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Micrografting and Heat Treatment Combination for Eliminating Virus of CTV-infected *Citrus*

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This study was conducted to eliminate viruses from citrus-infected plants using micrografting and thermotherapy. Six citrus cultivars including a 'Setoka' hybrid were used as plant sources. The TAS-ELISA technique demonstrated that several plants were CTV positive. However, no CTV symptoms were detected in plants obtained from shoots and treated at a high temperature of 40°C during the day and night and micrografted for two weeks with old trifoliate orange rootstock *in vitro*. Indexing of CTV, SDV, and CTLV for RT-PCR analysis of the eleven citrus seedlings, including 'Setoka', 'Samdajosang', 'Pungkwang', 'Shiranuhi', and 'Ehimekashi dai28go' was virus free following the micrografting and thermal therapy.

Key words : Citrus, CTV, virus free plant, RT-PCR, TAS-ELISA

Introduction

Citrus is one of the worldwide fruit crops that can be taken as fresh fruit or as juice. *Citrus* which is vegetatively propagated is subjected to virus infection during grafting and cultivation. Twenty species of viruses have been reported to infect citrus plants in the world, and in Korea, four species were identified such as *satsuma dwarf virus* (SDV), *citrus tristeza virus* (CTV), *citrus tatter leaf virus* (CTLV), and *citrus mosaic virus* (CiMV) [7]. These viruses significantly affect both the quality and quantity of fruits including the scion of *Citrus* through multiple infections [6]. Among those viruses, CTV was considered the most important viral pathogens of citrus with 2000 long flexuous filaments, phloem-limited, with monopartite, largest single-stranded and positive-sense RNA genome of 19.3 kb organized into 12 open reading frames (ORFs). Aphids naturally spread the virus but it is also easily transmitted by grafting [2].

Germplasn conservation can be assessed in terms of useful traits for citrus breeding and of the economic impact on citrus productivity. In citrus breeding procedure, new progenies are grafted on symptomless virus infected rootstocks accidently and symptomless CTV infected scion sources have been grafted on virus free rootstocks for propagation. Therefore, a secured germplasm propagation and conservation must be required for eradication of viruses *in vitro* [6, 12]. Complete elimination of virus is still difficult in obtaining virus - free plants in *Citrus* despite the use of diverse methods such as shoot apex culture, thermotherapy, antivirals treatment, callus culture, protoplast fusion, and nucellar clone. Hence, the effect of micrografting and combination of thermotherapy for eliminating virus in Citrus and new citrus progenies will be examined.

Materials and Methods

Plant materials

One hundred fifteen cultivars (Fig. 1), including mandarin hybrids 'Setoka' from the Citrus Research Station (CRS) in Korea were used as plant materials in this experiment. TAS ELISA and SDV Chromato techniques were performed on leaves from field tree and used as a source of explants as well as the leaves from young acclimatized plantlets produced through thermotherapy coupled with *in vitro* shoot tip grafting.

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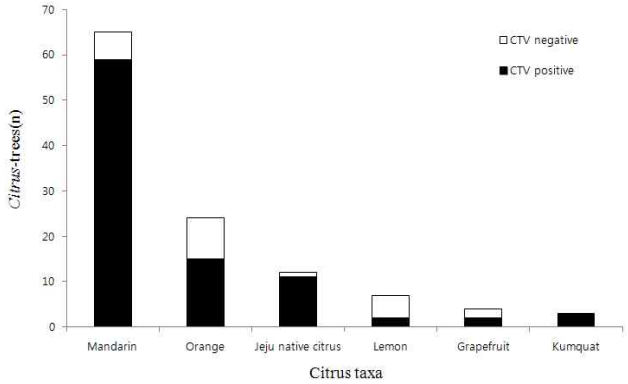

Fig. 1. Detection of CTV of citrus trees originated from germplasm in CRS by TAS-ELISA.

