Evaluation of the Nonpathogenic *Agrobacterium vitis* Strain ARK-1 for Crown Gall Control in Diverse Plant Species

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Abstract

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The nonpathogenic strain of *Agrobacterium* (= *Rhizobium*) *vitis* ARK-1 is a biological agent able to effectively control grapevine crown gall. In this study, treating apple, Japanese pear, peach, rose, and tomato by soaking the roots in a cell suspension of ARK-1 before planting into soil infected with tumorigenic *Agrobacterium* spp. reduced the number of plants developing crown gall tumors. Meta-analyses of the results from six field trials of apple, four field trials of Japanese pear, and four field trials of peach, from 2010 to 2013, showed integrated risk ratio (IRR) after treatment with ARK-1 to be 0.38 for apple crown gall, 0.16

for Japanese pear crown gall, and 0.20 for peach crown gall, indicating that the disease incidence was significantly reduced by ARK-1 treatment. Meta-analyses of the results from three greenhouse trials of rose and three greenhouse trials of tomato showed IRR after treatment with ARK-1 to be 0.29 for rose crown gall and 0.16 for tomato crown gall, indicating that the disease incidence was significantly reduced by ARK-1 treatment. These results indicated that control by ARK-1 covers five different species of host plants and tumorigenic (Ti) strains of *Agrobacterium* species.

Crown gall disease is one of the most economically important soilborne bacterial plant diseases in the world (14). Crown gall is identified by overgrowths appearing as galls on roots and at the base or “crown” of woody plants such as pome fruit (e.g., apple, pear), stone fruit (e.g., cherry, apricot), and nut (e.g., almond, walnut) trees (14). Crown galls are also formed on ornamental woody crops such as roses, Marguerite daisies, and *Chrysanthemum* spp. as well as on vines and canes such as grapevines and raspberries (14). Occasionally, galls are observed on field crops such as cotton, sugar beets, tomatoes, beans, and alfalfa, but the disease does not have an economic impact on such crops (14). In Japan today, crown gall diseases occur not only on grapevines but also in many commercial plantings of apple, peach, Japanese pear, and rose. Crown gall of apple in particular is a most important disease affecting the production of nursery stock and causing substantial economic losses in Japan (24). Further, tomato crown gall was recognized in the United Kingdom in 2005 (40), indicating the possibility that tomato crown gall will be found in other countries.

Crown gall is mainly caused by *Agrobacterium tumefaciens* (Ti; where “Ti” means “tumorigenic”) [= *Rhizobium radiobacter* (Ti), *A. tumefaciens* biovar 1] and *A. rhizogenes* (Ti) [= *R. rhizogenes* (Ti), *A. tumefaciens* biovar 2], which are gram-negative, bacilli-form bacteria that are normally associated with the roots of many different plants in the field (14). On the other hand, grapevine crown gall is caused by *A. vitis* (Ti) [= *Rhizobium vitis* (Ti), *A. tumefaciens* biovar 3] (3,5,38). Experimental inoculations indicate that plants representing over 93 families are susceptible to crown gall (14). The pathogenicity genes are mostly located on large tumor-inducing plasmids (pTi), and during infection, a part of the plasmid (T-DNA) is inserted into the nuclear DNA of the plant (8).

The nonpathogenic *A. rhizogenes* strain K84 has been used successfully to control crown gall in many plant species (26,31,35). An agrocin produced by *A. rhizogenes* K84 (agrocin 84) is thought to be the primary factor in the control (26). However, K84 does not control crown gall of grapevine caused by *A. vitis* (Ti) because *A. vitis* (Ti) is insensitive to agrocin 84 (4,19,20,22,23). However, its continued success is threatened by the ability of pAgK84 to transfer conjugatively to Ti strains, which then become insensitive to agrocin 84 (36). Recombinant DNA techniques have been used to construct a new biological control strain, K1026, that is identical to K84 except for a 5.9-kb deletion overlapping the *Tra* region of pAgK84 (13). K1026 is unable to transfer its mutant agrocin 84 plasmid, designated pAgK1026, to other agrobacteria, specifically conjugal transfer of pAgK84 (13,34). However, it remains inhibitory to strains sensitive to agrocin 84 (13,34).

