Protection of Preinoculated Citrus Trees against Tristeza Virus in Relation to the Virus Concentration Detected by ELISA

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ABSTRACT. Isolate No. 145 was collected from a 13-year-old pummelo hybrid tree and selected because of its ability to protect against tristeza. It was symptomless on citrus tristeza virus (CTV) test plants as well as CTV-ELISA (enzyme-linked immunosorbent assay) negative. Preinoculation with this isolate provided full protection of trees of sweet orange or grapefruit on sour orange or trifoliate orange rootstock against the full CTV complex transmitted by *Toxoptera citricida* (Kirk.). In preinoculated trees, CTV concentration as detected by ELISA was very low and decreased with time. In contrast, there was marked increase in virus in the non-preinoculated trees similarly inoculated by the aphids. Virus present in budwood collected from the preinoculated trees caused only mild symptoms in Mexican lime and slight to no symptoms of seedling yellows on sour orange. In contrast, budwood collected from the non-preinoculated trees caused severe symptoms on the test plants. Preinoculation with vein enation virus was less protective for trees of sweet on sour orange rootstocks. However, some protection was noted and expected with sweet orange on trifoliate orange because of a gradual decrease in the CTV concentration.
Index word. Preimmunization.

Preimmunization with mild virus strains has been tried in several countries for controlling tristeza disease, and showed satisfactory performance. In Japan, tristeza disease has been a minor problem due to use of the highly resistant stock-scion combination of satsuma mandarin grafted on trifoliate orange as a standard combination. However, change in varieties from satsuma mandarin to other citrus has increased recently because of overproduction of satsuma mandarin. These newly planted cultivars soon become infected by tristeza virus (CTV) and severely stem-pitted and dwarfed, because the full tristeza complex including the seedling yellows component is widely distributed (7, 15) and the primary tristeza vector, *Toxoptera citricida*, is prevalent in Japan. Therefore, the use of an effective mild strain of virus to protect or immunize healthy plants from the tristeza disease is highly desirable.

Previously, the senior author collected certain virus sources which showed protection against seedling yellows tristeza virus (CTV-SY) (5). These included citrus vein enation virus (CVEV) as well as mild strains of CTV. Some of these have been tested in the field to evaluate their protective effect on various citrus cultivars. However, it will take several years or more to obtain the results. Therefore, several experiments using potted trees were carried out to evaluate the protective effect and to clarify characteristics of the mild virus strains during the course of the protection test.

MATERIALS AND METHODS

Preinoculated trees. Two-year-old potted trees of Valencia orange, Marsh Seedless grapefruit, and Yoshida navel orange, were used. The former two plants were virus-free nucellar seedlings and the latter originated from virus-free budwood produced by shoot-tip grafting. These were grown in a glasshouse kept aphid-free by screening and frequent sprays of pesticide.

Virus source for preinoculation. Budwood of isolate No. 145 was

collected from a 13-year-old pummelo hybrid tree in 1976 and grafted on trifoliate orange rootstock and held as a source plant in a screenhouse. When indexed, this source produced no symptoms on Mexican lime and sour orange and was negative for CTV using ELISA with anti-CTV-SP gamma globulin. The original tree planted in the field was free from tristeza symptoms until 1981. In contrast, most trees of similar hybrids became dwarfed and severely stem-pitted. However, small stem-pits were observed on the twigs of the original tree in 1982 and the CTV-ELISA test became positive.

Isolate No. 1605 was collected from a 15-year-old nucellar Valencia tree in 1978. This source produced no symptoms when indexed to Mexican lime, but showed a few small vein enation symptoms on sour orange leaves. The CTV-ELISA test has been negative. Isolate No. 1597 was also collected from a similar Valencia tree in 1978 and produced only vein clearing on Mexican lime but no symptoms on sour orange when indexed. The CTV-ELISA test was positive. The original tree in the field showed very small stem-pits on the twigs. This tree grew vigorously until 1981, however, in 1982, the tree fruited heavily and became stunted. Isolate HM-55 was collected in Hiroshima prefecture from an old and healthy tree of Hassaku (8) and this source carried mild CTV and CVEV (9).

