Discovery of a Strain of Tristeza Virus (K) Inducing No Symptoms in Mexican Lime

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ABSTRACT. In a rootstock trial, six kumquat cultivars were grafted on trifoliate orange and on Troyer citrange. All six cultivars did well on trifoliate orange, but only one thrived on citrange. This finding prompted us to search for potential viruses in our kumquat cultivars. In addition to indexing on indicator plants, we looked for inclusion bodies and found that two kumquat trees on trifoliate orange had inclusion bodies in the phloem, suggesting citrus tristeza virus (CTV) infection. ELISA (enzyme-linked immunosorbent assay) tests were positive for CTV using antibodies to the Reunion strain of CTV. Filamentous virions similar to CTV could be seen by electron microscopy in the phloem of the two kumquat trees and were purified from young shoots. The purified virions were ultrastructurally and serologically indistinguishable from typical CTV. These results prove that the two kumquat trees are infected with a strain of CTV referred to here as strain K. The two CTV-positive kumquat trees were among three trees introduced to Corsica from Morocco in 1959. These trees indexed negatively on Mexican lime seedlings in 1963 and were considered free from CTV. When it was realized in 1981 that two of the three trees were infected with CTV, they were again indexed on Mexican lime seedlings in Corsica and Bordeaux. No vein clearing or stem pitting was observed, but ELISA showed that the limes were infected with CTV. Because strain K is symptomless on Mexican lime CTV infection in the two kumquat trees was not detected earlier. Indexing by ELISA has shown that no spread of CTV has occurred from the two initial kumquat trees or their progeny.
Index words. kumquat, ELISA, inclusion bodies, electron microscopy, indexing.

Several lines of kumquat (Marumi K123, K124 and K154 ; Nagami K125 and K169 ; Meiwa K153) have been introduced into Corsica and have been propagated on two rootstocks: trifoliate orange and Troyer citrange. All kumquat lines did well on trifoliate orange, but some declined on Troyer citrange (K123, K124, K125, K153) or induced vein clearing on sweet orange (K153), as reported by Navarro, *et al.* (6). These results prompted us to determine the pathogen content of the kumquat lines (7). Citrus tristeza virus (CTV) was the first agent to be tested. Two techniques were used: staining for virus inclusion bodies by Azure A stain (2) and ELISA using IgGs against the Reunion strain of CTV (3). By these methods, one kumquat line (K123) was found to be infected with CTV. Virus particles similar to CTV could be seen by electron microscopy in the sieve tubes of ELISA-positive kumquat trees. This paper reports the first graft-transmission experiments carried out with this strain (strain K) and its purification. A companion paper (1) describes additional unusual properties of this strain of CTV.

EXPERIMENTS AND RESULTS

Mexican lime seedlings infected with CTV strain K are symptomless. The CTV-infected K123 kumquat line is an old line imported from Morocco in 1959. Three trees budded on rough lemon were imported: K123-T22, K123-T23 and K123-T24. In 1981, trees T22 and T24 were found positive for CTV by both inclusion body staining and ELISA, whereas tree T23 was negative. When the trees were indexed for CTV on Mexican lime seedlings in 1963, no symptoms of vein clearing or stem pitting were noticed. Repeated indexing on Mexican lime at San Giuliano and Bordeaux since 1981 has never revealed the slightest tristeza symptom. ELISA was used to verify whether the symptomless lime seedlings contained CTV. Sixty-four of 65 Mexican lime seedlings graft-inoculated with CTV-K-infected kumquat bark from trees T22 and T24 were found positive for CTV by ELISA within 4 yr after inoculation. None of the 64 plants showed CTV symptoms and/or stunting. In the same experiment, groups of five Mexican lime seedlings were

graft-inoculated with Mexican lime bark infected with CTV strain T1 or CTV strain T4 (4). Five of the seedlings inoculated with CTV-T1 and four of those inoculated with CTV-T4 were positive by ELISA and all nine ELISA-positive seedling showed stunting and typical symptom of CTV. Five uninoculated Mexican lime seedlings were used as a control.

