Susceptibility to citrus canker caused by *Xanthomonas axonopodis* pv. *citri* depends on the nuclear genome of the host plant

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Abstract Susceptibility to *Xanthomonas axonopodis* pv. *citri* of a citrus cybrid, in which the nuclear and cytoplasmic genomes were derived from *Citrus sinensis* and *C. unshiu*, respectively, was evaluated. Bacterial growth in the leaves of the cybrid was similar to that in *C. sinensis* after pin-prick inoculation throughout the experiment, whereas growth was greater than that in *C. unshiu* from 8 days after inoculation. Lesion expansion and pustules that later developed from the lesions on the cybrid and on *C. sinensis* also appeared to be greater than those on *C. unshiu*. The incidence of citrus canker disease caused by the bacteria on the cybrid trees was in the field was equivalent to that on *C. sinensis* but was severer than on *C. unshiu*. These results indicate that the nuclear genome of the cybrid plays a critical role in susceptibility to citrus canker disease. However, the pathogenicity gene *pthA* of the bacteria is not likely to be involved in the difference in susceptibility to the bacteria between *C. unshiu* and *C. sinensis* because their susceptibility to a *pthA*-deficient mutant of the bacteria also differs.

Keywords *Xanthomonas axonopodis* pv. *citri* · Citrus cybrid

Introduction

The causal agent of citrus canker disease, *Xanthomonas axonopodis* pv. *citri*, has pathogenicity gene *pthA*, which belongs to the *avrBs3/pthA* (avirulence/pathogenicity) gene family and is required for full virulence on citrus plants (Kanamori and Tsuyumu 1998; Swarup et al. 1992). The transient expression of *pthA* in the leaves of *Citrus* species results in citrus canker symptoms involving cell hypertrophy, division, and death, which indicates that *PthA* functions inside the plant cell as a specific plant signal (Duan et al. 1999).

X. axonopodis pv. *citri* has a broad host range among members of the Rutaceae, although differences in field resistance exist among *Citrus* species (Gottwald and Graham 2000). The resistance of citrus plants has been considered as not due to active physiological resistance because all plants were highly susceptible after artificial inoculations (Brunings and Gabriel 2003). However, another gene in the *avrBs3/pthA* family, *hssB3.0*, was recently isolated from *X. axonopodis* pv. *citri* and characterized as responsible for the host-specific suppression of virulence, which induces a defense response specifically on cultivars of *C. grandis* (Shiotani et al. 2000, 2007).

The *avrBs3/pthA* family is a group of highly conserved members prevalent in the genus *Xanthomonas*. The genes in this family confer two functions to the bacteria avirulence and virulence to the host plants with and without, respectively, the corresponding resistance gene (Gabriel 1999; Leach and White 1996). Both functions are highly dependent on the type III protein secretion system, which is

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encoded by the *hrp* gene cluster in the bacterial genome (Büttner and Bonus 2002). The functions of these gene products in the host plant have been deduced from their unique protein structure, which is composed of a central region of multiple, nearly identical repeated sequences of 34 amino acid residues, three nuclear localization signals (NLSs), and an acidic activation domain (AAD) in the carboxyl terminus (Ponciano et al. 2003). On the basis of the structure of the NLSs and the AAD, AvrBs3/PthA proteins are predicted to function in the plant nucleus (Szurek et al. 2001; Yang et al. 2000; Yang and Gabriel 1995). Mutations in the structures of both the NLSs and the AAD abolish the defense responses and reduce the ability of the pathogen to cause disease in various plant–pathogen interactions.

A citrus cybrid, which consists of the *C. sinensis* nuclear genome and the *C. unshiu* cytoplasmic genome, was produced to investigate cytoplasmic male sterility that probably originated from *C. unshiu* (Yamamoto and Kobayashi 1995). *C. unshiu* and *C. sinensis* have been considered to be moderately resistant and highly susceptible, respectively, to citrus canker disease (Matsumoto and Okudai 1990). The cytoplasm has been shown to be involved in cytoplasmic male sterility and vital plant functions such as photosynthesis, sugar and fatty acid metabolism, ATP production, and disease resistance (Kadowaki et al. 1990; Mazouz et al. 2002; Otani et al. 2002). Because there is little cytoplasmic information in citrus, this cybrid can be an ideal material for studying cytoplasmic involvement in resistance to citrus canker disease. We report here that the nuclei of host plants also play a critical role in this disease through experiments with this cybrid and its parent citrus plants.

Materials and methods

Plant materials

The citrus cybrid was produced previously by electrofusion between protoplasts derived from the nucellar embryo of *Citrus unshiu* MARC cv. Juman and a mesophyll cell of *C. sinensis* Osbeck cv. Washington naval orange (Yamamoto and Kobayashi 1995). Leaf morphology of the cybrid was similar to that of the mesophyll parent *C. sinensis*. Flower characteristics of the cybrid were almost identical to those of *C. sinensis* except for the number of stamens (Yamamoto et al. 2001). The cybrid consists of the *C. sinensis* nuclear genome and *C. unshiu* cytoplasmic genome. Scions of the cybrid and its parent plants used for the inoculation test were grafted onto potted two- year-old rootstocks *Poncirus trifoliata* Raf. (trifoliate orange).

