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Evaluation and Inheritance of Crown Gall Resistance in Apple Rootstocks

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To identify sources of resistance to crown gall disease and to investigate its inheritance pattern to descendants, we assessed the degree of resistance among seven apple rootstocks of 'JM5', 'JM7', 'M. 9', 'M. 27', 'G. 65', *Malus prunifolia* 'Mo 84a', and 'Morioka Seishi', two wild *Malus* accessions of *Malus sieboldii* 'Sanashi 63' and 'Mo-15', and its hybrids (147 individuals). The inoculation was tested using two *Agrobacterium tumefaciens* strains of Peach CG8331 (biovar 2) and ARAT-001 (biovar 1) as inocula. *M. sieboldii* 'Sanashi 63' and 'Mo-15' did not show any galls at the inoculated sites six months after inoculation with suspensions of the strain Peach CG8331. Galls developed on the other rootstocks with a frequency from 0.31 to 0.82. In an inoculation test with strain ARAT-001 as the inoculum, no galls were formed on *M. sieboldii* 'Mo-15', and the frequency of *M. sieboldii* 'Sanashi 63' was low, 0.19. The frequency in 'G. 65' inoculated with strain ARAT-001 was much lower than that with strain Peach CG8331, whereas that in other rootstocks showed similar or higher frequency compared to strain Peach CG8331. The results suggested that there is an interaction (specificity) for the frequency of gall occurrence between *A. tumefaciens* strain and apple rootstocks. Based on the results of our study, *M. sieboldii* 'Sanashi 63' and 'Mo-15' were regarded as the most resistant genotypes to the virulent strains of *A. tumefaciens* used in our study. Resistant hybrids with no galls were found in progeny derived from a cross between 'JM7' × 'Sanashi 63' against strains of *A. tumefaciens*; numbers of hybrids were 19 (16%) and 5 (4%) against strains Peach CG8331 and ARAT-001, respectively. In F_1 progeny between 'JM5' × 'G. 65', plants with no galls were not observed. These results indicate that crown gall resistance in *M. sieboldii* 'Sanashi 63' is heritable through its descendants.

Key Words: crown gall, dwarfing rootstock breeding, *Malus* spp., resistance.

Introduction

Crown gall disease, caused by *Agrobacterium tumefaciens*, induces tumors at the wounding sites of many dicots, including fruit trees. The most serious damage is caused to fruit trees, raspberry and grapevine plantations (El-Fiki and Giles, 1981; Moore and Cooksey, 1981). Recently, crown gall disease has been increasing in apple nurseries in Japan (Nekoduka et al., 2001). To succeed in growing intact saplings, soil fumigation is effective as a means of control, but it has been claimed that soil fumigation does not entirely prevent crown gall disease (Schroth et al., 1971). Use of resistant rootstocks is an alternative method for controlling crown gall disease. In a field experiment with grapevine, crown gall was reduced by using crown gall-resistant rootstocks (Sule and Burr, 1998).

In woody plants, including fruit trees, many studies of crown gall disease have been reported. Screening for a crown gall-resistant genotype has been reported in several fruit crops, and some crown gall-resistant accessions have been discovered (Beneddra et al., 1996; Bliss et al., 1999; Mahmoodzadeh et al., 2004; Stover et al., 1997; Szegedi et al., 1984). The inheritance pattern of crown gall resistance has been studied in grapevine and apricot (Szegedi and Kozma, 1984; Tsiantos et al., 2002); however, little is known about the source of resistance and the inheritance pattern of resistance for crown gall disease in apples.

Apple rootstock breeding at the Apple Research Station, National Institute of Fruit Tree Science (NIFTS) in Japan was initiated in the early 1970s in an effort to improve propagation ability, dwarfing ability, disease and pest resistance, and tolerance to wet soil conditions (Soejima et al., 2000); five dwarfing rootstock cultivars, 'JM1', 'JM2', 'JM5', 'JM7', and 'JM8', have been released to date. These promising rootstocks possess

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advantage over 'M. 9 EMLA' and/or 'M. 26 EMLA' such as high propagation ability by hardwood cuttings, resistance to crown rot and woolly aphid, and high productivity of scion cultivars (Soejima et al., 1998, 2000). However, all of the JM series rootstocks are known to be susceptible to crown gall. 'JM7' was reported to be extremely sensitive to natural infection with *A. tumefaciens* among major apple rootstocks in Japan (Nekoduka et al., 2001; Suzaki, 2005); therefore, the development of new rootstock varieties with resistance to crown gall is an important objective of apple rootstock breeding at NIFTS.

