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The pathogen of crown gall disease on flowering cherry and its sensitivity to strain K1026

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Abstract

Six strains of crown gall bacteria were isolated from flowering cherry. It was revealed by Otten paper electrophoresis that of the six strains, only BYH18-4 possessed the octopine type Ti plasmid, the remainder having nopaline type Ti plasmid. BYH5-1 was identified by physiological and biochemical tests to be *Agrobacterium tumefaciens* (originally biovar 1). The other five were *A. rhizogenes* (originally biovar 2). It was demonstrated with Stonier's method of double layer medium that flowering cherry crown gall bacteria exhibited different sensitivities to agrocin produced by biocontrol strain K1026. Strain K1026 on greenhouse-grown sunflower seedlings exerted a relatively potent inhibitory action on flowering cherry crown gall bacteria. Artificial inoculation showed that K1026 produced 67–99% inhibition of flowering cherry crown gall disease, compared with the treatment of inoculation with crown gall bacteria only.

Introduction

The host range of crown gall bacteria is wide, including over 600 species of trees with juicy fruits, stone fruits, kernel fruits, hard fruits, forests and flowers. In Mainland China, crown gall diseases of the grapevine (Ma et al., 1985; Wang et al., 1991), peach tree (Wang et al., 1996), cherry (Wang et al., 1998a), ni-plum (Li et al., 1996), hop (Ren et al., 1986), poplar (Zhang et al., 1998) and rose (Wang et al., 1998b) have been investigated. Crown gall disease also occurs on flowering cherry, sugar beet, pear, apple, haw, flowering crabapple, and walnut (Wang et al., 1991). The disease is spread when diseased plants are transplanted.

Since the 1970s research on prevention of crown gall disease has focused on its biological control. There is a complex system of interactions among plants, crown gall bacteria and biocontrol bacteria. Crown gall bacteria include *Agrobacterium tumefaciens* (originally *A. tumefaciens* biovar 1), *A. rhizogenes* (originally *A. tumefaciens* biovar 2), *A. vitis* (originally *A. tumefaciens* biovar 3) and *A. rubi*. Their Ti plasmid can be of the nopaline, octopine or agropine type, etc. according to the kind of opines that the genes on the Ti plasmids produce. Crown gall bacteria on the same host can belong to different species and plasmid types. Different species and plasmid types of crown gall bacteria have differences in host range. Different varieties of the same host may differ regarding resistance to crown gall disease. Crown gall bacteria and the reported biocontrol bacteria for crown gall disease belong to genus *Agrobacterium*, but originate from different hosts, and differ with regard to species and plasmid types. Different species and plasmid types of crown gall bacteria have differences in host range. Different varieties of the same host may differ regarding resistance to crown gall disease. The biocontrol effectiveness depends on the species and plasmid type of crown gall bacteria and biocontrol bacteria. Agrocin production is one of the mechanisms in inhibiting crown gall bacteria

(Kerr and Htay, 1974), but it is not the only mechanism. Owing to the diversity and complexity of crown gall bacteria and biocontrol bacteria, the type of crown gall bacteria on important host plants have to be elucidated, and selection of suitable biocontrol bacteria has to be made in order to achieve satisfactory biocontrol results.

Flowering cherry is extremely beautiful and have high appreciative value. Crown gall disease is detrimental to the development of flower cherry. It seriously affects the quantity and quality of blossoms. In some parks the incidence of crown gall disease in flower cherry is as high as 100%. On the basis of studying the strain and plasmid type of crown gall bacteria, K1026, which was Tra^- deletion mutant of pAgK84 in strain K84 and nontumorigenic, was used to investigate the possibility of biocontrol of crown gall disease in flowering cherry. This would provide a theoretical basis for the biocontrol of the disease in flowering cherry.

Materials and methods

Isolation of crown gall bacteria

Crown gall tissues (46 samples from 31 trees) of flowering cherry were collected in Beijing. The MW medium was prepared by dissolving 10 g mannitol, 5 g $NaNO_3$, 0.3 g KH_2PO_4, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 100 μg biotin, 2 ml 0.1% Fe-EDTA, 2 ml of a 0.1% crystal solution and 18 g agar in 1 l water. The improved 523 medium was prepared by dissolving 10 g sucrose, 10 g yeast extract, 0.3 g $MgSO_4 \cdot 7H_2O$, 2 g NaCl, 2 g K_2HPO_4, 5 g $CaCO_3$ and 15–20 g agar in 1 l water.

