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https://doi.org/10.5109/4363548

出版情報：九州大学大学院農学研究院紀要. 66 (1), pp.29-35, 2021-03-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **66** (1), 29–35 (2021)

Elimination of *Citrus Tristeza Virus* from Triploid Pummelo Cultivar [*Citrus maxima* (Burm.) Merr.] by *In Vivo* Micrografting on Seedlings of Three *Citrus* Cultivars

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(*Received October 31, 2020 and accepted November 4, 2020*)

Seedlessness in *Citrus* cultivars is one of the important characters accepted by consumers. Triploid pummelo contains no or a few seeds even though they are planted in a mixed block. Four triploid pummelo hybrids bred in Kyushu University are in the course of registration for new seedless cultivars release. *Citrus tristeza virus* (CTV) may be the catastrophic cause of decline of the production when the new pummelo cultivars release and expand. Therefore, virus test for the original trees and production of virus–free clones before the release are necessary. Micrografting with shoot apices of scions on the epicotyls of decapitated seedlings is the best ways to remove viruses in *Citrus*. In this study, shoot tips with 2, 4 or 6 leaf primordia of CTV–infected triploid pummelo 112A were grafted *in vivo* on 2-week-old seedlings of 'Hirado–buntan' pummelo, 'Natsudaidai' tangelo and 'Variegated Daidai' sour orange rootstocks to seek suitable rootstock seedlings and produce CTV–free clones. It was verified from this study that the larger the shoot tip size, the higher the rate for success in *in vivo* micrografting. All of the shoots derived through *in vivo* micrografting of shoot tips with 2, 4 or 6 leaf primordia were determined to be CTV–negative through immunochromographic assay 9–12 months after micrografting; however, 26.3 % of the shoots showed positive for CTV through RT–PCR test. The resulting virus free shoots (73.7 %) will be vegetatively propagated and proliferated for cultivar release.

Key words: *Citrus tristeza virus* (CTV), *in vivo* micrografting, triploid pummelo, virus–free.

INTRODUCTION

Pummelo plants [*Citrus maxima* (Burm.) Merr.], which belong to the subfamily Aurantioideae in the family Rutaceae, produce the largest fruit in *Citrus*. Pummelo fruit is popular throughout North and South America, Europe and Asia especially in South East Asia due to its attractive characteristics (Sirisomboon and Lapchareonsuk, 2012) such as fruit taste, fruit fragrance, longest longevity for fruit storage and resistance to citrus greening disease (syn. Huanglongbing, HLB) in the trees. Fruit of pummelo, however, contains too many seeds when the cultivars are not parthenocarpic or cultivated not in a single block but in a mixed block. On the other hand, fruit of triploid pummelo contains a few or no seeds even when they are planted in the mixed block. Because of their seedlessness that consumers prefer, triploid cultivars will be accepted by them in the markets. Four triploid hybrids bred in University Farm of Faculty of Agriculture, Kyushu University, are in the process of registration as new cultivars to release. Before the new cultivars release, virus test for the original trees and production of virus–free clones from the infected trees are necessary.

Citrus tristeza virus (CTV) that causes the reduction of yields in *Citrus* plants including pummelo can also be transmitted not only to healthy plants but also to tolerant ones (Soares *et al*., 2016) through scions, grafting tools or aphid species. Although pummelo is known as a resistant plant to CTV, the infected trees were found in Cameron Highlands in Malaysia (Ayazpour *et al*., 2011). Bernet *et al*. (2008) and Folimonova *et al*. (2008) reported that various levels of CTV resistance in pummelo plants were dependent on the difference in various strains of the CTV.