In this study, we assessed the degree of crown gall resistance among several apple rootstocks to identify sources of resistance to the disease and showed that crown gall resistance found in *Malus sieboldii* was a heritable trait in its descendants.

Materials and Methods

Plant materials

An apple rootstock genotype of 'JM7' and a wild *Malus* accession of *M. sieboldii* 'Sanashi 63' and 147 hybrid plants derived from two crosses of 'JM7' × 'Sanashi 63' and 'JM5' × 'G. 65' in the orchard of the Apple Research Station, NIFTS were investigated for resistance to crown gall in 2005 and 2006. In 2006, additional six rootstock genotypes of 'JM5', 'M. 9', 'M. 27', 'G. 65', *M. prunifolia* 'Mo 84a', and 'Morioka Seishi', and a wild *Malus* accession of *M. sieboldii* 'Mo-15' were investigated. In these two years, dormant shoots of all genotypes and hybrids were taken from the trees; these shoots were considered to be intact because of being planted in fumigated soil. For inoculation to shoots, three scions of each genotype and/or hybrid were grafted on rootstock 'JM7', which had been previously propagated by hardwood cutting in fumigated orchard soil one year earlier and potted in a greenhouse in March. Each scion contained three buds to grow three shoots on a rootstock. In June, when the shoots were about 30 cm long in many trees, inoculation was conducted.

For inoculation in soil, cuttings of 25 cm from dormant shoots from 21 seedlings, selected from a cross of 'JM7' × 'Sanashi 63' based on the diversity of the reaction to the crown gall, were rooted in pots with vermiculite in the early spring of 2006. Two months after cutting, seven to 32 rooted cuttings in each seedling were obtained.

Bacterial strains

Two virulent strains of *A. tumefaciens*, Peach CG8331 (biovar 2) and ARAT-001 (biovar 1), were used for the inoculation test. The strain Peach CG8331 was isolated from a peach tree in Yamagata, Japan, and kindly donated by Dr. Yuichi Takikawa of Shizuoka University. The strain ARAT-001 was isolated from an apple tree in Aomori, Japan, and kindly offered by Mr. Yoshifumi Fukushi of the Apple Experiment Station of the Aomori Prefectural Agriculture and Forestry Research Center. These bacterial strains were cultured with vigorous shaking in 3 mL of a YP broth (5 g yeast extract, 5 g peptone, and 5 g NaCl in 1 L distilled water, adjusted to pH 7.0) at 28°C for two days. Then, 0.7 mL of a YP broth containing proliferated bacterial cells and 0.3 mL of 50% glycerol in distilled water were mixed and preserved at −80°C. The virulence of these bacterial strains was preliminarily checked by the method described by Suzaki et al. (2004) using tomato 'Ponderosa' seedlings.

Preparation of inoculum and inoculation

For inoculation to shoots, frozen bacterial strains were thawed at room temperature and inoculated into 3 mL of a YP broth. The bacterial strains were then proliferated as described above. The number of living bacterial cells was measured using a dilution plate technique (Baker et al., 2006) and adjusted to a 10^7 or 10^9 colony-forming unit (cfu)/mL for inoculation.

The prepared bacterial suspensions were taken up into sterilized disposable syringes (5 mL, Terumo, Tokyo, Japan) with a sterilized needle (0.55 × 25 mm, Terumo), the needle was inserted into the internode of each growing shoot, and then one drop of bacterial suspension was injected, as illustrated in Figure 1. Needles were replaced for every injection. Inoculated apple genotypes were grown in a greenhouse until they were rated for crown gall formation.

In 2005, bacterial suspensions with different concentrations, 10^7 and 10^9 cfu/mL, of *A. tumefaciens* strain Peach CG8331 were used for the inoculation test.