Previously, we reported that a nonpathogenic *A. vitis* strain, VAR03-1, isolated from grapevine nursery stock in Japan produced a bacteriocin and greatly inhibited the formation of tumors in grapevine, rose, tomato, sunflower, and apple caused by several strains of *A. radiobacter* (Ti), *A. rhizogenes* (Ti), and *A. vitis* (Ti) isolated from different plants and locations in Japan (16,20,24). VAR03-1 is genetically dissimilar from tumorigenic or nonantagonistic *A. vitis* strains in the repetitive sequence-based (rep)-PCR DNA fingerprint patterns and sequences of three different housekeeping genes (17,25).

Recently, a new antagonistic strain, the nonpathogenic *A. vitis* strain ARK-1, was identified (19). ARK-1 did not reduce tumor incidence on the stems of grapevines when ARK-1 was dead or only the culture filtrate was used, indicating that ARK-1 inhibits grapevine crown gall by a different mechanism than VAR03-1 (19). ARK-1 is endophytic in grapevine, and grapevine crown gall was controlled better with ARK-1 treatment than with VAR03-1 in field trials (18).

The ultimate goal in conducting this study is to evaluate strain ARK-1 as biopesticide. However, there is no evidence that treatment with strain ARK-1 is effective in controlling crown gall in plant species other than grapevine in the field. In this article, we report that ARK-1 reduced the frequency of crown gall of apple, Japanese pear, peach, rose, and tomato caused by the tumorigenic strains of *A. tumefaciens* (Ti) and *A. rhizogenes* (Ti). In this paper, we follow the nomenclature for *Agrobacterium* species adopted by Young et al. (41) to avoid confusion, although other equally valid naming systems have been proposed (1,2,28,33,37,42).

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures are available online.

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Materials and Methods

Biological control in field trials. The conditions of each field trial are shown in Table 1. Thirteen trials designed as randomized or systematic controlled trials of biological control of crown gall were carried out in three different experimental fields at the Okayama Prefectural Technology Center in Akaiwa City, Okayama, Japan: field A (trials 2010-A-a, 2011-A-a, 2012-A-a, 2013-A-a, 2011-A-p, 2012-A-p, 2013-A-p, 2011-A-j, 2012-A-j, and 2013-A-j), field B (trial 2010-B-a and 2011-B-p), and field C (trial 2010-C-a and 2012-C-j). The sizes of experimental fields A, B, and C were 625.0 m² (25.0 × 25.0 m), 30.0 m² (10.0 × 3.0 m), and 54.0 m² (18.0 × 3.0 m), respectively. Two-year-old nursery stock of apple (scion: *Malus pumila* cv. Fuji; rootstock: *M. prunifolia* cv. Marubakaido) grown from cuttings, 2-year-old nursery stock of Japanese pear (scion: *Pyrus pyrifolia* var. *culta* cv. Housui; rootstock: *P. betulaefolia* cv. Hokushi-mamenashi) grown from cuttings, and 2-year-old nursery stock of peach (scion: *Amygdalus persica* cv. Hakuho rootstock: *A. persica* cv. Ohatsu) grown from cuttings were prepared. One month before the start of each trial, a commercial organic fertilizer [Temporon, containing N (0.77%), P (0.09%), K (0.08%), ligno-cellulose, humic acid, Ca, Mg, Mn, and B; Mitsubishi-Shoji, Tokyo, Japan] was applied at a rate of 4.0 kg/m² and thoroughly incorporated into the soil of the fields. Eight strains of *A. tumefaciens* (Ti) and *A. rhizogenes* (Ti) isolated in Japan were selected as the pathogens (Table 2). Cell suspensions (mixtures of tumorigenic strains) were prepared from 48-h liquid cultures grown on potato semi-synthetic (PS) medium (18). Two weeks before the start of each trial, 20 liter/m² of each cell suspension (about 1 × 10⁸ cells/ml; strains ARAT001, ARAT002, NEAR8, and NEAR11 in apple crown gall trials; P-Ag-1 and P-Ag-6 in Japanese pear crown gall trials; and Pch-Ag-2 and PI-Ag-1 in peach crown gall trials) was poured onto the soil, and then the soil was broken up to a depth of 16 cm with a Punch-X F402-J cultivator (Honda Motor Co., Tokyo, Japan) to disperse the inoculum through the soil.