Preliminary experiments, in which seedlings of sour orange, Marsh grapefruit, Mexican lime were preinoculated with each mild virus source followed by a challenge inoculation with CTV-SY by tissue grafting, showed that isolate No. 145 was protective to Marsh grapefruit, somewhat protective to Mexican lime, but not to sour orange. Isolate No. 1605 provided good protection to sour orange, some protection to Mexican lime but none to Marsh grapefruit. Isolate No. 1597 was protective to sour orange and Marsh grapefruit, and somewhat protective to Mexican lime. HM-55 was also protective to sour orange but not to Marsh grapefruit seedlings.

Preinoculation and preparation of seedlings. Each plant mentioned above was graft-inoculated with each virus source and cut back to force new shoot growth. Budwood collected from the preinoculated trees 5 months after inoculation was grafted on trifoliate orange or sour orange rootstocks planted in 18-cm diameter pots. Similar virus-free budwood was also grafted on the same rootstocks as controls. Six to ten trees were prepared for each treatment.

Challenge inoculation. When the potted trees grew to 20-30 cm in height and were in flush, they were challenged with CTV by inoculation with the vector *Toxoptera citricida* Kirk., which had been feeding on newly developed shoots of satsuma mandarin or Valencia orange trees. These satsuma mandarin or Valencia orange trees originated from an old bud-line and carried the full CTV-complex including CTV-SY and CTV-SP. Five to seven adults and 10-15 nymphs of *T. citricida* on leaf pieces of inoculum tissue were transferred to the new shoots and allowed to feed for 24 hours. Each tree was challenged once. After challenge inoculation the trees were kept in a glasshouse controlled at 22-24 C for 2 months and then transferred into a screenhouse. One year later, the trees were cut back to 25 cm to force new shoot growth. When the shoots matured, they were measured for length and samples were collected for ELISA. These tests were made once or twice a year.

Detection of CTV concentration in trees. Petioles and midveins

collected from fully developed leaves of five different shoots from each tree were macerated in a mortar with 10 volumes of extraction buffer ((v/w) (phosphate-buffered saline with 0.05% Tween-20, 2% polyvinyl pyrrolidone and 0.05% thioglycolic acid), followed by a low-speed centrifugation. As a standard for the virus concentration, bark tissue was collected from 2-month-old twigs of a SwO nucellar seedling infected with CTV-SY complex. The bark tissue was chopped, and the small pieces were blended and then subdivided into 0.5 g portions. The standard sample was stored in a freezer at –60°C.

The procedure for ELISA (enzyme-linked immunosorbent assay) was basically the double antibody-sandwich method according to Clark and Adams (3) and Bar-Joseph *et al.* (2). The anti-CTV serum was collected from a rabbit after two intra musclar injections plus an additional intravenous injection with purified CTV-SP extracted from albedo of Hassaku fruit infected by Hassaku dwarf disease. A set of the test samples, the serially diluted standard samples, and the virus-free sample were arranged on each plate. After development of color reaction, an optical density measurement of the substrate solution was made at 405 nm wave length, and the concentration of CTV relative to the standard was calculated.

Indicator plant reaction of the virus recovered from trees after challenge inoculation. Three to four years after the challenge inoculation, budwood collected from each tree was indexed to seedlings of Mexican lime and sour orange. The index plants were observed for one year in a glasshouse controlled at 22-24°C.

RESULTS

Experiment 1. The first experiment was started in 1978 using sweet orange and grapefruit trees, which had been preinoculated with the No. 145 source and grafted on sour orange or trifoliate orange rootstocks. After the challenge inoculation with the CTV-complex, all of the preinoculated sweet orange/sour orange and grapefruit/sour orange trees grew very vigorously. The relative concentration of CTV as detected by ELISA was very low and decreased to the detectable level in the large numbers of the trees during 3 years (figure 1). In contrast, seven of ten sweet orange/sour orange and two of eight grapefruit/sour orange trees without preinoculation declined showing yellowing, dwarfing and die back. The relative concentration of CTV in the declined trees as detected by ELISA was always high and increasing. In the vigorous and non-preinoculated trees of sweet orange/sour orange and grapefruit/sour orange, the CTV-concentration was low and gradually decreased. However, the CTV-concentration was found to increase in some vigorous trees in 1982 (figure 1, symbol a). In these trees, CTV had not been detected by ELISA for 3 years after the challenge inoculation.