Disease was assessed in each of three trees, planted eight years before, in the orchard at the Kuchinotsu Citrus Research Station.

Bacterial strains

A strain KC21 of *Xanthomonas axonopodis* pv. *citri* and its derivative mutant KC21T14, in which the pathogenicity gene *pthA-KC21* was disrupted by Tn5 insertion, were used in this study (Shiotani et al. 2007). The bacterial strains were cultured in yeast–peptone (YP) medium at 27°C. For solid medium, 1.5% agar was added to these media. The final concentration of the antibiotics incorporated into media was 50 µg per ml for kanamycin for selecting resistant strain KC21T14. The gene *pthA-KC21* was deposited in GenBank under accession number AB206388.

Inoculation test

The sensitivity of the cybrid and its parents plants to *X. axonopodis* pv. *citri* were evaluated after pin-prick inoculation of attached leaves with the bacterial strains as previously reported (Shiotani et al. 2000). Bacterial strains were grown on Wakimoto's potato semi-synthetic medium with 1.5% agar (PSA medium) (Wakimoto 1967) at 27°C for 1 day. Bacteria were suspended in 0.85% NaCl at a concentration of 10^8 cells/ml, and a drop of the suspension was placed on an attached, mature leaf of each citrus plant. Immediately after an insect pin (0.5 mm in diameter) was inserted into the leaves through the bacterial suspension, the inoculum drops were wiped off with sterile cotton. The inoculated plants were then grown in a greenhouse. The diameter of circular lesions, including cork tissue and water-soaked margin, was measured after inoculation. For observing bacterial growth *in planta*, the populations in the lesions at 0, 1, 2, 3, 4, 8, 16, and 32 days after pin-prick inoculation were determined after removing the lesion with a cork-borer. The excised tissue was macerated in 0.85% NaCl, and the suspension was plated on a semiselective XCSM medium (Shiotani et al. 2000).

Disease development in the orchard

Disease development was investigated in July 2003 on 20 shoots for each tree selected in order of lesion abundance, with the following index: of 0, no lesions; 1, 1–3 lesions; 3, 4–10 lesions; 5, 11–20 lesions; 7, more than 20 lesions.

Disease severity (DS) was calculated from the sum of the index numbers (SI) as follows:

$$DS = SI / (20 \times 7) \times 100$$

Results and discussion

Susceptibility to citrus canker disease depends on the nuclear genome of the host plant

The sensitivity of the cybrid and its parents plant, *Citrus unshiu* (cv. Juman) and *C. sinensis* (cv. Washington navel orange), to *X. axonopodis* pv. *citri* was evaluated after pin-prick inoculation of attached leaves with the bacterial strain KC21. Water-soaked lesions were elicited on all citrus plants within 4 days after inoculation. Pustules later developed from the lesions; however, those on *C. unshiu* appear to have developed less than those on the cybrid and *C. sinensis* (Fig. 1). In all the plants tested, populations of strain KC21 increased similarly for the first 4 days after prick inoculation to approximately 3.4×10^5 CFU per lesion (Fig. 2a). After 4 days, the rate of multiplication on *C. unshiu* differed from those on the cybrid and *C. sinensis*. After 16 days, the bacterial populations decreased on *C. unshiu*, but not on the cybrid nor *C. sinensis*. No significant

difference in the bacterial population was observed between the cybrid and *C. sinensis* throughout the experiment. These results indicate that the susceptibility to citrus canker disease in the leaf tissue of the cybrid plant, which is equivalent to that of *C. sinensis*, might depend on the nuclear genome of the species. Disease severity on the cybrid trees in the orchard was also similar to that on *C. sinensis* trees (Table 1). Citrus cultivars and species with greater frequency, size, and duration of leaf flushes are more susceptible to citrus canker in the field than are less vigorous cultivars or those with more rapidly maturing foliage (Gottwald et al. 1993). Because no difference in vigor has been observed between the cybrid and *C. sinensis* trees, the identical susceptibility of the cybrid leaf tissue and *C. sinensis* to *X. axonopodis* pv. *citri* appeared to be related directly to the field susceptibility of the cybrid trees to citrus canker. Cytoplasm can contribute disease resistance (Mazouz et al. 2002; Ohtani et al. 2002); however, the moderate resistance to citrus canker of *C. unshiu* might be independent of its cytoplasmic genome.