Thermotherapy experiments

Infected 'Setoka' hybrid plants grownin pots inside the green house were completely defoliated by hand and were placed in 3 different levels of temperature such as 30/30℃, 40/40℃ and 50/40℃ during day and night time for efficient level of temperature and virus elimination. A 3 cm long or shorter flushes was also used for the micrograting sources and shoots were collected from the fresh flushes of the chamber grown plants at 40/40℃ for one week.

Preparation of rootstock

Trifoliate orange (*Poncirus trifoliata*) was used as a rootstock. The seeds were extracted from the fruits, washed in tap water, surface dried at room temperature, and applied with fungicide to avoid fungus attack and placed in polyethylene bags for storage at 4℃ until used [9]. The seeds were peeled, disinfected by immersing in 10% Sodium hypochlorite solution containing 2-3 drop of 0.1% Tween-20 for 10 min and were thoroughly rinsed with sterile distilled water and one seed per test tube was cultured in MS medium [10]. The pH of the medium was adjusted at 5.7±0.1 prior to autoclaving at 121℃ for 15 min [11]. The cultures were kept under etiolated conditions at 27℃ and seedlings were grafted after two weeks of development.

Micrografting of scion

Two week old seedlings were decapitated leaving about 2 cm of epicotyl under the laminar flow system. Cotyledons were detached and roots were cut to a length of 2-3 cm. Primodial leaf was placed on the vascular ring tissue at 1cm under top of the decapitated epicotyls. The micrografts were aseptically cultured in MS medium without agar but the bottom was supported with agar medium. The concentration of sugar at 7% was also added in the media. The grafted cultures were kept at constant temperature of 27℃ and 16h daily exposure to light using fluorescent lamp.

Virus test

Direct tissue blotting immunoassay (TAS-ELISA, Agdia Inc., USA) was carried out for mass detection of CTV virus. The degree of chromatic appearance was observed in TAS-ELISA after applying sap of a leaf to a nitrocellulose membrane, reacting with antibody, and putting substrate. SDV test was done by SDV chromato (Mizuho Medy Corp, Japan). Virus-free plantlets obtained from micrografting were used as the control and were identified by reverse transcription polymerase chain reactions (RT PCR). The presence of CTV was confirmed by RT-PCR, which had higher sensitivity than TAS-ELISA. The total RNA was extracted from each leaf sample using TRIzol® LS reagent (Invitrogen Corp. USA). The cDNA product was made by TOPscript^{TM} cDNA Synthesis kit (Enzynomica Corp., KOR) diluted 5-fold with distilled water. Subsequently, PCR amplification was performed in a 17 μl DEPC (diethyl procarbonate) distilled water (Bioneer Corp., KOR), HotStart PCR PreMix (Bioneer, KOR), 1 μl of each primer (Forward and Reverse) and 1 μl of the cDNA product. Virus specific primer set was used (Table 1.) The PCR program for CTV analysis consisted of an initial denaturation for 2 min at 9 5℃; followed by 34 cycles of 1 min at 94℃, 55℃, and 1 min 30 sec at 72℃; and a final extension of 10 min at 72℃. PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. SDV and CTLV assay were done at different temperature s (Table 1) as we mentioned above.

Results and Discussion

Virus indexing

Some *Citrus* and *Fotunella* genera grown in the field and in the glass house were seriously infected by viruses (Fig. 1). About 92 (80%) out of 115 samples from germplasm collection were observed CTV-positive using TAS-ELISA. *Citrus* genera such as sweet orange, mandarins, lemons, grapefruits were figured out to be 62.5%, 90.8%, 28.6%, and 50.0% CTV infected, respectively. Whereas, *Fotunella* genera such as kumquats was observed to be 100% infected. Kim et al [7] reported that the infection rates of CTV, SDV, and CTLV in Jeju Island citrus orchards were found to be 69.8%,