Cell suspensions of strain ARK-1 were prepared from 48-h slant cultures grown on PS agar (PSA) medium (PS medium with 15 g agar) and adjusted to OD₆₀₀ = 0.1 (corresponding to about 10⁸

cells/ml). Roots of plants were pruned to half and soaked for 1 h in a cell suspension of strain ARK-1 or water, and then 13 to 42 plants per treatment were planted in each plot. The arrangement of each plot was random or systematic within each field. The ground of each plot was covered by a sheet of non-woven fabric to suppress weeds after planting. Tumor formation on roots and stems of plants was investigated after 6 to 8 months. The rainy season in Okayama, Japan, is from June to July. The temperature ranged from 10 to 37°C during the study period, and no severe damage by weather or insects was observed.

The disease incidences in the six field trials of apple, three trials of Japanese pear, and four trials of peach were subjected to meta-analysis according to a random effect model by the DerSimonian-Laird method (9,10) because the field trials were performed with different plot sizes, numbers of plots, plants, farms, and years. Meta-analysis is a set of statistical procedures for synthesizing research results from a number of different studies (9,10) and is also useful for integrating the results of several biological control trials in plant pathology (18,21,32). The DerSimonian-Laird method can incorporate variations among studies (9,10). An estimate of a statistical effect, such as the difference in disease severity in plants with or without treatment, is collected from each study along with a measure of the variance of the estimate of the effect. The size of the effect of ARK-1 treatment was calculated as an integrated risk ratio (IRR). Risk ratio (RR) is the ratio of the probability of developing a disease in the exposed group to that in an unexposed group (39), and is defined in this study as RR = (proportion of plants developing tumors with the antagonist treatment) / (proportion of plants developing tumors with water treatment). In evaluating the effect of a treatment, a low RR shows a high effectiveness.

Biological control in greenhouse experiments. Assessment of biological control was carried out using tomato seedlings and rose nursery stock based on methods reported previously (14,16). We used 1- to 2-month-old seedlings of tomato (*Solanum esculentum* cv. Ponderosa) and used rose nursery stock grown from cuttings for 1 year (scion: *Rosa hybrida* cv. Rote Rose, rootstock: *R. multiflora* cv. Multiflora Rose).

We prepared cell suspensions of the nonpathogenic strain ARK-1 from 48-h slant cultures grown on PSA medium and adjusted to

