Virus isolates and source plants. Sixteen container-grown seedlings were inoculated with bark chips from an uninfected, T36-, and/or T30-CTV-infected source tree. They were maintained in an aphid-free greenhouse and served as the acquisition hosts for the single aphid transmission experiments. GS-1 and GS-2 were replicate ‘Duncan’ grapefruit seedlings infected with the T36 isolate of CTV. The original source of T36-CTV was a container-grown C. excelsa Wester on rough lemon (C. jambhiri Lush) rootstock tree. The CTV isolate in this tree (termed T36) causes severe vein clearing, stunting, and stem-pitting of ‘Mexican’ lime, mild seedling yellows symptoms on ‘Eureka’ lemon [C. limon (L.) Burm.f.] and sour orange seedlings, and quick decline of sweet orange trees on sour orange rootstock. OS-1 and OS-2 were replicate ‘Valencia’ sweet orange seedlings infected with CTV isolate T36. GM-1 and GM-2 were replicate ‘Duncan’ grapefruit seedlings infected with the T30 isolate of CTV. The original source of T30 was a container grown C. excelsa on rough lemon rootstock tree. The CTV isolate in this tree causes mild symptoms (vein-clearing) on ‘Mexican’ lime, no seedling yellows on ‘Eureka’ lemon or sour orange seedlings, and no symptoms on sweet orange trees on sour orange rootstock. OM-1 and OM-2 were replicate ‘Valencia’ sweet orange seedlings infected with CTV isolate T30.

GFS-1 and GFS-2 were ‘Duncan’ grapefruit seedlings that were bark chip inoculated from two field grapefruit trees on sour orange rootstock that had been first inoculated with T30 and then challenge inoculated with T36, 12 months later. GFS-1 and GFS-2 were two field ‘Valencia’ sweet orange trees on sour orange rootstock that had been first inoculated with T30 and then challenge inoculated with T36, 12 months later. GU-1 and GU-2 were replicate ‘Duncan’ grapefruit and ‘Valencia’ sweet orange seedlings, respectively.

Receptor indicator plants. ‘Mexican’ lime seedlings were used as the receptor indicator plants. Seeds of ‘Mexican’ lime were sown in trays filled with custom soil mix (Conrad Fafard, Inc.), and grown under greenhouse conditions for 6 to 12 months. The seedlings were then transplanted into plastic pots containing the same soil mix, with one to three plants per pot. Seedlings with new flush were used as receptor indicator plants for single BrCA transmission of CTV. After inoculation with CTV by single BrCA, the receptor indicator plants were grown in an insect-free greenhouse, and tested by in situ immunostaining (ISIA) (Lin et al., 2000) with CTV monoclonal antibodies 17G11 and MCA13.

Brown citrus aphids. BrCA colonies were collected, maintained, and characterized as previously described (Lin et al., 2002). Virus-free colonies were established by feeding on uninfected ‘Duncan’ grapefruit or ‘Valencia’ sweet orange seedlings as described (Lin et al., 2002).

Single aphid transmission. ‘Duncan’ grapefruit and ‘Valencia’ sweet orange seedling source plants containing the T30, T36, or T30 plus T36 isolates of CTV or uninfected seedlings were pruned several weeks before aphid acquisition feeding to stimulate flushes of new growth. When the leaves on the new
flushes had completely expanded, groups of 200 to 400 virus-free aphids were transferred onto the new flushes for a 24-h acquisition access feeding. One hundred single aphids were then transferred from each source plant, using a small paintbrush, to a new flush on one hundred healthy ‘Mexican’ lime receptor seedlings (one aphid per receptor seedling). Only alate aphids were used. The aphids were allowed 24 h on the receptor seedlings for inoculation access feeding. After the inoculation access feeding, pesticides (Marathon) or 1 soap : 1 oil : 30 water mixed were sprayed to kill the aphids. The receptor ‘Mexican’ lime seedlings were kept under insect-free green house conditions (23 to 30 °C) for 4 to 6 months. Since BrCA alate can travel several meters from one plant to another, the receptor plants were caged individually. In addition, donor plants, receptor plants on which aphids were feeding, and receptor plants after insecticide treatment were housed separately. Control, uninoculated, uninfected plants were housed with the donor and receptor plants to signal contamination.

**Virus analysis.** Both acquisition and receptor plants were assayed for CTV infection by ISIA using monoclonal antibodies (MAb) 17G11 and MCA13. MAb 17G11 reacts with most Florida isolates of CTV including both T30 and T36. MAB MCA13 reacts with most Florida decline-inducing isolates of CTV (including T36), but not with nondecline-inducing isolates of CTV (including T30). The ISIA procedure has been previously described (Lin et al., 2000).

Each receptor plant was sampled three times, and each sample was assayed independently. A plant was considered positive if any of the three samples was positive. Each receptor plant was assayed again 6 weeks after the initial assay. On rare occasions when the repeat assay failed to confirm the initial assay, a third assay was performed.

**Results**

The source trees for aphid transmission could be placed in three virus infection categories based on ISIA. These included infection with decline-inducing CTV (reacted with MAb MCA13), infection with non-decline-inducing CTV (reacted with MAb 17G11, but not MCA13), and uninfected (reacted with neither MAb) (Table 1). ISIA results are consistent with the virus originally introduced into the source trees by bark chip inoculation. The ISIA cannot distinguish between a tree infected with only decline-inducing CTV and a tree infected with both decline-inducing and non-decline-inducing CTV.