8.6%, and 9.3%, respectively. In addition to 4-year-old progenies (Table 2), CTV-positive plants were observed in different rootstocks by TAS-ELISA. A top grafting of the progenies for reduction of juvenility in a 50-year-old satsuma mandarin intermediate rootstock (71.4% infected) tend to higher the rate of the CTV infection than on 10-year-old trifoliate orange rootstocks (41.2%). However, strong CTV symptom was not observed in micrograft such as withering and similar result was obtained in other study [6]. The progenies might be due to infected mild strain CTV, cross protection or resistance of the trifoliate orange rootstock effect [6]. Depending on a virus strain in scion cultivar and rootstock, CTV can induce one of the three main syndromes such as "severe stunting", "stem pitting" and "seedling yellows", but some mild strains are unnoticeable due to lack of visible symptoms [1]. By nature, CTV usually exists as a mixture of strains [6]. Virus symptoms that occurred in some citrus trees had similar characteristics all over the world. Some of them are considered to have multiple infections [6] with more than two strains of viruses, and mostly appearing with no symptoms on the individuals. In this study, all scions of progenies were not SDV virus symptoms such as boat or beat shaped in leaves [6]. Results of visual observation have been correlated with SDV infection diagnosed by SDV chromato (Table 2).

Thermotherapy and *in vitro* micrografting

CTV-free shoots of cv. 'Setoka' mandarin were obtained

Table 1. Primers for amplification of CTV, SDV and CTLV sequences

Virus	Primer sense	Primer sequence (5′→3′)	Primer name	Product (bp)	TM (℃)	Ref.
CTV	(+)	5′-TACCGTCCCCAAACCAACTA-3′	CTVpo1	738	55	[8]
	(-)	5-CATGGCAGGTTATACAGTAC-3	CTVpo2			[8]
	(+)	5-CGAGGTATCATTCTTCGAGC-3	CTV52	640	55	[5]
	(-)	5-CGCCATAACTCAAGTTGCG-3	CTV32			[5]
SDV	(+)	5′-ACTAGGGATAGCGCCCTAG-3′	SDV FW146	420	55	[4]
	(-)	5′-GGACCGATATTGGGCCAT-3′	SDV RV448			[4]
	(+)	5′-TGCACGGTCTCTCACTCAGGG-3′	SDVPP2-2	800	60	H
	(-)	5′-TCAGCGCTTGTGCCTGGTGG-3′	SDVPP2-2			H
CTLV	(+)	5′-CTCATGATTTGTTTATAATGC-3′	CTLV 3318	1,206	55	[3]
	(-)	5′-CTCAARTACCAYCCACAGAAC-3′	CTLV 4524			[3]
	(+)	5′-ACCTTAGAAGTGACAAATCG-3′	CTLV 140	662	55	[3]
	(-)	5′-ATGCCACTACAGGTGAAAGG-3′	CTLV 802			[3]

H: The primer set was self-developed by Jae Wook Hyun.

Table 2. Level of CTV and SDV infection in 4-year-old citrus progenies by ELISA assay

Rootstock source	Status of virus infection [zy]	
	CTV	SDV
Satsuma mandarin grafted on trifoliate orange	15/21 (71.4%) [x]	0/21
Trifoliate orange	7/17 (41.2%)	0/7
T-test	ns	-

[z]Number of positive(+) progenies / total number of grafted progenies.
[y]CTV and SDV were checked by TAS-ELISA and respectively.
[x]Virus positive ratio.

Table 3. Comparative analysis of the eradication of CTV in 'Setoka' hybrid by different heat treatment

Variety	Control [Z]	Level of temperature in the chamber		
		30/30℃	40/40℃	50/40℃
		One week		
'Setoka'	0/12(0%)	0/11(0%) [Y]	23/26(88.5% [W]) [X]	Not survived

[Z]CTV was checked by TAS-ELISA.
[Y]Number of pathogen-free plants / total number of grafted plants.
[X]Number of pathogen-free shoots/ total number of shoots in infected plants.
[W]CTV-free rate.

