene introgression had occurred between the cultivated rose and the wild *Rosa* species was measured using a PCR technique designed to identify whether the *KSN* locus interruption is present in wild *Rosa* species.

Table 2. Oligonucleotides used for PCR

Primer Name	Gene	Sequence	Size (kb)
KSNIF3	*KSN*	5′-CATATTATGGCATAGGGTGTGGC-3′	1.2
KSNInsR3		5′-TGTAATCTGTAGGAGATCCCATGC-3′	
RhGAPDH-237F	*GADPH*	5′-TGTCATCTCTGCCCCAAGTAAGG-3′	0.9
RhGAPDH-724R		5′-CAACATCCTCATCGGTGTAACCC-3′	

Materials and methods

Isolation of genomic DNA from germinated plants

Seeds collected from wild rose species were sowed in peat moss and maintained in a glasshouse (25–30°C) for 1–12 months. Genomic DNA was isolated from young leaves (25 mg) of each seedling by using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, USA).

PCR-mediated detection of the KSN locus interruption

Genomic DNA (3 ng) was used as a template for PCR amplification. Primers were used to amplify a 1.2 kb fragment of the locus and as a positive control, a 0.9 kb fragment of the *Rosa*×*hybrida GAPDH* gene (Table 2; Genbank accession no: AB370120). Amplification was performed in a 50 μl reaction with 0.25 mM dNTPs, 0.4 μM each primer, 1×TaKaRa Ex Taq™ reaction buffer and using 2.5 U of Ex Taq™ DNA polymerase (Takara Bio Inc., Otsu, Japan). Reactions were initially denatured at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min for amplification of the *KSN* locus, and 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min for amplification of the *GAPDH* gene, before a final extension step of 72°C for 7 min. Reaction products were electrophoresed through a 0.8% (w/v) agarose gel using 1×TAE running buffer (40 mM Tris-HCl, pH 7.6, 50 mM acetic acid, 50 mM EDTA) and were imaged under UV light after ethidium bromide staining. A negative result was obtained when no DNA fragment of the correct size was visible after 30 cycles of amplification, and where a DNA fragment was visible; it was considered a positive result.

Results

Locations for analysis of potential gene flow

Four geographic locations were identified where *Rosa*×*hybrida* cultivation was occurring in the vicinity of wild *Rosa* species. Within each location, seeds were collected from wild *Rosa* plants at up to twelve different sites (Table 3). Location A was the Keisei Nursery in Sawara, Chiba (Figure 1A). At this location, seeds were collected from six different sites at distances up to 200 m from the cultivation area. The grounds of a rose grower in Northern Okayama were examined as Location B (Figure 1B), with seven different sites of seed collection up to 100 m from the cultivation area. Locations C and D were university gardens at the Hokkaido University in Sapporo and the Gifu University in Gifu respectively (Figure 1C, D). Another location investigated was at NISSHOKU Corporation in Tsuyama, Okayama. Seeds were collected from twelve wild flower collection sites that were placed from 3, 5, 10, 20, 50, 100 and 300 m from *Rosa*×*hybrida* 'Lavande' (Figure 1E). The rose plants, both cultivated and wild *Rosa* species, have been growing at the locations used for between 15–25 years except for location E.

Table 3. A summary of the locations at which wild Rosa species were found growing in the vicinity of cultivated roses

Location	Description	Seed Collection Sites	Distance between wild and cultivated roses (m)
A	Keisei Nursery Sawara, Chiba	6	20–200
B	A rose Grower, Kaga, Okayama	7	5–130
C	Hokkaido University Botanical Gardens, Sapporo, Hokkaido	3	3–1,800
D	Gifu University, Gifu, Gifu	3	2–0
E	NISSHOKU Corporation Tsuyama, Okayama	12	3–300

Collection of seeds from wild Rosa species

Table 4 summarizes the number of seedling analyzed at each site, and from which wild *Rosa* species the seed was collected. Seeds from wild *R. multiflora* Thunb. and *R. luciae* Rochebr. et Franch. ex Crép. were collected from Location A, while only *R. multiflora* Thunb. seeds were collected from Location B. Seeds from both *R. multiflora* Thunb. and *R. rugosa* Thunb. were collected from Location C, Location D and Location E. Seeds were extracted from mature hips in September or October and stored at 2°C for 3 months before sowing.