Fresh crown gall tissue placed in an eppendorf vial was broken up with a stainless steel rod. A small amount of sterile water was added and the suspension was allowed to stand. The supernatant was taken and streaked on MW medium. Colonies exhibiting characteristics of *Agrobacterium* were selected. Single colonies were purified three times on MW medium and then transferred to a slant of improved 523 medium and kept in a refrigerator.

Determination of pathogenicity

Bacteria isolated from flowering cherry and sunflower seedlings (5–10 cm tall) were used. Sunflower stems were injured with a needle. Bacteria were smeared on the stem. Cotton wool moistened with water was used to wrap the stem and keep it moist. Tumor formation was observed 2 weeks later. Pathogenic bacteria were kept for future use.

Determination of plasmid type (Otten and Schilperoort, 1978)

The tumors which had grown on the stem for about 20 days were excised, and 1 g tissue was placed in an eppendorf vial. Juice was pressed out of the tissue using a dissecting needle. Following centrifugation the supernatant was transferred with a capillary to filter paper. Nopaline, octopine and arginine standards, all from Sigma, were used as controls. Electrophoresis in formic acid : acetic acid : water (5 : 15 : 80, v : v : v) at 400 V for 1.5 h was carried out before the filter paper was dried in an oven at 40–60 °C, stained with phenanthrene quinone–ethanol mixture, and dried again and observed under an ultraviolet lamp for the presence of fluorescent spots before photography.

Identification of bacterial strains

The regular identification methods (Lippincott et al., 1982; Wang et al., 1996) was adopted. *A. tumefaciens* IAM12048, *A. rhizogenes* IAM13570 and *A. vitis* IAM14140 from Institute of Applied Microbiology, University of Tokyo, were used as standards for identifying the isolated pathogenic bacteria.

Sensitivity of crown gall bacteria to agrocin 1026 (A 1026)

Incubation medium G was prepared by dissolving 5 g glucose, 3 g sodium glutamate, 0.3 g $MgSO_4 \cdot 7H_2O$, 5 mg VB_1, 100 μg biotin, 3 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 g sodium citrate, 5 mg ferrous sulfate, and 10 g agar in 1 l distilled water. Soft YEB incubation medium contained 0.7% agar in YEB incubation medium (NA with 1 g yeast extract, 5 g sucrose, and 0.5 g $MgSO_4 \cdot 7H_2O$). K1026 strain was provided by Prof. Deqin Ma, Institute of Microbiology, Chinese Academy of Sciences. The tests were repeated three times; each had three plates, and three points were on each plate.

In accordance with the double layer culture medium method of Stonier (1960), activated biocontrol bacteria were inoculated on plates containing incubation medium G, three spots per plate. The bacteria were grown at 28 °C for 3 days. The growing biocontrol bacteria were killed with chloroform vapor. Crown gall

bacteria, which had been cultured on a slanting surface for 48 h were used to make a suspension of bacteria. A 0.2 ml aliquot was added to 3 ml soft YEB agar which had been cooled down to 50 °C, mixed rapidly, and immediately poured into culture plates containing chloroform-killed biocontrol bacteria, to form an evenly thin layer. After incubating at 28 °C for 16–24 h, observations for the appearance of bacteria inhibition rings were made.

Sensitivity of crown gall bacteria on greenhouse-grown sunflower seedlings to strain K1026

K1026 and crown gall bacteria, which had been cultured for 48 h on a slanting surface, were used to prepare bacteria suspensions (10^9 cfu/ml). Biocontrol strain was mixed with various crown gall bacteria (1 : 1, v : v). The stem of a sunflower seedling (5–10 cm tall) was injured by stabbing with a needle. Cotton wool soaked with the mixture was used to wrap the wound and keep it moist. Inoculation of each strain of crown gall bacteria only and inoculation of biocontrol bacteria only were used as controls and observations for tumor formation were made 2 weeks later. Each strain was tested on three plates, and three inoculation points were made on each plate.