Table 4. SDV detection by the cultivar of scions obtained from micrografting along with the thermortherapy in heat charmber

Cultivar	RT-PCR (SDVPP2-2)	RT-PCR (FW146+RV488)	SDV chromato
Hybrid			
'Ehimekashi dai28go'	-[z]	-	-
'Setoka'	-	-	-
'Shiranuhi'	-	-	-
Nucelalr breed			
'Haraejosaeng'	-	-	-
'Pungkwang'	-	-	-
'Samdajosaeng'	-	-	-

[z] +, positive and -, negative. SDV positive cDNA was used as infected mandarin plant.

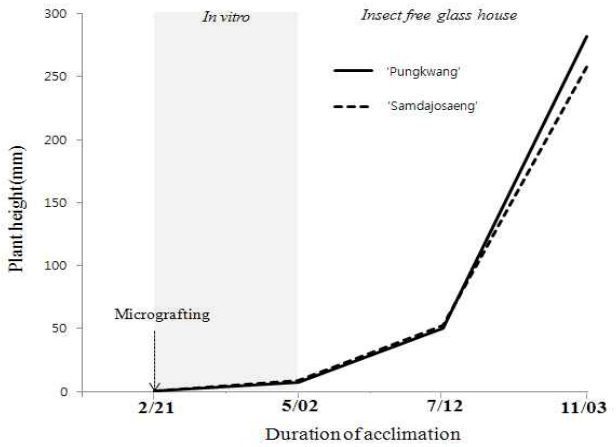

Fig. 2. Comparative analysis of the developmental pattern in madarin type 'Samdajosaeng' and orange type 'pungkwang' of the micrografed trees. Shoot-tip grafted stem-scion ('Pungkwang' and 'Samdajosaeng' / trifoliate orange, months after micrografting) with flush, side-grafted on 2-year-old trifoliate orange rootstock by banding with stretched parafilm on 2nd May.

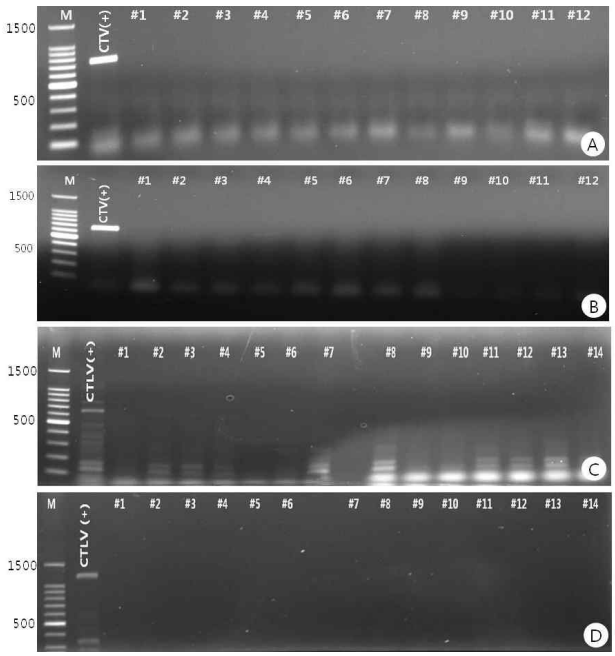

Fig. 3. Agarose gel analysis of micrografted trees and sources by CTV po1+CTVpo2 (738 bp; A), CTV 52+CTV32 (640 bp; B), CTLV3318+4254 (1,206 bp; C) and CTLV802+140 (662 bp; D). Lanes marked 'M' show DNA size markers in 100 bp increments. Numbers on the left of the figure indicate the size of the marker DNA. A and B: #1: 'Setoka (*In vitro*)'; #2~#8: 'Samdajosaeng'; #9 and #10; 'Shiranuhi'; #11: 'Pungkwang'; #12; 'Ehimekashi dai28go', C and D: #1~ #7: 'Samdajosaeng', #8 and #9: 'Shiranuhi'; #10: 'Setoka' (in the heat chamber); #11: 'Setoka' (*In vitro*), #12: 'Harejosang' (in heat chamber), #13: 'Ehimekashi dai28go'; #14: 'Pungkwang'.