Table 1. The conditions of 14 field trials

Trial	Plants	Cultivar	Plot size (m)	Plot arrangement	Total no. of plants/treatment	No. of plots/treatment	No. of plants/plot	No. of rows/plot	The date of planted/investigated
2010-A-a	Apple	Fuji	7.0 × 1.0	Systematic	33	3	11	1 row spaced 50 cm between plants	14-Apr/13-Dec
2010-B-a	Apple	Fuji	5.0 × 1.0	Systematic	21	3	7	1 row spaced 50 cm between plants	14-Apr/15-Oct
2010-C-a	Apple	Fuji	9.0 × 1.0	Randomized	42	3	14	1 row spaced 50 cm between plants	15-Mar/15-Oct
2011-A-a	Apple	Fuji	8.0 × 1.0	Randomized	39	3	13	1 row spaced 50 cm between plants	14-Mar/4-Oct
2012-A-a	Apple	Fuji	6.0 × 1.0	Randomized	28 to 29	3	9 to 10	1 row spaced 50 cm between plants	6-Apr/6-Nov
2013-A-a	Apple	Fuji	7.0 × 1.0	Randomized	32 to 33	3	10 to 11	1 row spaced 50 cm between plants	6-Apr/3-Dec
2011-A-p	Peach	Hakuho	9.0 × 1.0	Randomized	39	3	13	1 row spaced 60 cm between plants	14-Mar/4-Oct
2011-B-p	Peach	Hakuho	5.0 × 1.0	Systematic	13	2	6 to 7	1 row spaced 60 cm between plants	1-Mar/4-Oct
2012-A-p	Peach	Hakuho	9.0 × 1.0	Randomized	38	3	12 to 13	1 row spaced 60 cm between plants	6-Apr/6-Nov
2013-A-p	Peach	Hakuho	8.0 × 1.0	Randomized	32 to 33	3	10 to 11	1 row spaced 60 cm between plants	6-Apr/3-Dec
2011-A-j	Japanese pear	Housui	9.0 × 1.0	Randomized	39	3	13	1 row spaced 60 cm between plants	14-Mar/4-Oct
2012-A-j	Japanese pear	Housui	8.0 × 1.0	Randomized	30 to 32	3	10 to 11	1 row spaced 60 cm between plants	6-Apr/6-Nov
2012-C-j	Japanese pear	Housui	9.0 × 1.0	Randomized	38 to 39	3	12 to 13	1 row spaced 60 cm between plants	6-Apr/6-Nov
2013-A-j	Japanese pear	Housui	8.0 × 1.0	Randomized	31 to 32	3	12 to 13	1 row spaced 60 cm between plants	6-Apr/3-Dec

OD₆₀₀ = 0.1 (corresponding to about 1×10^8 cells/ml) and cell suspensions of four tumorigenic strains (ARAT001, ARAT002, NEAR8, and NEAR11) (Table 1) adjusted to about 10^8 cells/ml (OD₆₀₀ = 0.1). The soil was inoculated with each pathogen by pouring 10^8 cells/ml cell suspensions onto the soil (500 ml/kg soil) to obtain a final concentration of about 5×10^7 cells/g soil. Each young plant was pulled from the soil and the roots rinsed in water until clean. Roots were then pruned and soaked for 1 h in a cell suspension of strain ARK-1 or sterile distilled water (as a control) at 25°C. After soaking, 40 tomato seedlings per treatment were planted 8 cm deep in pots (10 cm depth $\times$ 12 cm diameter, one plant per pot) filled with the infested soil; similarly, 15 rose nursery stocks per treatment were planted 10 cm deep in pots (12 cm depth $\times$ 15 cm diameter, one plant per pot) filled with the infested soil. Plants were then grown in a temperature-controlled greenhouse at 25 to 28°C, and tumor formation on roots and stems was assessed after 3 months for tomatoes and 5 months for roses. The tomato and rose experiments were each repeated three times (experiments 1, 2, and 3). The severity of crown gall disease was assessed as described above for the field experiments.

The disease incidences in the greenhouse trials of rose and tomato were subjected to meta-analysis according to a fixed effect model by the Mantel-Haenszel method (12,30) because the greenhouse trials were performed with the same numbers of plants, greenhouses, and years. With this method, an estimate of a statistical effect, such as the difference in disease severity for plants with or without treatment, is collected from each experiment along with a measure of the variance of the estimate of the effect. The size of the effect of ARK-1 treatment was calculated as an IRR.

Assessment of ARK-1 survival on roots of apple, Japanese pear, peach, rose, and tomato. To assess the survival of the biological control agent after inoculation onto the roots of apple, Japanese pear, peach, rose, and tomato, we employed the antibiotic-resistant mutant ARK-1sc in order to differentiate the inoculated agents from indigenous agrobacteria. ARK-1sc was a streptomycin (St)-copper sulfate (CuSO₄)-resistant mutant (St-CuSO₄-mutant) obtained by growing strain ARK-1 on St-CuSO₄-PSA medium (amended with 500 ppm St and 250 ppm CuSO₄) (18). We checked that the growth and biological effectiveness of ARK-1sc were as same as those of wild type (Supplementary Figures S1 and S2). We then determined the survival rate of the nonpathogenic strain ARK-1sc in the roots of apple, peach, Japanese pear, rose, and tomato.