Sensitivity of crown gall bacteria on flower cherry to strain K1026

The crown gall bacteria (BYH1-1, BYH18-1 and BYH19-1) and K1026 were separately cultured for 72 h at 10^9 cfu/ml. The roots of flowering cherry (Mountain cherry and Chinese cherry) seedlings were injured and treated by dipping the roots into the suspensions in two ways: inoculated with crown gall bacteria only, and co-inoculated with a mixture of crown gall bacteria and biocontrol bacteria (1 : 1, v : v). The seedlings were then transplanted to a sterile pot. The test was conducted in the spring and observations were made in the fall for tumor formation in the root region.

Results

Isolation and pathogenicity test

Typical *Agrobacterium* colonies appear round, protruding, grayish white, glossy and sticky. After streaking, isolation and purification, 50 isolates were obtained from 21 tumor samples. Inoculation of 50 isolates in sunflower revealed that six BYH1-1, BYH5-1, BYH18-1, BYH18-4, BYH19-1 and BYH19-5 could induce tumors.

Determination of plasmid type

Examination under ultraviolet light after Otten paper electrophoresis disclosed that BYH1-1, BYH5-1, BYH18-1, BYH19-1 and BYH19-5 synthesized nopaline and BYH18-4 synthesized octopine (Figure 1).

Identification of bacterial strains

Physiological and biochemical tests were carried out on six isolated strains of pathogenic bacteria and three standard bacteria strains. Results indicate that BYH5-1 is *A. tumefaciens* (biovar 1), and that BYH1-1, BYH18-1, BYH18-4, BYH19-1 and BYH19-5 are *A. rhizogenes* (biovar 2) (Table 1).

Sensitivity of crown gall bacteria to agrocin 1026 (A 1026)

A 1026 exerted a prominent inhibitory action on flowering cherry crown gall bacteria BYH5-1, BYH18-1 and BYH19-1 in incubation medium G. The diameter of the largest inhibitory ring was 48 mm. BYH1-1, BYH18-4 and BYH19-5 were insensitive to A 1026. There was virtually no agrocin production after 24 h of incubation, and the amount of agrocin produced after 72 h of incubation was significantly larger than that obtained after 48 h.

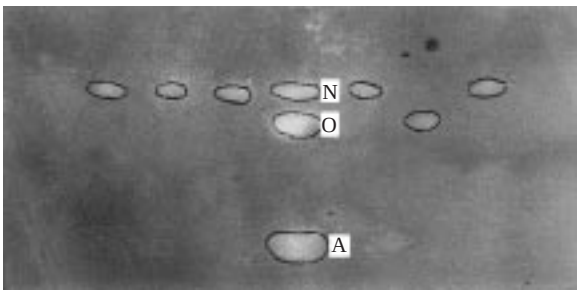

Figure 1. Paper electrophoresis of opines from crown gall bacteria of flower cherry. From left to right: BYH19-1, BYH18-4, ONA standards (O: Octopine, N: Nopaline, A: Arginine), BYH19-5, BYH5-1, BYH18-1.

Table 1. Physiological and biochemical tests for identificaton of crown gall bacteria on flower cherry

	Test strain						Standard bacteria strain		
	BYH5-1	BYH1-1	BYH18-1	BYH18-4	BYH19-1	BYH19-5	12048	13570	14140
Growth at 35 °C	+	−	−	−	−	−	+	−	+
Growth in 2% NaCl	+	−	−	−	−	−	+	−	−
Lime milk	Alkaline	Acidic	Acidic	Acidic	Acidic	Acidic	Alkaline	Acidic	Alkaline
3-Ketolactose production	+	−	−	−	−	−	+	−	−
Ferric ammonium citrate	+	−	−	−	−	−	+	−	−
Citrate utilization	−	+	+	+	+	+	−	+	−
Oxidase production	+	−	−	−	−	−	+	−	−
Acid production from erythritol	−	+	+	+	+	+	−	+	−
Acid production from ethanol	+	−	−	−	−	−	+	−	−
Acid production from melezitose	+	−	−	−	−	−	+	−	−
Alkali formation from malonate	−	+	+	+	+	+	−	+	+
Alkali formation from mucic acid	−	+	+	+	+	+	−	+	−

12048, 13570 and 14140 were, respectively, *A. tumefaciens*, *A. rhizogenes* and *A. vitis*.