Molecular analysis of wild rose genomic DNA

Genomic DNA was prepared from young leaves of seedlings derived from the 1,296 seeds collected from wild *Rosa* species. No genomic DNA samples analyzed contained the *KSN* locus that *Rosa*×*hybrida* contains (Table 4). Detection of the endogenous glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene served as an internal positive control. Amplification of an 0.9 kb fragment of the *GADPH* gene was achieved for all genomic DNA samples examined, indicating that the reaction conditions used were optimal, and that the genomic DNA used as template was amplifiable (Figure 2).

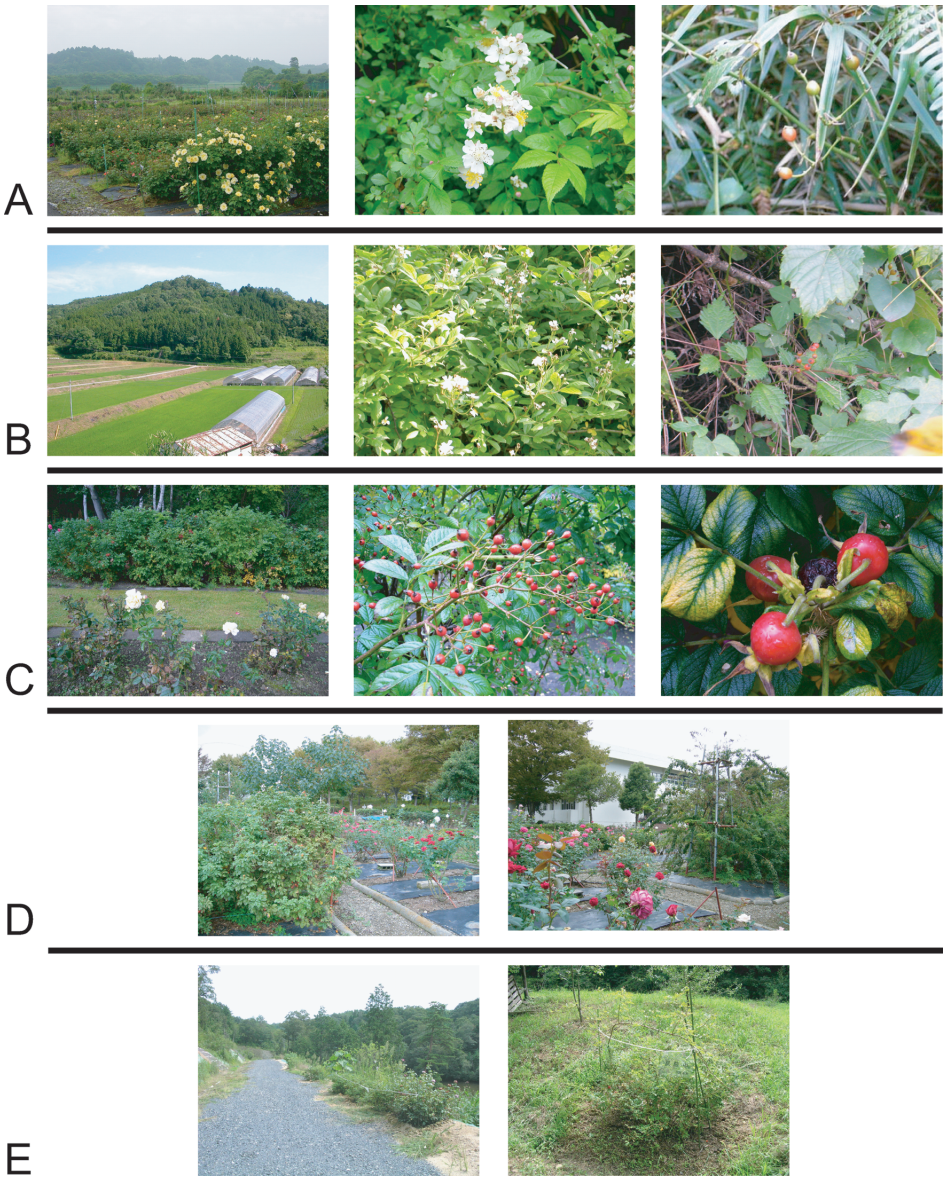

Figure 1. The five locations identified for collection of seeds from wild *Rosa* species growing in the vicinity of cultivated roses. Seeds from wild *Rosa* species were collected from (A) Keisei Nursery in Sawara, Chiba. From left; overview, *R. multiflora* Thunb. flowers, *R. multiflora* Thunb. hips. (B) A rose grower in Kaga, Okayama. From left; overview, *R. multiflora* Thunb. flowers, *R. multiflora* Thunb. hips. (C) Hokkaido University Botanical Gardens, Sapporo, Hokkaido, where wild and cultivated roses are grown in close vicinity in a rose garden. From left; overview, hips from wild *R. multiflora* Thunb., hips of *R. rugosa* Thunb. (D) Gifu University, Gifu. The wild *Rosa* species, *R. multiflora* Thunb. and *R. rugosa* Thunb. are grown within an area that has a perimeter of cultivated *Rosa* × *hybrida.* (E) NISSHOKU Corporation, Tsuyama, Okayama. *R. multiflora* Thunb. (left) and *R. rugosa* Thunb. (right).

Discussion

The mechanisms whereby gene flow may occur vary depending on the plant species, but include the possibilities of asexual propagation, wind or insect-mediated pollen dispersal and also seed dispersal (Warwick et al. 2009). In the case of rose, cultivation is achieved by vegetative propagation by cutting or grafting, and not seed. The first GM rose variety released in Japan is chimeric, and the pollen is non-transgenic (Nakamura et al. 2011), nevertheless the results of this study suggest that the probability of pollen-mediated gene flow from cultivated rose to wild rose populations in Japan is likely to be very low.