A cell suspension of ARK-1sc containing 1×10^8 cells/ml was prepared. Roots of the apple, Japanese pear, and peach nursery stock were pruned to half and then soaked for 1 h in the cell suspension of strain ARK-1sc; the fruit trees were then planted in concrete-framed plots (1.0 $\times$ 1.0 m/plot, 1.0 m tall, in the field) filled with soil (pH = 5.8, NO₃-N = 8.2 mg/100 g soil, P₂O₅ = 122 mg/100 g soil, K₂O = 41 mg/100 g soil, CaO = 222 mg/100 g soil, cation exchange capacity = 23.4 meq/100 g soil, organic matter content = 1.5%) on 14 April 2011 (five plants of one species per concrete-framed plot). These experiments were carried out in the

field. Roots of each of the five rose plants were pruned to half and soaked for 1 h in the cell suspension; roses were then planted in pots (12 cm depth $\times$ 15 cm diameter, one plant per pot) with soil 10 cm deep. Roots of tomato seedlings were pruned to half and soaked for 1 h in the cell suspension; tomato seedlings were then planted in pots (10 cm depth $\times$ 12 cm diameter, one plant per pot) with soil 8 cm deep. Rose and tomato plants were then grown in a temperature-controlled greenhouse at 25 to 28°C.

To determine the populations of ARK-1sc, root samples (1 g of fresh weight per plant) were collected from five plants of each plant species. Root samples from the apple, Japanese pear, and peach were collected 10 months after planting. Root samples from the roses were collected 5 months after planting, and root samples from the tomato plants were collected 3 months after planting. To remove adhering soil, each piece was scrubbed by hand and rinsed under tap water for 10 s and dried with paper towels. To thoroughly wash the surface of the roots, each sample together with 1 ml of sterile distilled water was incubated at 20°C with shaking (approximately 150 rpm) for 1 h. In order to isolate ARK-1sc from roots, the root sample collected after incubation was mashed with an autoclaved mortar and pestle in 1 ml of sterile distilled water, and then serial dilutions of the samples were plated on St-CuSO₄-PSA supplemented with 500 ppm tebuconazole (Bayer Crop-science, Tokyo, Japan) to avoid the influence of contamination by fungi on the roots. The plates were incubated at 27°C for 5 days. The observations were based on 10 plates of each dilution, and the numbers of colony forming units (CFU) of strain ARK-1sc were counted on each root sample. The bacterial population on the root was transformed as a logarithm (base 10) of CFUs per gram of root for analysis. To check the soils for natural populations of bacteria capable of growing on St-CuSO₄-PSA agar plate, soil samples (10 g of fresh weight per concrete-framed plot or pot) used in each experiment were collected and serial dilutions of the samples were plated on St-CuSO₄-PSA with tebuconazole as described above, and any suspicious bacterial colonies of strain ARK-1 were not grown (data not shown). The survival experiments were each repeated twice.

Data analysis. Meta-analyses were performed using EZR (15), which is a graphical user interface for R (The R Foundation for Statistic Computing, version 2.14.0).

Results

Biological control in field experiments. Meta-analysis of the six field trials performed from 2010 to 2013 regarding the biological control effect of strain ARK-1 on apple crown gall (Fig. 1A) showed that IRR was 0.38 [95% confidence interval (CI): 0.23–0.64, $P = 0.0003$], indicating that the disease incidence was significantly reduced by ARK-1. Meta-analysis of the four field trials performed from 2011 to 2013 regarding the biological control effect of strain ARK-1 on Japanese pear crown gall (Fig. 1B) showed that IRR was 0.24 (95% CI: 0.12–0.47, $P < 0.0001$), indicating that the disease incidence was significantly reduced by