According to Chand *et al*. (2016), use of the *Citrus* plants free from virus and virus–like pathogens was an effective method to increase the yield and protect the citrus industry from graft transmissible diseases. This suggests that production of CTV–free nurseries is essential to control this pathogen in the citrus orchards. Several methods were applied to eliminate viruses from infected plants, e.g., shoot tip culture, micrografting, thermotherapy and chemotherapy for *Citrus* (Abbas *et al*., 2008; Chand *et al*., 2013; Singh *et al*., 2008, Juarez *et al*., 2015), apple (Hu *et al*., 2015, 2017) and pistachio (Onay, *et al*., 2003). Among these methods, shoot tip culture is difficult in most woody plants including *Citrus* as far as the authors know in the literature. Thermotherapy is an effective technique to eliminate viruses from infected plants, because high temperature inhibits viral RNA synthesis resulting in the reduction of movement of virus particles to the apical meristem (Wang *et al*., 2008); the high temperature also affected on the growth and survival of apple plants (Hu *et al*., 2015). *In vitro* shoot tip grafting (Murashige *et al*.,

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1972) is a useful technique for eradication of viruses and virus–like pathogens that could not be eliminated by thermotherapy treatment; the virus free plants recovered from the shoot tips showed adult phase and did not have juvenile characteristics (Hartl–Musinov *et al*., 2006). Thus, the micrografting is the best way to restore the virus–free plants from infected ones. However, the protocol for the elimination of CTV in pummelo has not been reported so far.

In vitro micrografting requires expert skills, aseptic condition and acclimation of the micro–grafted plants to *ex vitro* environment or natural conditions. *In vivo* micrografting also requires expert skills when the grafting carry out; however, aseptic condition and acclimation of the micro–grafted plants are unnecessary. It was reported that in *in vivo* micrografting of *Citrus* plants the frequency of successful grafts was less than that of *in vitro* micrografting (George *et al*., 2008). This suggests that selection of appropriated rootstock seedlings for each of the scions would be important to increase the success rate. Shoot tips used for *in vivo* micrografting were usually collected from apical or axillary buds of actively growing shoots in a greenhouse, field, or *in vitro* (George *et al*., 2008; Juarez *et al*., 2015). For micrografting of the scions, very young seedlings derived from polyembryonic trifoliate orange are usually used. In tropical and subtropical regions and in summer and autumn seasons, however, it is difficult to use the seedlings as rootstocks for micrografting, because trifoliate orange is deciduous trees adapting in relatively cold temperate region and produces seeds with dormancy in early autumn. Lee *et al*. (2020) tested seedlings from 11 *Citrus* cultivars and concluded that 'Hirado-buntan' pummelo seedlings are the best rootstock for micrografting of satsuma mandarin.

In the present study, we firstly checked whether these triploid pummelo trees were infected with CTV or not. Second, suitable rootstock seedlings resulting in the high success rate of *in vivo* micrografting of the triploid pummelos were determined. Lastly, we revealed the shoot apex size resulting in high success rate for *in vivo* micrografting and examined the elimination of CTV in the successful grafts with the aid of PCR and immunochromographic assay.

MATERIALS AND METHODS

Plant materials

Three diploid *Citrus* cultivars, monoembryonic 'Hirado-buntan' pummelo [*C. maxima* (Burm.) Merr.; 2n=2x=18], polyembryonic 'Natsudaidai' tangelo (*C. natsudaidai* Hayata) and polyembryonic 'Variegated Daidai' sour orange (*C. aurantium* Linn.), whose seedlings showed high potential for adventitious bud formation on the decapitated epicotyls and the high successful *in vivo* micrografting rates for satsuma mandarin scions (Le *et al*., 2020), were used for the production of 2–week–old rootstock seedlings in this study.

Four triploid pummelos [*C. maxima* (Burm.) Merr.; 2n=3x=27] 'Juto–peiyu', 'Chosan–peiyu', 'Shunpei–peiyu' and 'Kokko–peiyu', were used to detect viruses and then to eliminate the viruses through micrografting. The four triploid pummelos with high fruit quality were selected from triploid seedlings derived from small seeds of open–pollinated 'Banpeiyu' pummelo in Ibusuki Experiment Station of Faculty of Agriculture, Kyushu University located at Ibusuki, Kagoshima Prefecture, Japan 39 years ago. 'Juto–peiyu', 'Chosan–peiyu' and 'Shunpei–peiyu' are in the process of registration as new cultivars, while 'Kokko–peiyu' is scheduled to be applied as a new cultivar within five years. They were 7– to 35–year–old trees grown in the orchard of Kyushu University Farm located at Sasaguri, Fukuoka Prefecture, Japan.