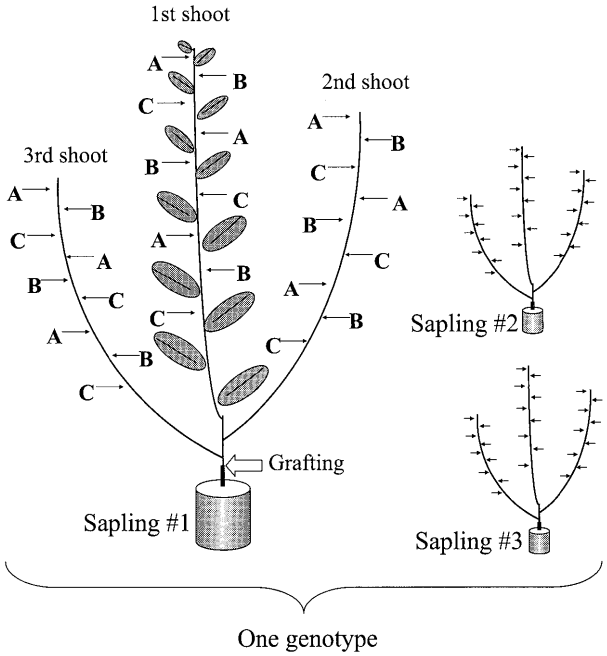

Fig. 1. Inoculation sites on a shoot with three distinct inocula. A and B: independent *A. tumefaciens* strains (2006) or different concentrations of strain Peach CG8331 (2005), C: sterilized distilled water.

In 2006, bacterial suspensions of *A. tumefaciens* strain Peach CG8331 and strain ARAT-001, adjusted to 10^9 cfu/mL, were prepared and used for the inoculation.

The bacterial strain Peach CG8331, for inoculation in soil, was cultured in a 2 L of YP broth, then, harvested by centrifugation (3000 rpm for 5 min at 4°C) and washed once in sterilized water. Washed bacterial cells were suspended in 2 L of sterilized water for use. The concentration was adjusted to 10^9 cfu/mL as described above. Lower parts of the cuttings were wounded using pruning scissors at four sites at 1 cm intervals and immediately soaked in the bacterial suspension overnight. These cuttings were then planted in pots filled with sterilized orchard soil and the inoculated sites of the cuttings were kept below the soil level. The cuttings were grown in a greenhouse until assessment of resistance levels to crown gall.

Evaluation of resistance levels to crown gall

Six months after inoculation, each inoculated site was visually assessed to determine whether a crown gall had formed, and the number of crown galls at the inoculated sites was counted for each genotype and/or hybrid. The frequency of crown gall occurrence was determined for each genotype and/or hybrid. The maximum diameter of each gall was also recorded. The size of galls was determined as follows; small: < 2.5 mm; moderate: 2.5–5.0 mm; large: > 5.0 mm. Resistance levels of each genotype and/or hybrid were classified into 3 classes on the basis of the frequency of gall occurrence as follows; resistant: 0; moderately resistant: 0–0.3; susceptible: > 0.3.