Different rates of lesion expansion between on leaves of the cybrid and *C. sinensis* were observed (Table 2). This observation suggests that this difference depends not on their nuclear but on the cytoplasmic genomes. However, this difference in lesion expansion does not mean that the

Fig. 1 Canker symptoms on abaxial leaf surface of *Citrus sinensis*, *C. unshiu*, and the cybrid between them. Symptoms developed 40 days after prick inoculation with strain KC21 and its derivative *pthA-KC21*-deficient mutant KC21T14 of *Xanthomonas axonopodis* pv. *citri*. Bar represents 1 mm

Fig. 2 Time course of bacterial growth in the leaves of citrus plants. Leaves of *Citrus sinensis*, *C. unshiu*, and their cybrid were prick inoculated with strain KC21 (**a**) of *Xanthomonas axonopodis* pv. *citri* and its derivative mutant KC21T14 (**b**), in which the pathogenicity gene *pthA-KC21* was disrupted by Tn5 insertion. Leaves were sampled for 32 days after inoculation. The values are means from five repetitions, and vertical bars represent the standard error at each sampling time

Table 1 Severity of citrus canker disease on *Citrus* species and their cybrid

Plant	Disease severity ^a
<i>C. unshiu</i>	3.1 ± 0.1b
<i>C. sinensis</i>	4.8 ± 0.5a
Cybrid	5.0 ± 0.4a

^a Disease severity on each plant was investigated in July 2003, on 20 shoots for each tree selected in order of lesion abundance, index: of 0, no lesions; 1, 1–3 lesions; 3, 4–10 lesions; 5, 11–20 lesions; 7, more than 20 lesions. The mean value and standard error of three trees is presented. Those followed by unlike letters differ significantly at $\alpha = 0.01$ according to the Tukey–Kramer honestly significant difference test

susceptibility to citrus canker differs between these plants because bacterial growth on these two was similar for 32 days after inoculation (Fig. 2a).

Table 2 Lesion diameters on leaves of *Citrus* species and their cybrid 40 d after prick-inoculation with either wild-type strain KC21 and its Tn5 mutant KC21T14 of *Xanthomonas axonopodis* pv. *citri*

Plant	Lesion diameter (mm) ^a	
	KC21	KC21T14
<i>C. unshiu</i>	2.63 ± 0.06c	1.69 ± 0.27b
<i>C. sinensis</i>	3.56 ± 0.05a	2.34 ± 0.20a
Cybrid	3.07 ± 0.07b	2.23 ± 0.13a

^a Each value represents the mean diameter (mm) and standard error of 16 lesions for each strain on attached leaves. Those followed by unlike letters differ significantly at $\alpha = 0.01$ according to the Tukey–Kramer honestly significant difference test

Pathogenicity gene *pthA* of *X. axonopodis* pv. *citri* is independent of differences in susceptibility of *C. unshiu* and *C. sinensis* to the bacteria

The pathogenicity gene *pthA*, essential for *X. axonopodis* pv. *citri* to cause citrus canker disease, confers the ability to rupture the epidermis and elicit cankers on citrus. Thus, *PthA* may be involved in the difference in pustule development observed between *C. sinensis* and *C. unshiu*, in which nuclear genomes directly affect the susceptibility to citrus canker disease. To determine the involvement of *pthA* in the different pustule development, we produced a mutant KC21T14, derived from a strain KC21 of *X. axonopodis* pv. *citri*, in which pathogenicity gene *pthA-KC21* was disrupted by insertion of Tn5, was used to prick inoculate the leaves of *C. unshiu* and *C. sinensis*. The mutant KC21T14 elicited water-soaked lesions on their leaves, but no pustules were observed on the lesions throughout the experiment (Fig. 1). Also, the mutant grew significantly less in the lesions on both *Citrus* species than did the wild strain KC21 from 8 days after inoculation (Fig. 2b). Although lesions on the cybrid and *C. sinensis* expanded at different rates after inoculation with a wild strain KC21, the *pthA-KC21*-deficient mutant KC21T14 induced lesions of the same size on both (Table 2). This suggests that *pthA-KC21* might be associated in the different lesion expansion.

The differences in bacterial growth and lesion expansion were still observed between on *C. unshiu* and *C. sinensis* even after the inoculation with the mutant KC21T14 (Fig. 2b and Table 2). These results indicate that the pathogenicity gene *pthA-KC21* in the strain KC21 is independent of the difference in susceptibility to the bacteria between these *Citrus* species although this gene contributes to eliciting the distinctive canker symptoms (Brunings and Gabriel 2003). In addition, the bacterial population and lesion expansion on the cybrid after inoculation with mutant KC21T14 was the same as those on

C. sinensis not on *C. unshiu*. These results strongly suggest that the nuclear genome of *C. sinensis* confers greater susceptibility to *X. axonopodis* pv. *citri*.

Strain KC21 harbors three genes in the *avrBs3/pthA* family other than *pthA-KC21*: *pB3.1*, *pB3.7*, and *hssB3.0* (Shiotani et al. 2007). Of these, *hssB3.0* is involved in the host-specific suppression of virulence, so that a defense response is elicited in *C. grandis*. Because all these family genes encode NLS sequences that are identical to those of *pthA*, their protein products could also be transferred into the nucleus of host plants. However, there is no evidence that any of the *avrBs3/pthA* family genes are involved in the moderate resistance of *C. unshiu* to *X. axonopodis* pv. *citri*. According to the gene-for-gene theory (Gabriel 1999), the gene product of *hssB3.0* might be recognized by a cognate resistance gene. Further studies will need to be done to elucidate the resistance gene in *Citrus* genus.

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