Micrografting

Three or four well–developed axillary buds on upper half of the spring shoots with the length of about 5 cm were used as scions for *in vivo* micrografting in the four triploid pummelo cultivars. The spring shoots were collected from trees expected to be infected with *Citrus tristeza virus* in the orchard of Kyushu University Experimental Farm. Under a stereoscopic microscope, shoot apices with 2, 4 or 6 leaf primordia were excised from the axillary buds of spring shoots. Shoot apex with 6 leaf primordia, which were difficult to count exactly, was estimated with the length of 0.6 mm.

The perfect seeds of the three cultivars for the preparation of rootstock seedlings were collected from open–pollinated fruits. Mature embryos obtained from the perfect seeds in each cultivar were placed on wet filter paper and incubated in a growth chamber at 25±2°C for about two weeks. The vigorously germinating embryos with a root of about 1.5–2.0 cm in length were transplanted to the pots containing a mixture of four kinds of soils [Kanuma pumice : Bora pumice : Akadama soil (red clay ball): leaf mold = 1:1:1:1 v/v/v/v] and carried to a greenhouse for raising the seedlings. Six seedlings were allowed to grow per each pot with a volume of 600 ml.

Two–week–old rootstock seedlings were decapitated at 1.0–1.5 cm of epicotyl from the cotyledon axillary node with a sharp razor blade. The shoot tips of triploid pummelo trees determined to be infected with CTV was, then, placed with the cut end on the cambium of the cut surface of rootstock epicotyl and wrapped in expanded parafilm. The graft unions or grafts were carried to a growth chamber and maintained at 25°C for 30 days before transferred to the greenhouse. Minimum and maximum air temperature in the greenhouse was 10°C in winter and 38°C in summer throughout this experiment from 2018 to 2019. Number of days to initation of scion growth was recorded two days intervals. Cotyledons axillary shoots were removed as soon as possible for the promotion of scion growth. The success rate for scions on each kind of rootstock seedlings was examined 30 days after micrografting (DAM). Observation was carried out from 20 to 26 graft unions for each combination.

The successful micrografting rate (%) in each scion-rootstock combination was calculated as follows: No. of developing green scions 30 DAM/No. of seedlings grafted×100. Number of full–expanded leaves in each

scion–rootstock combination was counted three months after micrografting. The height of growing scion from grafted position to shoot apex was also measured.

Detection of viruses in mother trees and micrografted plants

Detection of viruses in mother trees

Leaves collected from branches located at middle position of triploid pummelo trees growing in the orchard of Kyushu University Farm were used to detect viruses via immunochromatographic assay. Three leaves were collected from each of trees in the four triploid pummelos scheduled to release as new cultivars.

CTV was detected by immunochromatographic assay (Agdia–Bioford, Catalog no. STX78900) according to the manufacturer's instructions as follows: Grind 0.15 g leaves with 3 ml of extract buffer and collect the extract with mesh sample bags. Insert the end of the strip into the pouch and keep the strip to remain in the extract for a maximum of 30 minutes or until both the control line and test line appear.

SDV and ASGV were detected by using quick check (Mizuho Medy Co. Ltd., Japan) according to the manufacturer's instructions, i.e., a kit for assaying SDV based on the immune chromatography method (Kusano *et al*., 2007). Each leaf (0.1 g) was ground with 0.5 mL of extract buffer in a tube. The grind leaf extract was dropped into a test plate and the test plate was kept for 15 minutes at 25°C. Positive reactions were confirmed by the band seen on the test line of the test plate chromatograph.

Detection of viruses in micrografted plants

To confirm the elimination of target virus in the plants that grow from the micrografted shoot tips, the leaves were collected from each of them 4 to 6 months and 9 to 12 months after micrografting and checked for CTV by immunochromatographic assay as mention above. The plants, which showed a negative reaction with CTV via immunochromatographic assay, were further checked by RT–PCR of T36 gene regions in CTV (Kano *et al*., 2006) according to the procedure described by Ohta *et al*. (2011) and Kano *et al*. (2006). Reverse transcription was performed using a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio Inc., Japan) according to the manufacturer's instructions. After the reaction, PCR amplification was performed with forward primer FKS01 (5'AAGGTTACGAGGAGGCAACC3') and reverse primer RKS02 (5'ACTCGAAGGGCGTTAGTACG3'). PCR products were electrophoresed on a 2% agarose gel and stained with Midori Green Advance DNA Stain (Nippon Genetics Co. Ltd., Japan). Actin was use for internal control and distilled water was used for negative control in this experiment.