Results and Discussion

Difference in the frequency of crown gall occurrence among apple rootstocks

All of the inoculation sites that received sterilized distilled water as a control exhibited no gall formation (data not shown). When suspensions of strain Peach CG8331 at a concentration of 10^7 cfu/mL were used in the inoculation test, the frequency of gall occurrence was very low, less than 0.2, in more than 90% of the tested genotypes (data not shown). This indicated that the concentration of the suspensions, 10^7 cfu/mL, was inadequate for evaluating the sensitivity of apple rootstocks to *A. tumefaciens*; therefore, we adopted the results of an inoculation test with higher concentration of suspensions, 10^9 cfu/mL, for the evaluation of crown gall resistance. The frequency of crown gall occurrence and mean diameter of galls on shoots were quite different among apple rootstocks (Table 1). *M. sieboldii* 'Sanashi 63' and 'Mo-15' had no galls at the inoculated sites six months after inoculation with bacterial suspensions of *A. tumefaciens* strain Peach CG8331. In contrast with *M. sieboldii*, galls developed at the inoculation sites on shoots of 'JM7', 'JM5', 'M. 9', 'M. 27', 'G. 65', *M. prunifolia* 'Mo 84a', and 'Morioka Seishi'. The frequency of gall occurrence ranged from 0.31 to 0.45 in 'JM5', *M. prunifolia* 'Mo 84a' and 'Morioka Seishi', and the frequency was high, ranging from 0.67 to 0.77, in 'JM7', 'M. 9', 'M. 27', and 'G. 65'. Small galls ranging from 1.50 to 2.00 mm was formed on 'G. 65', 'M. 27' and *M. prunifolia* 'Morioka Seishi'. 'JM5' and 'JM7' formed galls of moderate size, 2.67 and 3.67 mm, respectively. Large galls were formed on 'M. 9' and *M. prunifolia* 'Mo 84a', ranging from 5.80 to 6.44 mm. The result indicated that 'M. 9' with large galls of high frequency was highly sensitive to strain Peach CG8331.

In the inoculation test with strain ARAT-001 as the inoculum, no galls were formed on any of the inoculated shoots of *M. sieboldii* 'Mo-15' at six months post-inoculation. Gall formation was observed at a few sites of inoculation on growing shoots of *M. sieboldii* 'Sanashi 63' and 'G. 65'. The frequency of gall occurrence was relatively high, 0.62 to 0.72, in 'JM7', 'M. 9', and 'M. 27'. An extremely high frequency of gall occurrence, more than 0.9, was observed in 'JM5', *M. prunifolia* 'Mo 84a', and 'Morioka Seishi'. The galls formed in *M. sieboldii* 'Sanashi 63' and 'G. 65' were small, 1.67 and 1.33 mm, respectively. Moderately sized galls were

Table 1. Frequency of gall occurrence and gall size among apple rootstock genotypes inoculated with virulent strains of *A. tumefaciens*. Each genotype was inoculated at 27 sites on growing shoots (9 sites × 3 saplings).

Genotype	Peach CG8331		ARAT-001	
	Frequency	Mean diameter (mm)	Frequency	Mean diameter (mm)
JM5	0.40[z]	2.67 ± 1.48	1.00	5.60 ± 1.38
JM7	0.67	3.67 ± 0.77	0.72	4.85 ± 1.10
M. 9	0.77	5.80 ± 2.20	0.62	4.88 ± 1.71
M. 27	0.67	2.00 ± 0.58	0.67	4.25 ± 1.60
G. 65	0.75	1.83 ± 0.24	0.19	1.33 ± 0.33
M. prunifolia 'Mo 84a'	0.45	6.44 ± 2.31	1.00	8.25 ± 1.27
M. prunifolia 'Morioka Seishi'	0.31	1.50 ± 0.27	0.96	2.67 ± 0.29
M. sieboldii 'Sanashi 63'	0	0	0.19	1.67 ± 0.67
M. sieboldii 'Mo-15'	0	0	0	0

[z] Frequencies on 'JM7' and *M. sieboldii* 'Sanashi 63' are the mean value of inoculations with strain Peach CG8331 (10^9 cfu/mL) in 2005 and 2006. Others were observed in the inoculation in 2006.

observed on *M. prunifolia* 'Morioka Seishi', 'JM7', 'M. 9', and 'M. 27', ranging from 2.67 to 4.88 mm, while large galls were formed in 'JM5' and *M. prunifolia* 'Mo 84a', with mean diameters of 5.60 and 8.25 mm, respectively.

In the case of 'G. 65' inoculated with strain Peach CG8331 and *M. prunifolia* 'Morioka Seishi' inoculated with strain ARAT-001, the frequency of gall occurrence was high, but the mean diameter was relatively small, indicating that the frequency of gall occurrence and gall size did not always coincide well. Iwanami et al. (2006) revealed that gall size was affected by the growth condition of plants and environmental factors such as shoot length and the inoculation position of each growing shoot, whereas the frequency of gall occurrence was scarcely affected. They concluded that for evaluating resistance to crown gall, gall frequency was a more reliable index than gall size. Based on the results of Iwanami et al. (2006), we adopted the frequency of gall occurrence as the mainly index for the evaluation of crown gall resistance.