Sensitivity of crown gall bacteria on greenhouse-grown sunflower seedlings to bacteria strain K1026

K1026 elicited a pronounced inhibition of crown gall bacteria BYH1-1, BYH5-1, BYH18-1, BYH18-4 and BYH19-5 from flowering cherry. Inoculation of a mixture of crown gall bacteria and biocontrol bacteria resulted in total inhibition (i.e., no gall formation) or partial inhibition (i.e., smaller galls). BYH19-1 was insensitive. Strains BYH1-1 and BYH18-4 were not sensitive to agrocin K1026 on plates, but gall production was inhibited by K1026 on sunflower.

Sensitivity of crown gall bacteria on flowering cherry to strain K1206

When mixtures of crown gall bacteria only were inoculated, the incidence of crown gall in mountain cherry was 100%, the gall number per plant was 9 and the gall weight per plant was 49.49 g. When crown gall bacteria and K1026 were co-inoculated, the corresponding data were 33%, 0.3 and 0.8 g respectively. There was a statistically significant difference between the group inoculated with crown gall bacteria only and the group co-inoculated with crown gall bacteria and biocontrol bacteria. The percentage of inhibition for the three aforementioned criteria were, respectively, 67.0%, 96.7% and 98.4%. In Chinese cherry, the incidence of crown gall disease, the gall number per plant and gall weight per plant when crown gall bacteria only were inoculated were, respectively, 100%, 8.0 and 28.58 g. When crown gall bacteria and K1026 were co-inoculated, the data were, respectively, 25%, 0.2 and 0.2 g. The percentages of inhibition for the three aforementioned criteria were, respectively, 75%, 97.5% and 99.3% (Figure 2).

Discussion

The sensitivity of flowering cherry crown gall bacteria to biocontrol bacteria K1026 on plates, on sunflowers seedlings and on flowering cherry plants were the same, suggesting that agrocin production is not always the basis for *Agrobacterium* biocontrol and that biocontrol effectiveness is also related to host plants. K1026 was the derivative of K84, which was isolated from peach rhizosphere, and peach has a close relationship with flowering cherry. The crown gall bacteria tested in this study were also isolated from flowering cherry. The results suggest the existence of two mechanisms in biological control of crown gall bacteria (i) inhibition

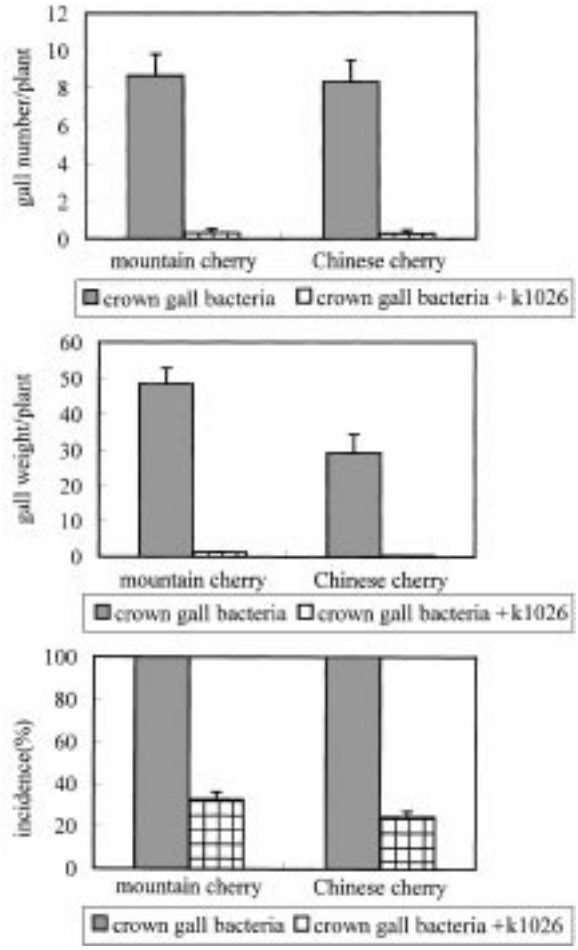

Figure 2. Inhibitory effect of strain K1026 on incidence of crown gall disease, gall number and gall weight in mountain cherry and Chinese cherry.

by agrocin and (ii) competition and/or colonization at the infection site of host plants.

The crown gall bacteria of flowering cherry are mainly *Agrobacterium* rhizogenes (biotype 2) and the Ti plasmids of most strains are of the nopaline type. Crown gall disease of flower cherry could be controlled by strain K1026.

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