Table 2. Bacterial strains used in this study

Bacterial strain	Pathogenicity ^a	Opine type	Description (supplier)
<i>Agrobacterium tumefaciens</i> (Ti) (= <i>Rhizobium radiobacter</i> (Ti), <i>A. tumefaciens</i> biovar 1)			
ARAT001	Ti	Nopaline	Isolated by K. Suzaki from galled apple trees in Japan (K. Suzaki) (24)
<i>A. rhizogenes</i> (Ti) (= <i>R. rhizogenes</i> (Ti), <i>A. tumefaciens</i> biovar 2)			
ARAT002	Ti	Nopaline	Isolated by K. Suzaki from galled apple trees in Japan (K. Suzaki) (24)
NEAR8	Ti	Unknown	Isolated by K. Kondo from galled apple trees in Japan (K. Kondo)
NEAR11	Ti	Unknown	Isolated by K. Kondo from galled apple trees in Japan (K. Kondo)
Pch-Ag-2	Ti	Nopaline	Isolated by H. Sawada from galled peach trees in Japan (H. Sawada)
PI-Ag-1	Ti	Unknown	Isolated by H. Sawada from galled Japanese plum trees in Japan (H. Sawada)
P-Ag-1	Ti	Nopaline	Isolated by H. Sawada from galled Japanese pear trees in Japan (H. Sawada)
P-Ag-6	Ti	Unknown	Isolated by H. Sawada from galled Japanese pear trees in Japan (H. Sawada)
Nonpathogenic <i>A. vitis</i> (=Nonpathogenic <i>R. vitis</i> , <i>A. radiobacter</i> biovar 3)			
ARK-1	N		Isolated by A. Kawaguchi from nursery stock of grapevine in Japan; biological control agent for crown gall (19)

^a Ti: Tumorigenic. N: Nonpathogenic.

ARK-1. Meta-analysis of the four field trials performed from 2011 to 2013 regarding the biological control effect of strain ARK-1 on peach crown gall (Fig. 1C) showed that IRR was 0.20 (95% CI: 0.09–0.41, $P < 0.0001$), indicating that the disease incidence was significantly reduced by ARK-1. The heterogeneity between studies was tested for each meta-analysis. The I^2 value and P value were 39.6% and 0.1414 for the six field trials of apple crown gall, 1.8% and 0.3831 for the three field trials of Japanese pear crown gall, and 0% and 0.4051 for the four field trials of peach crown gall, indicating the absence of heterogeneity between the field trials.

Biological control in greenhouse experiments. Meta-analysis of the three independent greenhouse experiments regarding the biological control effect of strain ARK-1 on rose crown gall (Fig. 2A) showed that the IRR was 0.29 (95% CI: 0.12–0.73, $P = 0.0082$), indicating that the disease incidence was significantly reduced by ARK-1. Meta-analysis of the three independent greenhouse experiments regarding the biological control effect of strain ARK-1 on tomato crown gall (Fig. 2B) showed that the IRR was 0.16 (95% CI: 0.10–0.27, $P < 0.0001$), indicating that the disease incidence was significantly reduced by ARK-1. The heterogeneity between studies was tested in each meta-analysis. The I^2 value and P value were 0% and 0.5435 for the three rose crown gall experiments and 4.4% and 0.3514 for the three tomato crown gall experiments, indicating the absence of heterogeneity between the greenhouse experiments.

Survival of ARK-1 on roots of apple, Japanese pear, peach, rose, and tomato. The bacterial population on the roots of fruit tree nursery stock 10 months after inoculation with strain ARK-1sc was 1.0×10^4 CFU/g (fresh weight) for apple, 4.2×10^3 CFU/g (fresh weight) for Japanese pear, and 5.5×10^3 CFU/g (fresh weight) for peach (Fig. 3). The bacterial population on rose roots

was 1.5×10^4 CFU/g (fresh weight) 6 months after inoculation, and 4.8×10^5 CFU/g (fresh weight) on tomato roots 3 months after inoculation (Fig. 3).