Galls occurred at inoculated sites on the trunk of cuttings in the soil. The frequency of gall occurrence at the inoculation sites on the shoot above the soils was positively correlated with that in the soil ($r = 0.684$, Fig. 2). A high correlation of the frequency of gall occurrence between above and below ground has also been reported in apple rootstock (Stover and Walsh, 1998); therefore, we regarded the frequency of gall occurrence on shoots as representing resistance (or susceptibility) levels of the whole plant to virulent *Agrobacterium* strains.

The frequency in 'JM5', *M. prunifolia* 'Mo 84a', and 'Morioka Seishi' inoculated with strain ARAT-001 was high compared with those inoculated with strain Peach CG8331, whereas the frequency in 'G. 65' inoculated

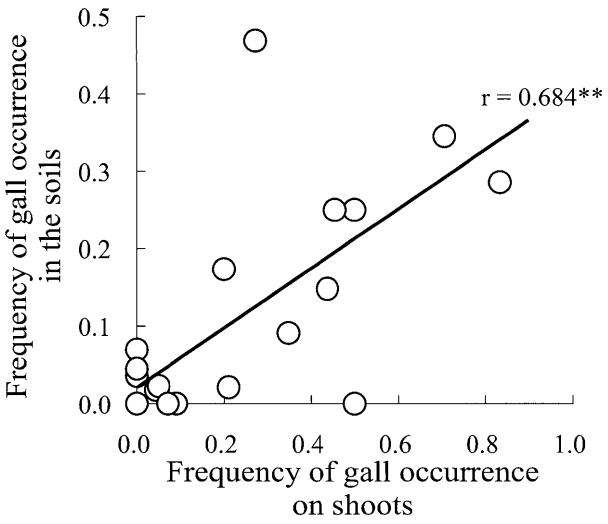

Fig. 2. Relationship of the frequency of gall occurrence at inoculation sites on shoots of grafted trees and on trunks of rooted cuttings below ground among 21 seedlings of apple rootstocks. **: significant at $P < 0.01$ by t-test.

with strain ARAT-001 was much lower than that inoculated with strain Peach CG8331. Other accessions showed similar reactions to both strains. These results suggested that strain ARAT-001 seemed to be slightly more virulent to genus *Malus* than strain Peach CG8331; however, the results also suggested an interaction (specificity) of the frequency of gall occurrence between the *A. tumefaciens* strain and apple rootstock; 'G. 65' exhibited a moderately resistant reaction to strain ARAT-001 but was susceptible to Peach CG8331. Such a strain specificity of *Agrobacterium* resistance (susceptibility) was also reported in grapevine (Szegedi et al., 1984). The presence of a specific interaction for crown gall resistance between *A. tumefaciens* strain and host plants shows that the use of rootstock, resistant to a wide range of strains, will overcome the disease efficiently. In this context, *M. sieboldii* 'Sanashi 63' and 'Mo-15' are indeed valuable resistance sources for the crown gall-resistant breeding of apple rootstocks since they are regarded as the most resistant genotypes to the virulent strains of *A. tumefaciens* used in this study. However, a further inoculation test on the resistance of *M. sieboldii* will be necessary using various virulent strains of *A. tumefaciens* different from those used in this study. Almost nothing has been understood about host plant factors involved in crown gall resistance in *M. sieboldii* 'Sanashi 63' and other accessions. Recently, Suzaki et al. (2004) developed highly specific primer pairs for detecting Ti plasmid in *Agrobacterium* strains isolated from apple saplings. These primer pairs are a powerful tool for detecting *Agrobacterium* strains infecting apple saplings and may offer suggestions for studying the mechanism of crown gall resistance in *Malus* accessions.