by thermotherapy in the heat chamber. After the treatment at a temperature of 40/40℃, plants were tested for the presence of tristeza-virus using TAS-ELSA technique. Results showed that 88.5% of 'Setoka' hybrid was CTV-negative. CTV-free plants of cv. 'Setoka' hybrid were then developed through the micrografting *in vitro*. The *poncirus* genus for rootstock was used. And the ternate compound leaf of trifoliate orange rootstocks and simple leaf of bud-wood could be distinguished easily [14, 15]. Sanitation was done and plants were tested for the presence of tristeza-virus using both TAS-ELISA and RT-PCR indexing tests. The results showed that 20% of 'Setoka' hybrid was CTV-negative. However, surface placement in the study might not be the best. In the future, a more specific and efficient method should be tested to develop and obtain virus-free plants amidst threatening of certain factors. In the study, the grafted plants were successfully acclimatized and survived with robust growth and observed to be a normal phenotype. Certified methodology was obtained in the conservation of plants in insect-proof glass house of nucellar variety of 7 'Samdajosaeng', 1 'Pungkwang', hybrid of 2 'Shiranuhi', and 1 'Ehimekashi dai28go'. Robust growth of CTV-free plants of cv. "Samdajosaeng" and "Pungkwang"

nucellar varieties were observed from shoot-tip grafted stem-scion with flush, side-grafted on 2-year-old trifoliate orange rootstock by banding with stretched parafilm on May (Fig. 2). No CTV symptoms on micrografted plants have been found.

Detection of viruses by RT-PCR

Furthermore, it will be necessary to use RT-PCR based for sensitive and reliable CTV diagnosis since the detection will be obscure due to a low concentration and seasonal variations of the virus from different sources. RT-PCR-based detection of CTV was done using primer pairs CTV PO1+PO2 and CTV52+32, respectively. In RT-PCR assays, one distinct amplification pattern was observed (Fig. 3). Simplex RT-PCR analyses revealed that all micrografted plants were not subjected to amplification and none infected. The same results were obtained in RT-PCR and in TAS-ELISA. The RT-PCR assays are proved to be a specific, can be reproduced and a reliable method for the simultaneous detection of CTV and can determine multiple virus infection analysis. The RT-PCR based detection of SDV and CTLV were done-using primer pairs SDV PP2-2 and FW146+RV488 for SDV, and CTLV3318+4524 and CTLV802+140 for CTLV, respectively. It revealed that all the micrografted and grafted plants were not subjected to amplication. The same resuls of RT-PCR in SDV and TAS-ELISA were obtained. Also, there were no micrografted plants which observed any specific symptom of CTLV. The result obtained was in agreement to RT-PCR repeatedly. CTV positive plant of cv. 'Harejosaeng' in the heat chamber was found to be negative in the SDV and CTLV through the RT-PCR assay. Thus, micrografting in combination of thermotherapy method for the conservation of 11 virus-free plants in an insect-proof glass house (Fig. 4) was employed. Consequently, through this method, the CTV was easily eliminated and as a result, all viruses that fatally affected citrus from shoot tips even without antiviral substances were completely eradicated.

The specific temperature treatment, designed and set up in this work, is proved to be a reliable method for the efficient elimination of CTV. Combination of micrografting method using citrus shoot tip can be routinely adopted for the propagation of pathogen-free citrus plants.