RESULTS

Virus check test for scions and rootstock seedlings

CTV, SDV and ASGV were not detected through immunochromatographic assay in three trees for 'Juto–peiyu', four trees for 'Chosan–peiyu', four trees for 'Shunpei–peiyu'. One tree for 'Kokko–peiyu', however, showed positive reaction for immunochromatographic assay only for CTV (Table 1, Fig. 1). Therefore, the one tree with the ID number of 112A was selected as a donor tree for elimination of CTV.

Regarding the trees of tree cultivars for rootstock seedlings, CTV was detected in all trees of 'Hirado–buntan', 'Natsudaidai' and 'Variegated Daidai' by immunochromatographic assay; however, their embryos and 2–week–old seedlings were CTV–negative.

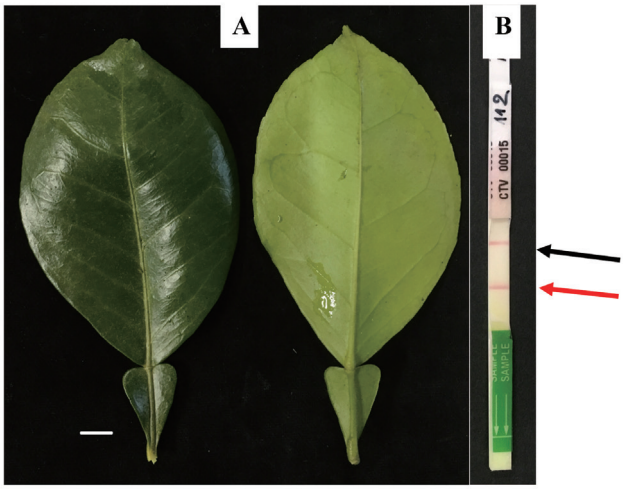

Fig. 1. Detection of viruses in triploid pummelo via immunochromatographic assay. (A) Leaves of CTV–infected tree 112A; (B) Immunochromatographic assay of the leaves of CTV–infected tree 112A. An upper arrow indicates a control line for CTV, while a lower arrow indicates the test line showing the positive response for CTV. Horizontal white bar=5 mm.

Table 1. Positive (+) or negative (–) reaction of 12 trees for three viruses in four triploid pummelos via immunochromatographic assay

Identification number of pummelo trees for 'Juto–peiyu' (106), 'Chosan–peiyu' (110), 'Shunpei–peiyu' (111) and 'Kokko–peiyu' (112)	Positive (+) and negative (–) responses of triploid pummelo for indicated viruses		
	Citrus tristeza virus (CTV)	*Satsuma dwarf virus* (SDV)	*Apple stem grooving virus* (ASGV)
106A, 106B, 106C	–	–	–
110A, 110B, 110C, 110D	–	–	–
111A, 111B, 111C, 111D	–	–	–
112A	+	–	–

Success rate in micrografting

The rates for successful micrografting ranged from 4.4% to 16.0% for shoot tips with two leaf primordia, 3.8% to 17.4% for those with four leaf primordia and 4.4% to 34.6% for those with six leaf primordia (Fig. 2 and Table 2). There was no remarkable difference in the successful micrografting rates between summer (July to August) and autumn (September) grafting on 'Natsudaidai' and 'Variegated Daidai' rootstock seedlings. The maximum success rate was 34.6% for micrografting of shoot tips with six leaf primordia on Natsudaidai seedlings in summer, followed by 17.4% for micrografting of those with four leaf primordia on 'Variegated Daidai' albino seedlings in summer and 16.0% for micrografting of those with two leaf primordia on 'Variegated Daidai' albino seedlings in autumn (Table 2).