Indexing on Mexican lime and sour orange seedlings showed that the virus from all of the non-preinoculated and declined trees of sweet orange/sour orange produced severe symptoms of CTV-SY; *i.e.* severe vein-clearing, stunting and stem pitting of Mexican lime and severe yellowing and stunting of sour orange (figure 1). Budwood collected from the non-preinoculated and declining grapefruit trees produced severe CTV-SP symptoms; *i.e.* severe vein-clearing, stem pitting and stunting of Mexican lime but not yellowing nor stunting of sour orange seedlings. In contrast, budwood collected from the preinoculated trees produced no symptoms or mild

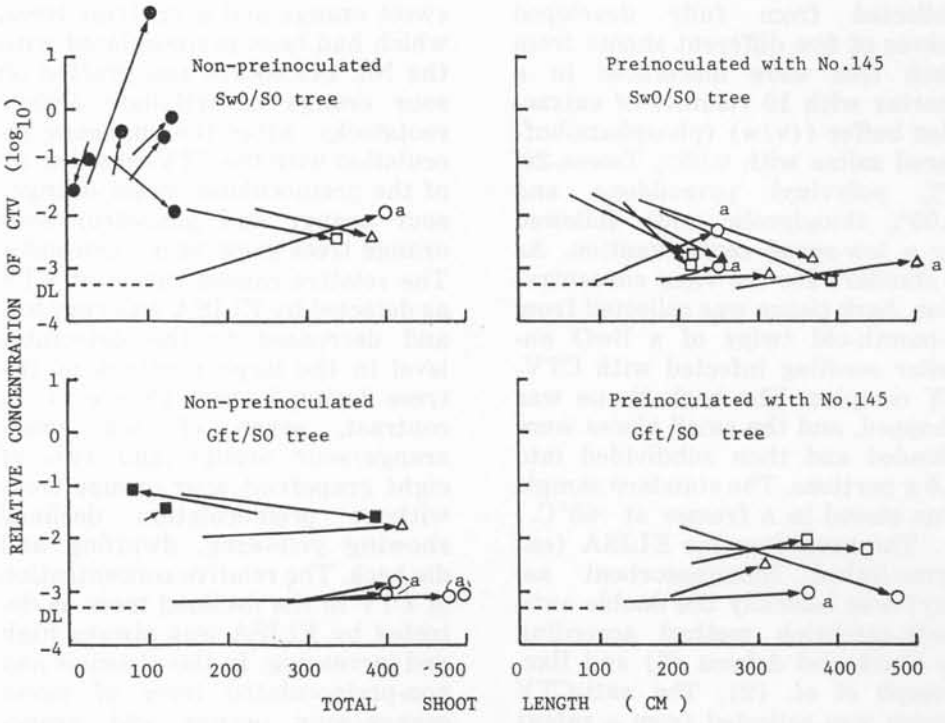

Fig. 1. Changes in shoot growth and CTV concentration of sweet orange (SwO) or grapefruit/sour orange (Gft/SO) trees preinoculated with No. 145 after challenge inoculation, and index plant reactions of recovered virus from the trees. ○ = no symptom on Mexican lime (ML) and SO; △ = only vein clearing of ML; □ = vein clearing and small stem pits on ML (CTV-SP symptom) and no symptom on SO; ■ = severe vein clearing, stem-pitting and stunting of ML (severe CTV-SP symptom) and no symptom on SO; ▲ = CTV-SP symptom and slightly yellowing or stunting of SO (SY symptom); ● = severe CTV-SP and SY symptoms; symbol a indicates CTV-ELISA negative until September, 1981; ← = transition of the obtained data from May, 1980 to November, 1982; DL, detectable level by ELISA.

symptoms of CTV-SP such as slight vein-clearing and stem-pitting of Mexican lime and no symptoms on sour orange with the exception of one budwood-source of the sweet orange/sour orange tree. The latter budwood, however, produced slight yellowing and stunting of sour orange seedlings. Budwood collected from non-preinoculated and vigorous trees produced mild CTV-SP symptoms.

All of the preinoculated trees grafted on trifoliate orange rootstock grew vigorously. The CTV concentration in the large numbers of the trees was low and decreased gradually. In three trees of the preinoculated sweet orange/trifoliate orange where CTV had not been detected by ELISA until 3 years after the challenge inoculation, CTV became detectable in 1982 (figure 2, symbol a). Non-preinoculated trees of sweet orange/trifoliate orange and grapefruit/trifoliate orange also grew vigorously in the early years. Towever, some of them became stunted.