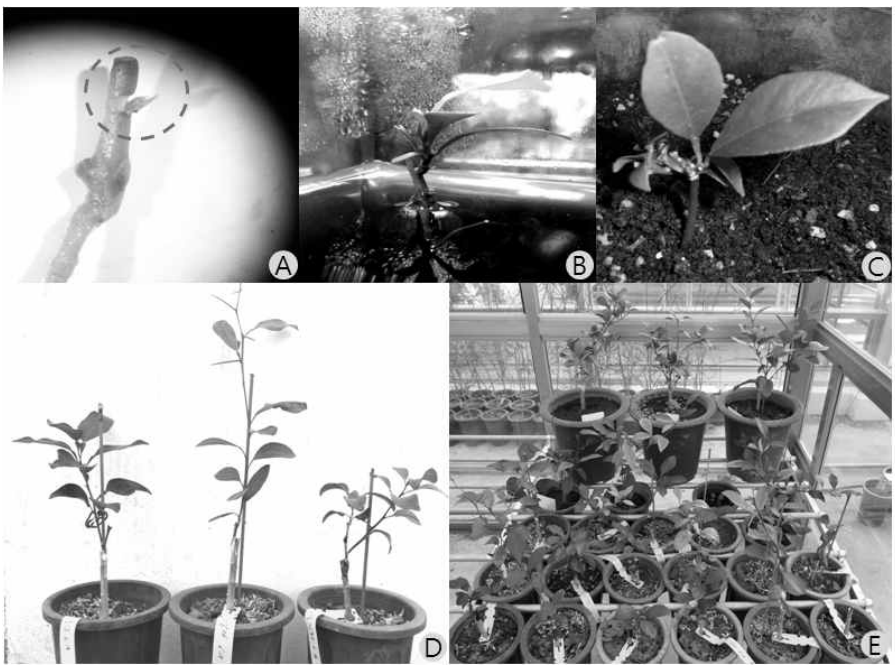

Fig. 4. Micrografting shape for virus-free stock. A, the shape of leaf primordia of 'Setoka' hybrid shoot-tip micrografted in of trifoliate orange (*Poncirus trifoliata*) rootstock; B, leaves of sprout from 'Setoka' hybrid shoot-tip micrografted in trifoliate orange (*P. trifoliata*) rootstock; C, the acclimating staus of 'Ehimekashi dai28go' hybrid / trifoliate orange was planted in artificial soil in a insect free glass house; D, Shoot-tip grafted stem-scion ('Pungkwang', 'Samdajosaeng' and 'Shiranuhi' hybrid / trifoliate orange, months after micrografting) with flush, side-grafted on 2-year-old trifoliate orange rootstock by banding with stretched parafilm; E, Micrografted with along the thermotherapy under a insect free glass house.

Acknowledgments

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초록 : CTV 바이러스 보균 감귤나무로부터 열처리와 경정접목을 통한 바이러스 제거

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본 연구는 바이러스에 감염된 감귤나무로부터 열처리와 경정접목을 병행 처리하여 바이러스를 제거코자 수행하였다. '세토카' 교잡종을 포함한 6품종을 재료로서 사용하였으며 이를 조사된 모든 개체에서는 외관적으로 바이러스 감염 증상이 관찰되지 않았으나 TAS - ELISA 검정을 통해 전원 CTV에 감염되어 있음이 확인되었다. '세토카'를 대상으로 특정 온도 시험을 수행한 결과, 주・야간 40℃ 온도 조건에서 높은 비율로 바이러스가 제거되었다. 항혈청법으로 할 수 있었으며, 그 처리에서 획득된 무균의 신초를 이용하여 기내 파종된 탱자 대목에 경정접목하였다. 경정 접목된 모든 개체에서는 바이러스 병징이 발견되지 않았고 TAS-ELISA와 SDV 크로마토법에 의해서도 무균이 확인되었다. CTV, SDV와 CTLV를 대상으로 한 RT-PCR 검사를 통해서도 극소량의 바이러스조차 발견되지 않았다. 따라서, 열처리와 경정접목을 통해 '세토카', '삼다조생', '풍광', '부지화' 및 '에히메카시 다이 28고' 품종에서 총 11개의 무균의 식물체를 획득하였다.