Discussion

The nonpathogenic *A. vitis* strain F2/5 has been studied as a biological control agent of grapevine crown gall and is effective against *A. vitis* (Ti) strains (6). However, the biological efficacy of F2/5 is specific to grapevine, and F2/5 is not effective on other host plants such as tomato, sunflower, and devil's backbone (*Kalanchoe daigremontiana*) (8). In contrast, the nonpathogenic *A. vitis* strain VAR03-1 is effective not only on grapevine, but also on tomato, rose, and apple, as we have already reported (16,20,24). In this study, ARK-1 seemed to protect a wider variety of plant species than did F2/5 and VAR03-1: ARK-1 was found to be effective not only on grapevine but also on tomato, rose, apple, Japanese pear, and peach. Moreover, ARK-1 reduced the number of tumors caused by several Ti strains of *A. vitis* (19), *A. tumefaciens*, and *A. rhizogenes* when the plant roots were soaked in cell suspensions of ARK-1 before planting in soil infected with the pathogens, indicating that ARK-1 protects against a number of Ti strains of several *Agrobacterium* species. The nonpathogenic *A. rhizogenes* strain K84 was shown to be effective in controlling crown gall on peach, almond, raspberry, cherry, plum, rose, apple, euonymus, boysen-

Fig. 1. Integrated evaluations based on meta-analyses of effects of the nonpathogenic *Agrobacterium vitis* strain ARK-1 on crown gall of nursery stock of (A) apple in six field trials, (B) Japanese pear in four field trials, and (C) peach in four field trials. The center of each lozenge marks the value of the integrated risk ratio. Each gray square marks the value of the risk ratio, and the size of the square indicates the weight of each field trial. The spread (horizontal line) indicates the 95% confidence interval.

Fig. 2. Integrated evaluations based on meta-analyses of effects of the nonpathogenic *Agrobacterium vitis* strain ARK-1 on crown gall of (A) rose nursery stock in three greenhouse experiments and (B) tomato seedlings in three greenhouse experiments. The center of each lozenge marks the value of the integrated risk ratio. Each gray square marks the value of the risk ratio, and the size of the square indicates the weight of each field trial. The spread (horizontal line) indicates the 95% confidence interval.

Fig. 3. Survival of strain ARK-1 on roots of apple, Japanese pear, peach, and rose rootstocks, and tomato seedlings after inoculation. Columns show means ($n=5$); error bars show standard deviation.

berry, tomato, and chrysanthemum in greenhouse and field tests (11,27,29). However, K84 does not control crown gall on grapevine (4,19,20,22,23), and there are no reports that K84 or any other bacterial strain could control crown gall of pear including Japanese pear. Thus, ARK-1 is the first biological control method reported to succeed in controlling crown gall of Japanese pear. We will conduct further field experiments to investigate which other species of plants ARK-1 can colonize to control crown gall.

The IRR values 0.38, 0.24, and 0.20 indicate that the incidence of apple, Japanese pear, and peach crown gall disease, respectively, during treatment with ARK-1 was decreased to 38, 24, and 20% of the incidence without ARK-1 and that the control effect in each is high in the field. Thus, the IRR values indicate that ARK-1 is useful against this soilborne disease in the field. There are no reports of any biological control agent that is better at inhibiting tumor formation on these fruit trees in the field than ARK-1. Moreover, the IRR values 0.29 and 0.16 indicate that the incidence of rose and tomato crown gall disease, respectively, during treatment with ARK-1 was decreased to 29 and 16% of the incidence without ARK-1 and that the control effect in each is also high. Although roses are usually grown in commercial greenhouses currently in Japan, they are now also grown in the field, and we will have to investigate biological control of rose crown gall in field trials in the near future.