Frequency of crown gall occurrence in F_1 progeny

Resistant hybrids with no galls were found in progeny derived from a cross between 'JM7' × 'Sanashi 63'; 19 (16%) and 5 (4%) plants showed no galls after inoculation with strains Peach CG8331 and ARAT-001, respectively (Fig. 3A, B). F_1 plants exhibited various degrees of gall occurrence; nevertheless, most plants seemed to show frequencies similar to either 'JM7' or 'Sanashi 63'. This tendency was more prominent in the results of inoculation with strains Peach CG8331 than ARAT-001. In F_1 progeny between 'JM5' × 'G. 65', no plants with no gall occurrence were observed (Fig. 3C, D). Most plants showed a relatively high frequency of gall occurrence, more than 0.5. The percentage of highly susceptible plants with more than 0.6 frequency of gall occurrence in the F_1 of 'JM5' × 'G. 65' was higher than that of 'JM7' × 'Sanashi 63'.

As was the case in rootstock accessions, there was a specific interaction in the frequency of gall occurrence between the *A. tumefaciens* strain and F_1 plant; i.e., different reactions due to the difference of inoculated strains were observed on many F_1 plants. In the F_1 of 'JM7' × 'Sanashi 63', 43 plants showed resistance or

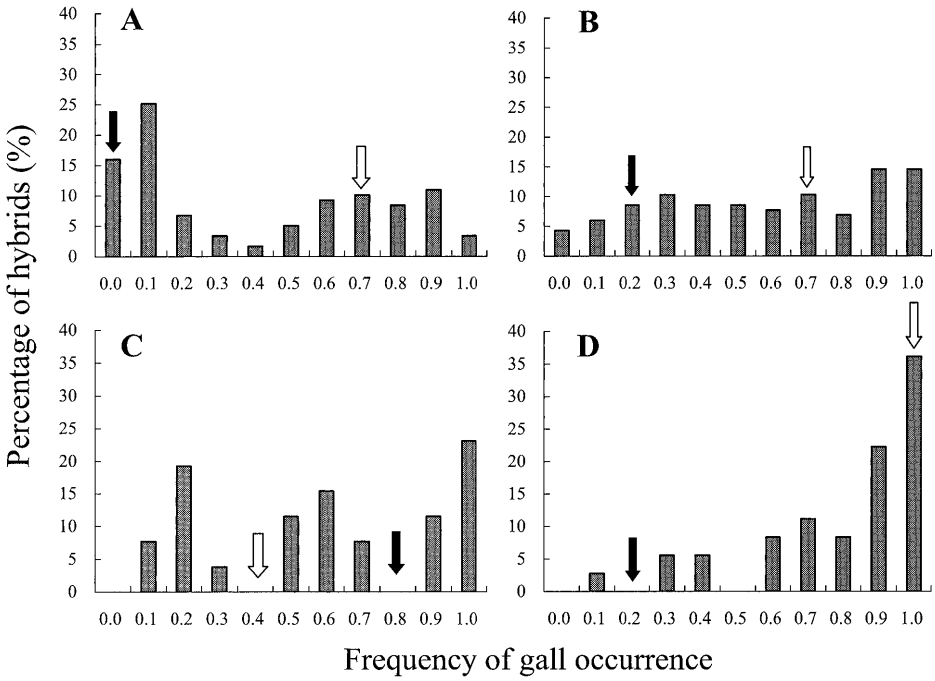

Fig. 3. Distribution of the frequency of gall occurrence in F_1 progeny derived from 'JM7' and *M. sieboldii* 'Sanashi 63' inoculated with (A) *A. tumefaciens* strain Peach CG8331 or (B) strain ARAT-001 and in F_1 progeny derived from 'JM5' and 'G. 65' inoculated with (C) strain Peach CG8331 or (D) strain ARAT-001. The frequency of each individual represents the mean of measurement in 2005 and 2006. Opened and closed arrows indicate the frequency of 'JM7' and *M. sieboldii* 'Sanashi 63' (A and B) and/or 'JM5' and 'G. 65' (C and D), respectively.

moderate resistance to strain Peach CG8331 whereas they were susceptible to ARAT-001. In the F_1 of 'JM5' × 'G. 65', eight plants were moderately resistant to strain Peach CG8331 while susceptible to ARAT-001. Although such a specific interaction was obvious in not a few of the hybrids, two plants were resistant to both strains in the F_1 of 'JM7' × 'Sanashi 63'; they could be valuable candidates as new apple rootstocks resistant to crown gall.