Almost all survived shoot tip scions began to grow to form shoots with expanded leaves 11 to 30 days after grafting, while unsuccessful shoot tips turned into brown and died. The days to initiation of the pummelo scion growth was short in Natsudaidai (13.0–18.0 days) and 'Variegated Daidai' seedlings (14.0–17.5 days) in summer micrografting. Number of leaves per growing shoot on different rootstock seedlings six months after micrografting were a few (2–4 leaves) and not significantly different among different scion–rootstock combinations. In most cases, the length of 6–month-old shoots after micrografting on 2–week–old 'Natsudaidai' seedlings was higher than those on 2–week–old 'Hirado–buntan' seedlings and 'Variegated Daidai' albino seedlings (Table 2).

Fig. 2. Elimination of CTV in triploid pummelo 112A 4–6 months after *in vivo* micrografting. (A) A leaf of CTV–free triploid pummelo 112A 4–6 months after *in vivo* micrografting; (B) immunochromatographic assay of the CTV–free shoot of 112A. An arrow indicates a control line for CTV. There is not visible test line. Horizontal white bar=5 mm.

Virus test for growing plants after micrografting

All of 19 plants obtained after micrografting were determined to be CTV–negative via immunochromographic assay (Fig. 2), whereas the rate of virus–free plants was 73.7% (14 of 19 plants) when RT–PCR test was carried out for the 19 plants. In this case, five of the

Table 2. Effect of shoot apex size of triploid 'Kokko–peiyu' pummelo and different rootstock seedlings from three cultivars on success in in vivo micrografting and elimination of CTV

Seed parent for rootstock seedlings (Time of grafting)	Scion with indicated number of leaf primordia	% success in micrografting 30 DAG[a] (No. scions growing / No. micrograftings)	Days to initation of scion growth (± SE)	No. leaves per micrografting 6 MAG[b] (± SE)	Length of developing scion (cm) 6 MAG[b] (± SE)	% of CTV–free plants (No. CTV–free plants / No. plants tested)
Hirado-buntan (July to August)	2	9.1 (2/22)	11.0 ± 1.0	3.0 ± 0.0	0.8 ± 0.0	100 (1/1)
	4	5.0 (1/20)	28.0 ± 0.0	–	–	–
	6	5.0 (1/20)	30.0 ± 0.0	3.0 ± 0.5	0.5 ± 0.0	100 (1/1)
Natsudaidai (July to August)	2	7.7 (2/26)	13.0 ± 1.0	3.0 ± 1.0	0.4 ± 0.1	100 (2/2)
	4	3.8 (1/26)	18.0 ± 0.0	3.0 ± 0.0	0.8 ± 0.0	100 (1/1)
	6	34.6 (9/26)	14.9 ± 2.2	4.4 ± 0.7	1.3 ± 0.4	100 (6/6)
Natsudaidai (September)	2	4.8 (1/21)	12.0 ± 0.0	4.0 ± 0.0	1.4 ± 0.0	100 (1/1)
	4	9.5 (2/21)	23.0 ± 5.0	2.0 ± 0.0	0.6 ± 0.0	100 (1/1)
	6	5.0 (1/20)	16.0 ± 0.0	4.0 ± 0.2	1.8 ± 0.0	100 (1/1)
Variegated Daidai (July to August)	2	4.4 (1/23)	14.0 ± 0.0	2.0 ± 0.0	0.5 ± 0.0	100 (1/1)
	4	17.4 (4/23)	17.5 ± 3.5	4.0 ± 1.2	0.7 ± 0.2	100 (2/2)
	6	4.4 (1/23)	14.0 ± 0.0	4.0 ± 0.0	0.7 ± 0.0	100 (1/1)
Variegated Daidai (September)	2	16.0 (4/25)	21.0 ± 3.0	–	–	–
	4	8.0 (2/25)	24.0 ± 2.0	4.0 ± 0.0	0.5 ± 0.0	100 (1/1)
	6	8.0 (2/25)	27.0 ± 1.0	–	–	–

[a] DAG: days after micrografting.
[b] MAG: months after micrografting.