In another experiment, groups of five Mexican lime seedlings were used to study the time required for CTV-K, CTV-T1 and CTV-T4 to pass from bark inoculum to Mexican lime seedlings. Seedlings were graft-inoculated with bark infected with the respective CTV strains. The bark inoculum was removed 2, 4, 6, 8 and 15 days after grafting. ELISA was used to determine the presence of CTV in the seedlings 6 weeks after graft inoculation. No CTV transmission was obtained within 4 days after inoculation. After 6 days, 2 of 5, 3 of 5 and 2 of 5 seedlings, respectively, inoculated with CTV-K, CTV-T1 and CTV-T4 were ELISA positive. After 8 days all inoculated seedlings were infected. No CTV symptoms were observed in CTV-K infected seedlings while characteristic symptoms developed on the seedlings carrying CTV-T1 or CTV-T4.

Finally, symptomless Mexican lime seedlings infected with CTV-K seemed to contain as much virus as symptomatic Mexican lime seedlings infected with CTV-T1 or CTV-T4, as judged from the number of inclusion bodies observed and the ELISA readings, which were between 0.8 and 1.0 OD at 405 nm.

These experiments demonstrated that CTV-K infects and multiplies to normal titers in Mexican lime, but that it does not induce tristeza symptoms or stunting in these seedlings. This result explains why CTV-K was not detected in 1963 when the K123 kumquat trees were indexed on Mexican lime seedlings in Corsica.

Transmission of CTV strain K by bud propagation. Until it was recognized in 1981 through ELISA and inclusion body staining that two of the three initial K123 kumquat parent trees were infected with CTV, all three trees were used indistinguishable as sources of budwood for propagation on trifoliate orange. The percentage of infected trees in various kumquat orchards has been determined by ELISA. Twenty-five of 35 (71%) were found infected in the San Giuliano Station, 40 of 114 (35%) in one commercial orchard and 22 of 87 (25%) in another orchard. These percentages probably reflect the proportion of budwood taken from the two infected parent trees to that taken from the single healthy tree and indicate that CTV-K is easily graft-propagated. To prove this, buds from CTV-K-infected tree K123-T22 and from uninfected tree K123-T23 were respectively propagated on two groups of six trifoliate orange seedlings. All the six trees propagated from CTV-K infected buds were CTV positive by ELISA and inclusion body staining, whereas none of the budlings propagated from CTV-free budwood were positive.

Graft-inoculation of kumquat trees on trifoliate orange by various strains of CTV. Buds from CTV-free tree K123-T23 were propagated on trifoliate orange and groups of eight trees were graft-inoculated with infected bark of kumquat tree K123-T22, CTV-T1-infected Mexican lime bark, and CTV-T4-infected Mexican lime bark, respectively. Eight and six trees were grafted with bark from the CTV-free tree K123-T23 and healthy Mexican lime bark (negative controls), respectively. ELISA was done 3 months after graft-inoculation and inclusion body staining after an additional 3 months to determine presence of CTV. All trees inoculated with CTV-T1, or CTV-T4 were found positive, whereas seven of eight trees grafted with CTV-K-infected bark gave positive reactions. Control trees were all negative.

ELISA reactions of CTV-strain K and CTV from Reunion. CTV-K was purified by the technique of Gon-

salves *et al.* (5) from bark of K123-kumquat trees shown CTV-positive ELISA, and compared with CTV purified from a Reunion source. Morphologically and spectrophotometrically, the two strains were very similar. With IgGs against CTV-Reunion, strain CTV-K gave almost the same optical density readings in ELISA at 405 nm as CTV-Reunion. Virus concentration ranged from 500 to 0.5 ng/well assuming an extinction coefficient E (0.1%/cm) at 260 nm of 3.0.

Lack of evidence for natural transmission of CTV-K. Many trees (sweet orange, Clementine, etc.) on sour orange growing next to K123 kumquat trees known to carry CTV-K were examined by ELISA for possible CTV-K contamination. All assays were negative. In one commercial K123 kumquat orchard, all trees were indexed by ELISA for CTV infection in 1981 and again in 1986. All trees that were found CTV-negative in 1981 were still negative in 1986.

CONCLUSION

These data show that two of the three parent trees of Marumi kumquat K123 are infected with a strain of CTV referred to as CTV strain K. No evidence for natural transmission of CTV-K has been obtained. CTV-K is easily propagated with buds of infected kumquat trees in the field. CTV-K can also be readily graft-inoculated into healthy kumquat trees or Mexican lime seedlings. Since none of the many Mexican lime seedlings infected with CTV-K showed any tristeza symptoms or stunting indexing on Mexican lime is not suitable for diagnoses of CTV-K. ELISA or microscopy must be used to index for this strain.

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