Elimination of Tatter Leaf-Citrange Stunt Virus from Satsuma Mandarin by Shoot-tip Grafting following Pre-heat-treatment

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ABSTRACT. An excellent variety of 'Niyu' satsuma mandarin carried tatter leaf-citrange stunt virus (TL-CSV). Elimination of TL-CSV from satsuma mandarin had been difficult because of its intolerance against heat treatment and failure to obtain TL-CSV-free trees by shoot-tip grafting (STG). Pre-heat-treatment of the potted Niyu satsuma at 35 C for 19-32 days or 40/30 C for 9 days plus an additional 35/30 C for 13-20 days and subsequent STG using a shoot-tip of 0.2 mm in length produced TL-CSV-free trees.

Index words. tatter leaf virus, elimination, satsuma mandarin.

Budunion crease caused by tatter leaf-citrange stunt virus (2) was recognized in several varieties or budlines grafted on *Poncirus trifoliata* rootstock in Japan (5, 6). In recent years certain viruses, including tatter leaf-citrange stunt virus (TL-CSV) have become widespread due to the popular practice of variety renewal by top-working. A local variety Niyu satsuma produces excellent quality fruit, but fruit set was subject to fluctuation because of TL-CSV even if the trees were approach-grafted with the resistant rootstock *C. junos* Sieb. ex. Tan. Elimination of TL-CSV from carrier plants was accomplished by heat treatment at 40/30 C (daytime/night temperatures) for more than 60 days (3, 7, 13). However, satsuma mandarin budwood was intolerant of those temperatures (7). Shoot-tip grafting (STG) developed by Murashige *et al.* (8) and improved by Navarro *et al.* (9) was effective for elimination of viruses, viroids, and *Spiroplasma citri* except for TL-CSV (14, 15). This paper reports the elimination of TL-CSV from Niyu satsuma by STG following short periods of heat treatment.

MATERIALS AND METHODS

Pre-heat-treatment. A two-year-old potted tree of Niyu satsuma grafted on rough lemon rootstock was used. This variety carried the virus or viruses causing tatter-leaf of *C. excelsa* and stunting, zig-zag shoots and severe mottle of Rusk citrange, and also seedling yellows tristeza virus (CTV-SY). After removing most of leaves, the tree was held in a glasshouse at 25-30 C to force new shoot growth. When new shoots were 3-5 mm in length, the tree was transferred to a growth-chamber illuminated at 10,000 lux for 12 hours each day and the temperature controlled at 35 C (Expt. 1). In the other experiment incubation temperature was alternated from 40 C to 30 C at 12-hour intervals (Expt. 2).

Grafting. After incubation, new shoots about 5-10 mm in length were collected. Following removal of large leaves the shoots were disinfected in 0.25% sodium hypochlorite plus 0.1% Tween-20 and rinsed 3 times in sterile distilled water. The technique of STG was done according to Navarro *et al.* (10). Twelve-day-old Troyer citrange seedlings grown aseptically from seed at 28 C in the dark were decapitated 2 cm above the cotyledons. The disinfected shoot-tip was trimmed to remove leaf primodia with a razor blade sliver attached to a handle. Finally, a small shoot-tip consisting of the apical meristem and three to four leaf-pri-

modia (about 0.2 to 0.4 mm in length) was excised with a razor blade sliver. The excised shoot-tip was placed on the trimmed rootstock. The grafted plant was placed aseptically on a filter-paper-platform in a test tube with roots immersed in Murashige and Skoog medium modified by Navarro *et al.* (9) for STG, and incubated overnight at 28 C in the dark. It was then transferred into a growth-chamber illuminated at 1,000-1,500 lux for 12 hours each day at a constant 28 C. When a new sprout appeared from the grafted tissue, the intensity of the illumination was increased to 10,000 lux. Successfully grafted plants were removed from the tube and cut off at the cotyledons, when the sprout grew to 1-3 mm in length. Then they were side- or approach-grafted to a 12 year-old potted rough lemon seedling to accelerate shoot growth.

Indexing. The successfully grafted plants were indexed for presence of tatter leaf-citrange stunt virus (TL-CSV) and tristeza virus (CTV). A fully developed and mature leaf or stem was collected from each plant and the tissues were side-grafted to a rough lemon seedling, which was top-grafted with a Rusk citrange or *C. excelsa* scion. The indexed plant was incubated in a glasshouse at 20-24 C for 6-12 months to force shoot growth. The developing shoots were periodically observed and cut back to force new shoot growth. Indexing of CTV was done by enzyme-linked immunosorbent assay (ELISA) basically according to Clark and Adams (4) and Bar-Joseph *et al.* (1) using anti-CTV-SP serum.