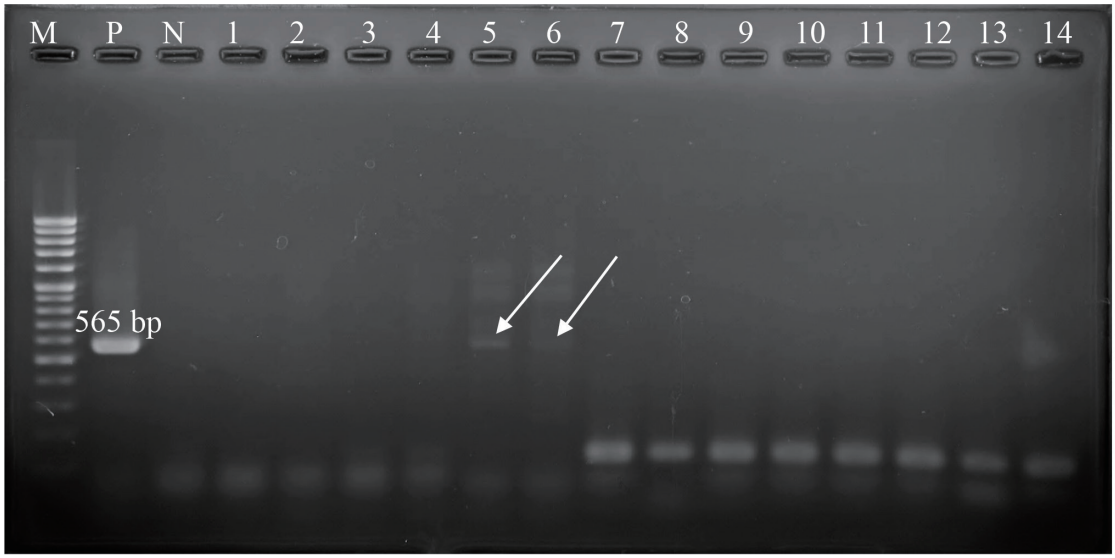

Fig. 3. Agarose gel electrophoresis of RT–PCR products from *in vivo* micrografted triploid pummelo 112A to detect presence or absence of CTV. Lane M: 100 bp ladder as marker; Lane P: virus–infected mother tree with a band of 565 bp; Lane N: negative control; Lanes 1–4: negative response of micrografted plants; Lanes 5–6: positive response of micrografted plants; Lanes 7–14: internal control (actin) of micrografted plants.

19 plants showed positive responses for CTV with the band of 565 bp (Fig. 3).

DISCUSSION

The four triploid pummelos, which are in the process of registration as new seedless cultivars with high fruit quality, have been established from small seeds of 'Banpeiyu' pummelo 39 years ago. During the 39 years of cultivation of the four triploid pummelo cultivars in a mixed field with 'Okitsu–wase' satsuma mandarin infected with CTV, SDV and ASGV (Le, 2019), the original 'Kokko–peiyu' tree has been infected with CTV. To release 'Kokko-peiyu' as a new cultivar, elimination of CTV is essential for the propagation. It has been obvious in the present study that the elimination of CTV from the tree is successful through *in vivo* micrografting on 2–week–old seedlings produced from seeds of 'Natsudaidai', 'Hirado–buntan' and 'Variegated Daidai'. It has been reported that depending upon the *Citrus* cultivars, rootstocks, time for infection in each virus and environmental conditions, the various virus disease symptoms such as stunting, slow or quick decline in trees, stem pitting and no symptoms have been observed (Garnsey *et al*., 1987; Huang *et al*., 2004). Thus, it is desirable to carry out virus test and use the present *in vivo* micrografting technique for elimination of the viruses in the other triploid pummelo cultivars before cultivar release.

Since the days to initiation of the growth of shoot tip after micrografting was short in 'Natsudaidai' rootstock seedlings (13.0–18.0 days) and 'Variegated Daidai' rootstock seedlings (14.0–17.5 days) in summer micrografting, these rootstock seedlings have higher ability to enhance micrografted scion growth than 'Hirado–buntan' rootstock seedlings in summer wherein trifoliate orange seedlings are very difficult to use.

By the first four months after micrografting, scions showed a poor growth, then formed new leaves and increased plant height. It seems that the difference in size of vascular tissues between triploid pummelo shoot tips and rootstock seedling epicotyls results in the poor contact and consequently slow growth rate of the grafted shoot tips. The numbers of days to initiate the growth of scion in summer micrografting are less than those in autumn (Table 2). The similar results have been reported in micrografting of satsuma mandarin (Le *et al*., 2020). These results suggest that the buds during non–dormancy period result in the acceleration of grafting process and scion growth. Okuda *et al*. (2005) have suggested that dormancy in satsuma mandarin trees with fruit in Japan takes place from September to October with the deepest dormancy in late September. After the spring shoots of satsuma form, they become mature from the middle of June (Inoue, 1989). Therefore, it seems that scions in autumn take long time to initiate their growth in Japan if the same phenomenon occurs in the triploid pummelo 'Kokko–peiyu'.