Budwood collected from the preinoculated sweet orange/trifoliate orange trees produced mild CTV-SP and no CTV-SY symptoms and budwood collected from the preinoculated grapefruit trees produced only mild CTV-SP symptoms. In contrast, collections from the non-preinoculated and high CTV-concentration trees of sweet orange and grapefruit produced se-

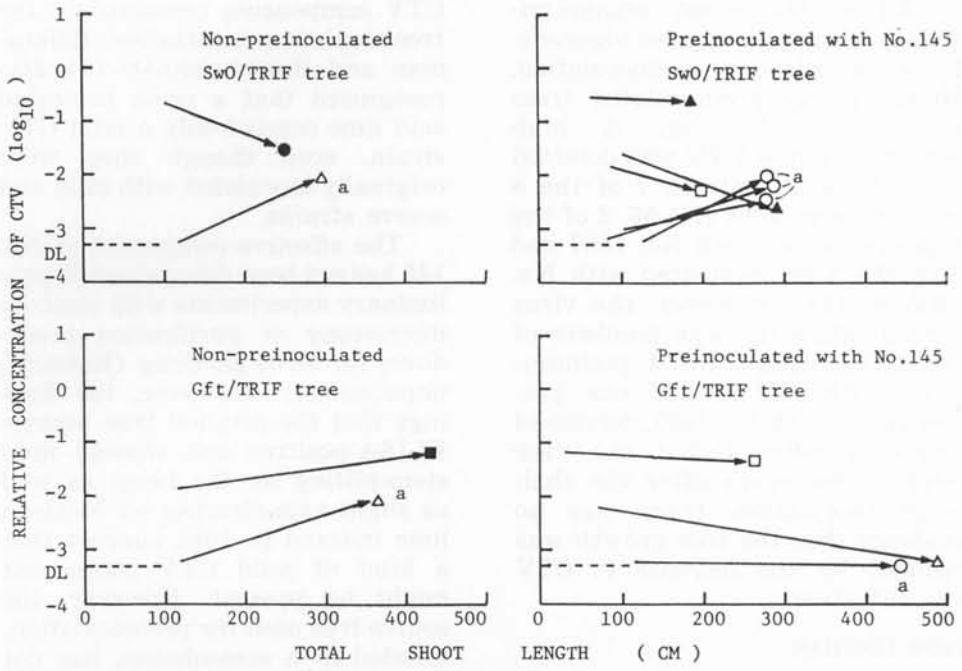

Fig. 2. Changes in shoot growth and CTV concentration of sweet orange (SwO) and grapefruit (Gft) trees on trifoliate orange (TRIF) rootstock preinoculated with No. 145 after a challenge inoculation of the full CTV complex and index plant reactions of recovered virus from the trees. Symbols are shown in Fig. 1.

vere CTV-SY and severe CTV-SP symptoms, respectively. The other non-preinoculated trees produced slight vein-clearing on Mexican lime.

Experiment 2. A similar experiment was repeated in 1981 using sweet orange/sour orange trees preinoculated with No. 145 and non-preinoculated trees. For 2 years after challenge inoculation with the full complex of CTV by *T. citricida*, 6 of 9 preinoculated trees grew vigorously and the others were somewhat stunted but did not decline. In contrast, 2 of 8 non-preinoculated trees died, 4 were severely stunted and yellow, and the other 2 were growing vigorously. This confirmed the protective effect of No. 145 against the full complex of CTV on sweet orange/sour orange trees.

Experiment 3. The third experiment started in 1979 using sweet orange trees which had been preinoculated with HM-55, No. 1597 or No. 1605 and grafted on sour orange or trifoliate orange rootstocks. Those trees grafted on sour orange were less protected against challenge inoculation by the CTV complex. That is, 8 of the 9 trees preinoculated with HM-55, 4 of the 7 trees preinoculated with No. 1597, 6 of the 6 trees preinoculated with No. 1605 and 5 of the 6 control trees were chlorotic. However, large numbers of the trees preinoculated with HM-55 and No. 1605 were growing more vigorously than the non-preinoculated trees.

The chlorotic trees showed high CTV-concentration in 1-2 years after the challenge inoculation. The CTV concentration decreased gradually in sweet orange/sour orange preinoculated with No. 1605 and in some preinoculated with HM-55. In the vigorous and non-chlorotic trees preinoculated with HM-55 and No. 1597, the CTV concentration decreased to a lower level.