In the progeny of 'JM7' × 'Sanashi 63', we found that many of the plants formed no crown gall after inoculation with virulent strains of *A. tumefaciens*, while all plants formed galls at various frequencies in the progeny of 'JM5' × 'G. 65'. This result clearly indicates that crown gall resistance in *M. sieboldii* 'Sanashi 63' is heritable by its descendants. In the 2-year successive inoculation tests with Peach CG8331, the frequency distribution of F_1 plants of 'JM7' (susceptible) × 'Sanashi 63' (resistant) was bimodal; F_1 plants could be classified into two categories, resistant (R) or moderately resistant (MR) and susceptible (S). When resistance levels of plants were categorized as above, the number of (R + MR) and S plants were 58 and 61, respectively (Fig. 3A). The (R + MR): S segregation in the progeny fitted to a 1 : 1 ratio ($\chi^2 = 0.075$, $P = 0.783$). Frequency distribution of F_1 plants of 'JM5' (S) × 'G. 65' (S) were somewhat complicated; frequencies in many plants of this progeny deviated from a range between those of parents (Fig. 3C). Many F_1 plants were regarded as S, but eight F_1 plants (29.6% of the progeny) could be categorized as MR. The result (Fig. 3A, C) suggested that a possible major gene could control the resistance found in *M. sieboldii* 'Sanashi 63' against the strain Peach CG8331, but additional minor genes were also responsible for the resistance. On the other hand, the frequency distribution of F_1 plants of 'JM7' (S) × 'Sanashi 63' (MR) to strain ARAT-001 for gall occurrence exhibited rather continuous distribution and 28 plants belonged to the MR class (Fig. 3B). Only a few F_1 plants derived from a cross of 'JM5' (S) × 'G. 65' (MR) were categorized as MR and the others as S (Fig. 3D). The frequency distribution might be skewed because of the relatively small number of progeny (27 hybrids). The result (Fig. 3B, D) suggested that minor genes conditioned an MR reaction in 'Sanashi 63' against ARAT-001 strain. Studies on the inheritance of crown gall resistance in *Vitis* using F_1 and self-pollinated hybrid plants showed a Mendelian dominant inheritance of resistance in *V. amurensis* to *A. tumefaciens* biovar 3 (= *A. vitis*) (Szegedi and Kozma, 1984). To demonstrate the possible involvement of a major resistance gene to crown gall in *M. sieboldii* and to reveal whether the resistant trait is dominant, more extensive inoculation tests will be necessary using several families originating from crosses among several resistant genotypes of *M. sieboldii* and highly susceptible apple rootstocks.

Although the genetic basis of resistance to crown gall in apple rootstocks is not entirely clear, we believe that there is sufficient genetic variability for crown gall resistance in apple rootstocks to breed for an increased

degree of resistance to the disease. Because moderately resistant hybrids were obtained even in the F_1 of 'JM5' × 'G. 65' crosses despite the fact that the frequency of hybrids was considerably low in this progeny. Especially impressive is *M. sieboldii* 'Sanashi 63' as a useful breeding material to develop crown gall-resistant rootstocks. *M. sieboldii* 'Sanashi 63' and a significant number of its offspring showed resistance or moderate resistance to *A. tumefaciens* strains, suggesting that a combination of *M. sieboldii* 'Sanashi 63' with dwarfing rootstocks, such as 'JM7' or 'JM1', may produce new apple rootstock candidates with crown gall resistance, high propagation ability by hardwood cuttings, and high productivity. A major disadvantage of *M. sieboldii*, however, as an apple rootstock is its vigorous effect on scion growth. Since the current fashion in much of the world is to grow apple trees on dwarfing rootstocks (Webster and Wertheim, 2003), to produce dwarfing rootstocks with improved horticultural traits and resistance (tolerance) against disease, pests, and unfavorable environmental conditions is very important in apple rootstock breeding programs worldwide, as is the case in the breeding program at NIFTS in Japan. With this background, more knowledge on the inheritance of dwarfing ability will be needed as well as other important traits to aid the future breeding of apple rootstocks.

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