The shape of leaves in grafted plants maintained the characteristics of mother plant such as large winged leaf and leaf hairs that are the markers for distinguishing shoots growing from a shoot tip from the adventitious shoots from the decapitated end of rootstock. Despite the same rootstock seedlings were used for micrografting with satsuma mandarin and for micrografting of triploid pummelo shoot tips, the days to initiation of scion growth was longer in triploid pummelo scion (11.0–30.0 days) than that in satsuma mandarin scion (7.5–19.0 days) (Le *et al*., 2019). It seems that the insufficient contact between shoot tip cambium of triploid pummelo and epicotyl cambium of rootstock seedlings also results in the slow growth of micrografted shoot tips. On the

other hand, it is well known that the growth rate of trees was noticeably influenced by rootstock cultivars (e.g., Morinaga and Ikeda, 1990). Although the extent of incompatibility between scion and rootstocks is a problem that will lead to their death (Darikova *et al*., 2011), the extent of incompatibility in the present *in vivo* micrografting is not a problem, since the three rootstock cultivars selected for triploid pummelo showed the same degree of success rates and growth in *in vivo* micrografting.

In the present study, the success rates for *in vivo* micrografting were low, but all derived plants through micrografting of shoot tips with 2, 4 or 6 leaf primordia were CTV–negative through immunochromatographic assay, and 71.4% of them were also CTV–negative through RT–PCR test (Table 2, Fig. 3). It seems that the degree of virus proliferation and the distribution of virus particle are dissimilar in different positions of the trees and between trees. These may result in the diversity of virus eradiation. Singh *et al*. (2019) have reported that the survival rate for *in vivo* micrografting with shoot tip size of 0.3–0.5 mm was 42.0% and all micrografted 'Khasi' mandarin plants showed negative for CTV via RT–PCR test. It has been suggested that the frequency for virus elimination depends on the concentration of target virus in shoot apex and the physiological condition of the mother plant (Verma *et al*., 2004). The present *in vivo* micrografting has carried out under very high temperature condition in summer and relatively high temperature condition in early autumn with more than 35ºC, under which virus activity and proliferation may be suppressed and the suppression is one of the reasons resulting in the increase of success rate in production of virus free triploid pummelo plants. As has been suggested by Verma *et al*. (2004), there are possibility that the virus free plants would contained very low concentrations of viruses undetectable by immunochromatographic assay. Hence, repetition of virus free test will be necessary for the further growing virus-free plants with some intervals in near future. It is also necessary to protect these virus-free plants from their vectors.

Conclusion

All plants obtained through *in vivo* micrografting of shoot tips with 2, 4 or 6 leaf primordia from CTV–infected triploid pummelo cultivar 'Kokko–peiyu' have been determined to be CTV–negative via immunochromographic assay and 73.7% of them also showed negative for CTV through RT–PCR. The success rate for micrografting and elimination of virus will increase by improvement of micrografting methods such as usage of low concentration of plant hormones. It is expected to apply this *in vivo* micrografting technique in summer or in tropical regions for recovering free plants from viruse–infected and/or *Candidatus*–infected pummelo and the other *Citrus* cultivars.

AUTHOR CONTRIBUTIONS

M. L. Le designed the study, performed preparing plant materials and virus tests, analyzed the data and wrote the paper. K. Sakai provided resources, participated virus check, designed the study, and edited the paper. Y. Mizunoe provided facilities and resources, and edited the paper. K. Kajiwara and S. Kajihara participated preparing plant resources and virus tests. Y. Ozaki provided facilities, supervised the work and edited the paper. A. Wakana designed the study, supervised the work, and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

ACKNOWLEDGEMENTS

This study is funded in part by the Can Tho University Improvement Project VN14–P6, supported by a Japanese ODA loan.

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