Meta-analyses of the 14 field trials showed strong evidence that ARK-1 was effective in controlling apple, Japanese pear, and peach crown gall, indicating that soaking for 1 h in a cell suspension of 1×10^8 cells/ml of ARK-1 is suitable for practical use in the field. Although the three meta-analyses were performed on different numbers of field trials and not performed at the same time in the same field, the results of the field trials comparing the effectiveness of ARK-1 in three different types of fruit tree indicated that treatment with ARK-1 was most effective in controlling Japanese pear crown gall. In the meta-analysis of the seven field trials investigating the biological control effect of ARK-1 on grapevine crown gall (18), the IRR was 0.15. These results indicate that—in order of usefulness—ARK-1 is very useful for controlling crown gall on grapevine and Japanese pear; useful for controlling peach crown gall; and somewhat less useful for controlling apple crown gall. The IRR value of 0.38 indicates that ARK-1 is effective for controlling apple crown gall, but the 95% CI was very wide (0.23 to 0.64), indicating that the control effect of ARK-1 treatment for apple crown gall may be uneven in the field. Thus, we must improve the method of ARK-1 treatment to more effectively control apple crown gall. A higher density cell suspension than that used in this study and a longer soaking time than 1 h might improve the control effect for apple crown gall. We have reported previously that the nonpathogenic *A. rhizogenes* strain K84 and the nonpathogenic *A. vitis* strain VAR03-1 could control apple crown gall (24). In the meta-analysis of the three field trials investigating the biological control effect of K84 and VAR03-1 (soaking for 1 h in a 5×10^8 cells/ml of each cell suspension) on apple crown gall (24), the IRR of K84 was 0.36 (95% CI: 0.24 to 0.55) and that of VAR03-1 was 0.16 (95% CI: 0.09 to 0.30). Although these assessments of biological control effect were not performed at the same time and condition of field trial, these results indicate that the ability of ARK-1 to control apple crown gall is similar to that of K84. Although IRR value of VAR03-1 is superior to that of ARK-1, applied cell suspension of VAR03-1 (5×10^8 cells/ml) is different from that of ARK-1 (1×10^8 cells/ml), indicating that the comparisons should be done in next field trial.

ARK-1 colonizes roots of grapevine and persists on the roots for 2 years or more (18). The assessment of ARK-1sc survival on the roots of apple, Japanese pear, peach, and rose nursery stock and on the roots of tomato seedlings indicated that the ARK-1 strain could not only establish populations in the rhizosphere of these types of nursery stock and seedlings but could persist on roots for up to 10 months on apple, Japanese pear, and peach rootstock, 6 months on rose rootstock, and 3 months on tomato seedlings. These results indicate that ARK-1 would have been able to colonize and persist

on the roots of apple, Japanese pear, peach, and rose rootstock and tomato seedlings in this study until the plants were dug up to investigate tumor formation. We have reported previously that colonization of K84 and VAR03-1 remained at about 7.0×10^3 CFU/g of root (fresh weight) and 8.0×10^2 CFU/g of root (fresh weight) for up to about 11 months, respectively (24). In this study, colonization of ARK-1 remained at about 1.0×10^4 CFU/g of root (fresh weight) for up to 10 months. Although these assessments of survival were not performed at the same time, these results indicate that the ability of ARK-1 to colonize and persist on roots of rootstock for apple trees is similar to that of K84 and superior to that of VAR03-1. The ability to colonize roots might affect the persistence of the control of crown gall. Thus, the controlling effect of ARK-1 might continue for no more than a year. The persistence of the control of crown gall in apple, Japanese pear, peach, rose, and tomato by ARK-1 must be investigated in further detail. Furthermore, new treatment methods should be developed to continue the effect of controlling crown gall in these five plant species after the nursery stock or seedlings have been soaked in the cell suspension of strain ARK-1 and planted. In future studies, we plan to pour the cell suspension of ARK-1 onto the soil around the base of the plants to control crown gall every year after planting.

In conclusion, this is the first study to report that a nonpathogenic *A. vitis* strain, ARK-1, can effectively control crown gall in five different plant species in greenhouse and field experiments. ARK-1 seemed to protect a wider variety of plant species than other antagonistic strains reported previously (7,20,23,24). To develop a new bactericide with ARK-1, it is necessary to investigate the applicability of ARK-1 to other kinds of plant species in the field.

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