The most likely explanations for the lack of introgression of the *KSN* locus is that cultivated roses are tetraploids (Rajapakse et al. 2001), whereas wild roses, except some *R. acicularis* Lindl., found in Japan are diploid (Erlanson 1929; Ueda and Akimoto 2001). In contrast to diploid rose, tetraploid rose varieties are self-compatible (Joung et al. 2009) and often set self-pollinated seed (Debener and Linde 2009). A correlation was noted by Ueda and Akimoto (2001) of increased self compatibility with higher ploidy level. In addition to the

Table 4. The summary of the wild rose seedlings collected at each location

Location	Site No.	Species	Seedling number analyzed	Seeds positive for *KSN* locus	%
A	1	*R. multiflora*	75	0	0
	2	*R. multiflora*	12	0	0
	3	*R. luciae*	9	0	0
	4	*R. multiflora*	95	0	0
	5	*R. multiflora*	17	0	0
	6	*R. multiflora*	22	0	0
			230		
B	1	*R. multiflora*	51	0	0
	2	*R. multiflora*	34	0	0
	3	*R. multiflora*	92	0	0
	4	*R. multiflora*	77	0	0
	5	*R. multiflora*	40	0	0
	6	*R. multiflora*	7	0	0
	7	*R. multiflora*	81	0	0
			382		
C	1	*R. multiflora*	23	0	0
	2	*R. rugosa*	73	0	0
	3	*R. rugosa*	25	0	0
			121		
D	1	*R. rugosa*	12	0	0
	2	*R. multiflora*	98	0	0
	3	*R. multiflora*	98	0	0
			208		
E*	1 (3 m)	*R. multiflora*	6	0	0
	2 (5 m)	*R. multiflora*	100	0	0
	3 (10 m)	*R. multiflora*	100	0	0
	4 (20 m)	*R. multiflora*	80	0	0
	5 (300 m)	*R. multiflora*	9	0	0
	6 (3 m)	*R. rugosa*	18	0	0
	7 (5 m)	*R. rugosa*	2	0	0
	8 (10 m)	*R. rugosa*	16	0	0
	9 (20 m)	*R. rugosa*	12	0	0
	10 (50 m)	*R. rugosa*	5	0	0
	11 (100 m)	*R. rugosa*	5	0	0
	12 (300 m)	*R. rugosa*	2	0	0
			355		
Total			1,296	0	0

* *R. multiflora* Thunb. or *R. rugosa* Thunb. plants were placed from *Rosa*×*hybrida* plants at shown distance.