RESULTS

The first experiment, in which a Niyu satsuma tree was incubated at 35 C and new growth subsequently removed for STG, showed that the pre-heat-treatment for 13 days was insufficient to eliminate TL-CSV from the shoot-tips of 0.2 mm in length, even if they were freed of CTV (table 1). TL-CSV in those trees caused severe symptoms such as zig-zag shoots, stunt, irregular leaves and severe mottle on Rusk citrange and tatter leaf symptoms on *C. excelsa*. Heat-treatment for 19 days and subsequent STG produced a plant free of both TL-CSV and CTV. Four other plants were found free of CTV but not of TL-CSV. The TL-CSV within those trees produced typical symptoms of TL-CSV but less stunt on Rusk citrange. Heat-treatment for 32 days and subsequent STG using shoot-tips of 0.2 mm in length produced 2 plants free of both TL-CSV and CTV.

In the second experiment, the plant was subjected to pre-heat-treatment at alternate temperatures of 40 C and 30 C at 12-hour-intervals. However, shoot growth of Niyu satsuma ceased completely and the sprouts gradually withered. Therefore, the incubation temperature was changed to 35/30 C after the 9-day incubation at 40/30 C. At the lower temperature regime many new sprouts developed. Excision of the shoot-tips was carefully done with lengths of 0.2 mm or less except for some shoot tips collected from the sprouts after incubation for 29 days. Pre-heat-treatment for 9 days at 40/30 C was insufficient to eliminate TL-CSV from the shoot-tip (table 1). Pre-heat-treatment for 9 days at 40/30 C plus an additional 6 days at 35/30 C was also insufficient. However, heat-treatment for 9 days at 40/30 C plus an additional 13 or 20 days at 35/30 C eliminated TL-CSV from shoot-tips 0.2 mm in length. But the tree grown from a 0.3-0.4 mm shoot-tip collected after heat treatment for 29 days was not free of TL-CSV. All of the successfully

TABLE 1
RESULTS OF INDEXING FOR PRESENCE OF TATTER LEAF-CITRANGE STUNT VIRUS (TL-CSV) AND CITRUS TRISTEZA VIRUS (CTV) IN TREES OF 'NIYU' SATSUMA MANDARIN DEVELOPED BY SHOOT-TIP GRAFTING FOLLOWING PRE-HEAT-TREATMENT

Expt. no.	Pre-heat-treatment		Size of shoot-tip	No. of successful grafts	No. of TL-CSV-free plants*	No. of CTV-free plants*
	Temp.	Period				
1	35 C	13 days	0.2-0.4 mm	3	0	1
	35 C	19 days	0.2 mm	5	1	5
	35 C	32 days	0.2 mm	2	2	2
2	40/30 C	9 days	0.2 mm	2	0	2
	40/30 C + 35/30 C	9 days 6 days	0.2 mm	1	0	1
	40/30 C + 35/30 C	9 days 13 days	0.2 mm	3	2	3
	40/30 C + 35/30 C	9 days 20 days	0.2 mm	3	3	3
	40/30 C + 35/30 C	9 days 20 days	0.4 mm	1	0	1

*TL-CSV was indexed on Rusk citrange and *C. excelsa*. CTV was indexed by ELISA.

grafted plants were free of CTV. The TL-CSV carried by the trees grown from shoot-tips after heat-treatment for 9-17 days produced severe symptoms when indexed on Rusk citrange. In contrast, the virus in a tree grown from the shoot-tip after heat-treatment for 29 days caused mottled leaves but no zig-zag shoots nor stunting when indexed on citrange plants.

DISCUSSION

For the elimination of TL-CSV from budwood of Meyer lemon, heat treatment for 6 weeks at 40/30 C plus an additional 2 weeks at 44/30 C was required, but the incubation for 16 weeks at 38 C or for 12 weeks at 40/30 C were insufficient (3). Miyakawa (7) also demonstrated that heat treatment for 90 days or more at 40/30 C was required for the elimination of TL-CSV from the budwood of certain citrus plants. TL-CSV was eliminated from Meyer lemon by preconditioning of budwood and treatment for 4-22 hours at 50 C in moist hot air (13). However, satsuma mandarin was intolerant against such heat treatment.

Roistacher *et al.* (14, 15) failed to eliminate TL-CSV from Meyer lemon budwood by STG and his third attempt also failed (personal communication). This experiment shows that combination of short heat-treatment and subsequent shoot-tip grafting could resolve the difficulty. Navarro *et al.* (11) also showed that psorosis-like pathogens could be eliminated by preconditioning plus STG. Okudai *et al.* (12) confirmed that pre-heat-treatment for 40 days or more at 40/30 C provided CTV-free shoot-tips of 5-13 mm in length and they utilized an easy method of STG combined with pre-heat-treatment to obtain CTV-free plant. These indicate that the combination of pre-heat-treatment and STG is the most reliable method for elimination of virus pathogens from citrus budwood.

ACKNOWLEDGMENTS

The author is grateful to Dr. T. Miyakawa, Mr. C. N. Roistacher and Dr. S. M. Garnsey for critical review of the manuscript.

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