Apple Stem Grooving Virus(ASGV)無毒のナシ植物体の作出

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Development of Pear Plants Free from Apple Stem Grooving Virus (ASGV) †1

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I Introduction

Virus diseases causing decline of vigor, yield and quality in fruit trees have become a serious problem. In *Pyrus* plants the incidence of graft transmitted diseases, e. g., pear necrotic spot (Kishi *et al.* 1972), pear vein yellows, pear ring pattern mosaic and quince sooty ring spot (Takanashi *et al.* 1980) were reported in Japan. In addition, pear trees have been found to be latently infected with apple stem grooving virus (ASGV) in Japan (Takanashi 1983). In South Africa, it has also been reported that ASGV is associated with the decline of Packham's Triumph pear on seedling rootstock (Van Siebert and Engelbrecht 1982).

As the mother stock trees of commercially important cvs. 'Hosui', 'Kosui' and 'La France' in our Research Station were found to be infected with ASGV based on indexing by sap inoculation to some herbaceous test plants (Takanashi unpublished data), attempts were made to obtain ASGV-free plants of the above three cultivars.

Thermotherapy has been considered to be an effective and convenient method for obtaining pathogen-free plant materials (Calavan *et al.* 1972, Nyland and Goheen 1969). However, certain pathogens can not be readily eliminated by the application of thermotherapy (Murashige *et al.* 1972), as in the case of ASGV in apple trees (Shu-Ching Huang and Millikan 1980). Therefore, the effect of meristem culture combined with thermotherapy was evaluated for this purpose.

II Materials and Methods

Plant materials and heat treatment

Trees of Japanese pear (*Pyrus serotina* 'Hosui' and 'Kosui') and European pear (*P. communis* 'La France') which were grown in the fields of the Fruit Tree Research Station (FTRS) at Tsukuba were used as original plants. Budwoods taken from these trees were grafted on pear seedlings in December, 1984. In the next spring, after preincubation for 1 week at 35 / 30℃ (day time /night time, each 12hrs), these grafted materials were incubated in a heat chamber and at a controlled temperature of 38 / 33℃ during 5, 10 or 15 weeks.

Tissue culture

The terminal part of the current shoots which grew during the heat treatment was excised over a 30 to 50 mm length and sterilized by stirring for 15 min in a 0.5% sodium hypochlorite solution containing a few drops of Tween 20, and thereafter rinsed 3 times in sterile distilled water. Apical tissues of the shoots (0.2 mm in length) were cut out and placed on half strength Murashige and Skoog's (MS) (1962) medium, supplemented with 5 μM 6-benzylaminopurine, 3% sucrose and 0.8% bacto agar (pH 5.8). Then, they were incubated at 28℃ under a 16hr-photoperiod using 3000-lux

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fluorescent light according to Hirabayashi *et al.* (1987). Proliferating shoots of 'La France' were cut out and dipped into 1 mM 3-indole butyric acid (IBA) for five seconds to induce rooting and placed on a sterile vermiculite medium containing MS solution. In the case of 'Hosui' and 'Kosui', the cultured shoots (20 to 30 mm in length) were directly cleft-grafted on green seedlings of pear which were considered to be pathogen-free, and covered with polyethylene bags to prevent drying.

Indirect ELISA using $F(ab')_2$ fragment

Newly expanded leaves of the plants obtained after meristem culture and those of the original plants were excised in spring and kept in a freezer at -80℃. ASGV was assayed by a modified ELISA procedure using $F(ab')_2$ fragment according to Yanase *et al.* (1986). The frozen leaves were homogenized in 10mM phosphate buffer (pH7.0) (20ml/g leaf fr.wt.) containing 0.5% Tween 20, 2% polyvinylpyrrolidone (PVP), 0.2% ovalbumin and 0.01 M sodium N, N-diethyldithiocarbamate trihydrate (DIECA) (buffer A). The procedure of ELISA was as follows : solution (250 μl) containing $F(ab')_2$ fragment of IgG against ASGV (1.0 μg / ml) diluted 1 : 250 in carbonate-bicarbonate buffer (pH9.6) was added to each well of a plate (Dynatech Immulon I) and incubated overnight at 4℃. After the plate was washed 3 times by filling with PBS-Tween, 250 μl of the test sample diluted in 1 : 20 w/v in buffer A was added, then incubated overnight at 4℃. The plate was washed as described before and then 250 μl of a solution of 0.02% antiserum and 0.005% enzyme-labelled protein A in PBS-Tween containing 0.1% bovine albumin (Fraction V) and 1% (w/v) crude sap of healthy *C. quinoa* was added and incubated overnight at 4℃. After the plate was washed, 250 μl of a peroxidase substrate solution was added, and the plate was then incubated for 1hr in the dark at room temperature. The reaction was stopped with 50 μl of 2.5M H_2SO_4. The results were assessed both visually and spectrophotometrically at 492 nm absorbance. The reactions giving an obvious brown color or absorbance values greater than 2 times the average ones for healthy samples (pear seedlings) were regarded as positive.

III Results

Data on the elongation of the current shoots during the heat treatment are presented in Table 1. There was no consistent relation between shoot elongation and the duration of the heat treatment. In

Table 1. Elongation of current shoots during heat treatment.