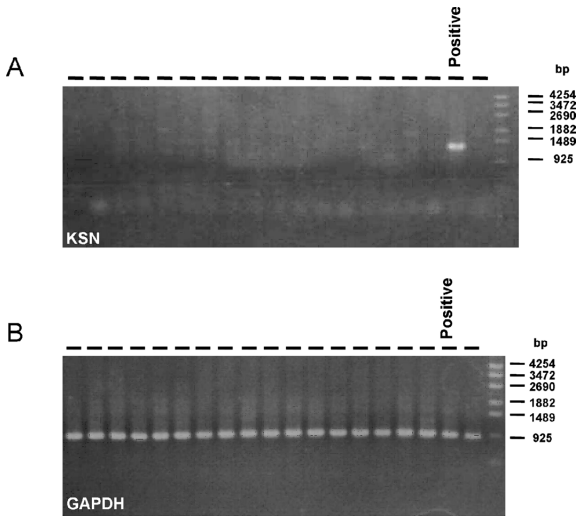

Figure 2. Agarose (0.8% w/v) gel electrophoresis of PCR products of the reactions used to amplify (A) the *KSN* locus and (B) the *GAPDH* gene fragment, from DNA extracted from young leaves of seedlings of the wild *Rosa* seeds analyzed. Positive; positive control.

effect of the different ploidy levels, fertilization, seed development and seed germination barriers exist in inter-specific crosses in rose (Debener and Linde 2009; Gudin 2000; Joung et al. 2009). It is therefore possible that hybridization events between cultivated and wild rose may have occurred but have not resulted in sustainable hybrid populations. Notwithstanding genetic barriers to hybridization, wild species have a short flowering period (Table 1), further reducing the possibility of out-crossing with cultivated rose, which flowers for most of the non-dormant stage of plant growth.

Debener (2005, The probability of outcrosses between cultivated and wild roses. Federal Centre for Breeding Research on Cultivated Plants (BAZ), Institute of Ornamental Plant Breeding (http://www.gmo-safety.eu/database/900.probalility-outcrosses-between-cultivated-wild-roses.html) has examined gene flow from cultivated rose in Germany. In these experiments a similar experimental approach was used to the present study, in that molecular (microsatellite) markers were used to look for gene flow to wild rose stands found in close proximity to large areas of cultivated rose production. Debener (2005) only found one case of possible gene transfer, and also concluded that gene flow from cultivated rose was a rare event. Under controlled conditions, it is possible to produce hybrids using *R. rugosa* Thunb. as the female and pollen from tetraploid hybrid tea rose (Sparinska et al. 2009).

The regulatory requirements that must be met before the release of a GM plant differ depending on the country and region. In Japan, ornamental species such as *Rosa*×*hybrida* are not considered as food or feed and the main regulatory requirement is to ensure that there are no adverse effects on conservation and sustainable use of biodiversity resulting from the introduction of the GM ornamental species. Impacts of gene flow on biodiversity range from concerns for the potential for generating more aggressive weed species to a reduction or even the extinction of rare wild species, where gene flow of alleles that provide an evolutionary advantage acts as a 'cohesive force that integrates species' (Ellstrand 2003).

The sample size may not have been sufficient to measure extremely low levels of hybridization but there are advantages in using molecular techniques, as the time and cost associated with determining whether gene flow has occurred can be greatly reduced. Hybridization events have been identified between the two diploid species *R. rugosa* Thunb. and *R. blanda* Aiton in eastern North America by the development of allele-specific primers to assay single nucleotide polymorphisms

(SNPs; Mercure and Bruneau 2008). It would not have been possible to identify the *KSN* locus in possible hybrids phenotypically, as recurrent flowering is a recessive trait (Debener 1999; Shupert et al. 2007). Furthermore, *R. rugosa* Thunb. has a low level of the recurrent blooming characteristic (Bruun 2005; Mercure and Bruneau 2008), which could lead to false phenotypic identification of possible hybrid events.

In this study, no evidence of gene flow was found after analysis of 1,296 seedlings from wild *Rosa* species including *R. multiflora* Thunb., *R. luciae* Rochebr. et Franch. ex Crép. and *R. rugosa* Thunb. in five different cultivation areas in Japan. All three species were historically used in the development of modern cultivated rose. This information, in conjunction with the fact we are unaware of any reports of natural hybrids between cultivated rose and wild rose species in Japan, suggests release of GM rose in Japan is most unlikely to have any impact on biodiversity as a result of gene flow.

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