At least one successful aphid transmission (out of 100 attempts) occurred from each source tree except the uninfected controls, from which no transmissions occurred (Table 1). Transmission from GS-1 (grapefruit inoculated with T36-CTV) resulted in one tree that reacted with both MABS 17G11 and MCA13 and one tree that reacted only with 17G11. This second tree was only infected with T30-CTV (as detectable by ISIA), indicating that this T36-CTV source tree was infected with both MCA13 reactive (T36-CTV) and MCA13-nonreactive (T30-CTV) virus, and the aphid vector could selectively transmit the T30-CTV from the mixture. Aphids did not separate any non-MCA13 reactive CTV from the remaining three source trees (GS-2, OS-1, or OS-2) believed to be inoculated only with T36-CTV.

Aphids were able to selectively transmit non-MCA13 reactive (nondecline-inducing) CTV from each of the seedlings (GFS and OFS) inoculated with both T30-CTV and T36-CTV resulting in ‘Mexican’ lime receptor plants that assayed positive for T30-CTV, but not T36-CTV.

Four source trees, two orange and two grapefruit, assayed negative for T36-CTV, but positive for T30-CTV by ISIA (OM-1, OM-2, GM-1, GM-2). Single-aphid transmission from these source plants resulted in several ‘Mexican’ lime seedlings that were infected with T30-CTV (reacted with MAB 17G11, but not MCA13), as expected.

However, four of the ‘Mexican’ lime indicators (one from each source plant), that were reactive with MAB 17G11, were also reactive with MAB MCA13. This suggests that GM-1, GM-2, OM-1, and OM-2 also contained a mixed infection of T36-CTV and T30-CTV, and the T36-CTV was not detectable in the source plant by ISIA. In spite of this, a single BrCA was able to acquire the T36-CTV and transmit it to an indicator plant.

**Discussion**

Previous results have shown that decline-inducing isolates of CTV can occur as mixtures in trees also infected with non-decline-inducing isolates of the virus. In some cases, the decline-inducing isolate is not immunologically detectable, but can be recovered by bark chip inoculation to indicator plants (Powell et al., 2002). We have now shown that BrCA can also acquire decline-inducing CTV from citrus plants in which it was not immunologically detectable. An explanation for this result is not clear. Perhaps the decline-inducing CTV is limited to a low number of cells in which it occurs at a transmissible concentration. Aphids may be able to acquire virus from these cells, but they may not have been included in an ISIA sample.

The transmission efficiency of CTV by BrCA from either grapefruit or sweet orange was low compared to some other reports (Broadbent et al., 1996; Costa and Grant, 1951; Lastra et al., 1992; Nickel et al., 1984; Yokomi et al., 1994). The reasons for this are unclear, but the same low transmission efficiency has been observed by other Florida virologists (R. Bransky and R. Lee, personal communications). The conclusion that aphids can transmit decline-inducing CTV from trees in which the virus is immunologically undetectable has significance for the citrus industry and CTV control. Currently most certification programs rely heavily on immunological detection to insure budwood sources are free of disease-causing CTV. In many cases nondecline-inducing isolates of CTV are ignored or deliberately propagated for cross-protection purposes. The observation that decline-inducing CTV can be recovered from trees, that would have passed these immunological tests, by aphids or grafting (Powell et al., 2002), suggests that procedures for certifying budwood sources as free of disease-causing CTV need to be reevaluated.

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**Table 1. Transmission of decline-inducing and nondecline-inducing isolates Citrus tristeza virus (CTV) from grapefruit and sweet orange seedlings with single brown citrus aphids.**

| Source plant | in situ Immunoassy of acquisition tree | Transmission |  |
|--------------|----------------------------------------|-------------|---|
|              | 17G11                                 | 17G11       | MCA13  |
| GS-1         | +                                     | +           | 2/100  | 1/100 |
| GS-2         | +                                     | +           | 1/100  | 1/100 |
| OS-1         | +                                     | +           | 3/100  | 3/100 |
| OS-2         | +                                     | +           | 3/100  | 3/100 |
| GFS-1        | +                                     | +           | 3/100  | 2/100 |
| GFS-2        | +                                     | +           | 3/100  | 1/100 |
| OFS-1        | +                                     | +           | 5/100  | 4/100 |
| OFS-2        | +                                     | +           | 3/100  | 1/100 |
| GM-1         | +                                     | +           | 2/100  | 1/100 |
| GM-2         | +                                     | +           | 1/100  | 1/100 |
| OM-1         | +                                     | +           | 3/100  | 1/100 |
| OM-2         | +                                     | +           | 4/100  | 1/100 |
| GU-1         | –                                     | –           | 0/100  | 0/100 |
| GU-2         | –                                     | –           | 0/100  | 0/100 |
| OU-1         | –                                     | –           | 0/100  | 0/100 |
| OU-2         | –                                     | –           | 0/100  | 0/100 |

GS = grapefruit with decline-inducing CTV; OS = orange with decline-inducing CTV; GFS = grapefruit, bud-inoculated with both decline-inducing and nondecline-inducing CTV; OFS = orange, bud-inoculated with decline-inducing and nondecline-inducing CTV; GM = grapefruit with nondecline-inducing CTV; OM = orange with non-decline-inducing CTV; GU = CTV negative grapefruit; OU = CTV negative orange. See Materials and Methods for details of source plants.

17G11 + source plant was infected with CTV. 17G11 = source plant was not infected with CTV as detectable by ISIA. MCA13 + source plant was infected with a decline-inducing isolate of CTV. MCA13 = source plant was not infected with a decline-inducing isolate of CTV as detectable by ISIA.

The number of successful aphid transmissions versus the number of attempts as detected by ISIA using MABS, 17G11 and MCA13.
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