All of the sweet orange/trifoliate orange trees grew vigorously even without preinoculation. However, non-preinoculated trees showed leaf curling. A high concentration of CTV was detected in 2 of the 6 controls, 7 of the 8 preinoculated with HM-55, 2 of the 6 preinoculated with No. 1597 and 8 of the 8 preinoculated with No. 1605 in 1981. However, the virus concentration in large numbers of the trees, except for 4 preinoculated with HM-55 and one preinoculated with No. 1597, decreased markedly when tested one year later. Three years after the challenge inoculation, there was no evidence that the tree growth was reduced by the increase in CTV concentration.

DISCUSSION

In the experiments of this paper, sweet orange or grapefruit trees grafted on sour orange or trifoliate orange rootstocks preinoculated with isolate No. 145 were markedly protected against the full complex of CTV transmitted by *T. citricida* and CTV concentration in these trees decreased markedly. Since isolate No. 145 initially was found negative for CTV by ELISA, subsequent changes of CTV concentration were probably due to CTV introduced by the aphids.

The virus recovered from the No. 145-preinoculated trees on sour orange and trifoliate orange rootstocks caused very mild symptoms on Mexican lime and slight to no yellowing and stunting of sour orange seedlings. In contrast, the virus recovered from the non-preinoculated and declined trees caused severe symptoms on sour orange and Mexican lime. This indicates that both components of CTV-SY and severe CTV-SP were apparently eliminated from the CTV complex in the No. 145 preinoculated tree, and only the mild CTV components remained in the tree at a low concentration. Balaraman and Ramakrishnan (1) also recognized that a cross protected acid lime carried only a mild CTV strain, even though they were originally inoculated with mild and severe strains.

The effective component of No. 145 has not been determined in preliminary experiments with electron microscopy or purification procedures for virus particles (Koizumi, unpublished). However, the findings that the original tree became ELISA-positive and showed mild stem-pitting on the twigs as well as slight vein-clearing on Mexican lime indexed in 1982 suggest that a kind of mild CTV component might be present. However, the source tree used for preinoculation, isolated in a screenhouse, has not shown any reaction. Since the original tree was virus-free when planted in the field, it is probable that the effective component was introduced into the tree by a vector in the early years after planting.

Wallace and Drake (11, 12), and McClean (6) reported that the residual components in sour orange or lemon trees after recovery following inoculation with the full complex of CTV were protective against the original CTV complex on sour orange or lemon seedlings. Wallace *et al.* (12, 13, 14) demonstrated that sweet orange/sour orange trees preinoculated with the residual components gave protection against tristeza in the field in California under natural infection. The effective component of No. 145 is different from the residual component because of the failure of protection of sour orange preinoculated with No. 145 against CTV-SY.

A vigorous tree individually infected with vein enation virus (CVEV) was occasionally found in the field and some isolates of CVEV could protect against CTV-SY in-

oculated by tissue grafting on sour orange seedlings (5). Sasaki (10) confirmed the protective effect of CVEV not only on Hassaku trees under the natural infection, but also on *Citrus junos* seedlings under the *T. citricida* transmission with CTV-SP. McClean (6) reported no evidence of interference to tristeza infection by CVEV on rough lemon trees. The latter might be due to a different host.

Preinoculation of CVEV (No. 1605) into sweet orange/sour orange trees did not protect against the CTV complex, even though the CTV concentration was somewhat decreased. In the trees of sweet orange/trifoliate orange, protection was evident because of the vigorous growth of the trees and the decrease of CTV concentration. HM-55, which carried the mild CTV and CVEV, was somewhat protective. Since the protection phenomena of HM-55 and No. 1605 resemble each other, the effective component might be common in the two virus sources, but further study will be needed to confirm it. Particles of CVEV were previously described as a small rod by Hooper and Schneider (4), however, this has not been confirmed in Japan by preliminary efforts of purification and electron microscopy (Koizumi, unpublished).

For evaluation of protective effect of the mild virus, quantitative determination of CTV by ELISA is useful. However, anti-CTV-SP gamma globulin used for ELISA could not distinguish between severe strains of the challenged CTV and the preinoculated virus. Therefore, it was indefinite whether the detected CTV in a tree was due to severe or mild strain until the virus was indexed to some indicator plants. To evaluate the protective effect of mild CTV by means of ELISA, a distinguishable CTV antiserum between mild strain and severe strain is needed.

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