Cultivar	Duration of heat treatment (weeks)	Elongation (cm)		
		0 - 5	5 -15	15-
		No. of plantlets		
Kosui	5	2	1	2
	10	1	0	3
	15	0	1	4
Hosui	5	5	0	2
	10	2	0	2
	15	1	1	6
La France	5	3	4	0
	10	13	1	0
	15	8	0	2

'Hosui' and 'Kosui', the shoots tended to be longer (>15cm) during the heat treatment applied for 10 and 15 weeks compared with that of 5 weeks. On the contrary, the shoots of 'La France' hardly elongated during the heat treatment applied for 10 and 15 weeks. In the current experiment, tissue cultures were also induced from shoots which hardly elongated during the heat treatment. Shoots of 'Hosui' and 'Kosui' propagated *in vitro* were directly cleft-grafted on green seedlings due to the difficulty of *in vitro* rooting of the *P. serotina* plants. In 'La France', rooting of the cultured shoots occurred easily by dipping in auxin (IBA), although most of the rooted shoots were damaged by fungi and other agents in the process of acclimatization. As a result, 15 plants were obtained through a series of treatments (Table 2).

Table 2. Number of plants obtained by heat treatment and meristem culture.

Cultivar	Duration of heat treatment (weeks)				
	0	5	10	15	
	No. of plants obtained				Total no.
Kosui	0	2	1	3	6
Hosui	0	1	1	3	5
La France	1	3	0	0	4

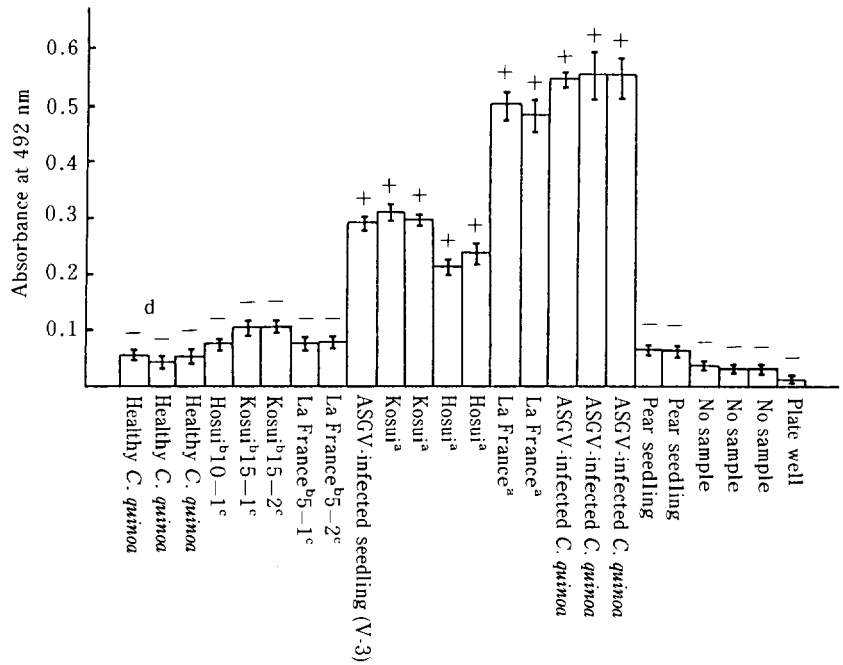

Fig. 1. Detection of ASGV in pear tree by modified indirect ELISA.

[a] Original trees infected with ASGV

[b] Treated plants free from ASGV

[c] 10-1 ; Duration of heat treatment (wks) − No. of plantlets

[d] Visual assessment

Differences in absorbance of spectrophotometry at 492 nm between healthy and ASGV infected plants are shown in Fig. 1. Absorbance in healthy *C. quinoa* plants and healthy pear seedlings gave a low value (0.05 – 0.06), while that in ASGV-infected *C. quinoa* plants and seedlings infected with V-3 which is one of the pear isolates of ASGV (Takanashi, 1983) gave a high value (0.29 – 0.55). A brown color could be seen in the well when the absorbance value exceeded 0.20. It was evident that the original pear plants grown in the fields of FTRS were infected with ASGV. Based on these results, the clones 'Hosui' 10–1, 'Kosui' 15–1, 'Kosui' 15–2, 'La France' 5–1 and 'La France' 5–2 which were subjected to a combination of heat treatment and meristem culture, were found to be free from ASGV (Fig. 1). Visual assessment of the color of the wells was consistent with the spectrophotometric absorbance readings.

IV Discussion

Shoot-tip culture has been used to obtain virus-free plants (Mori *et al.*, 1969 ; Mori, 1971). Morel and Martin (1952, 1955) demonstrated that meristems were not infected with the virus. However, Hollings and Stone (1964) showed that more than half of the meristem domes in carnation was infected with carnation mosaic virus. Walkey and Cooper (1972) showed that cherry leaf roll virus in tobacco was eliminated during tissue culture, unlike other viruses such as cucumber mosaic virus. Thus, the elimination of virus through tissue culture may depend on the virus type. In this case, shoot-tip culture combined with a high temperature treatment was found to be useful to eliminate such viruses (Walkey and Cooper, 1972), and this method could be applied to eliminate ASGV. Yamaga (1987) demonstrated that shoot elongation during heat treatment was important to eliminate ASGV in apple tree. However, we were able to obtain ASGV-free pear plants from *in vitro* propagated shoots which showed almost no growth during the heat treatment. In this experiment, *in vitro* plants obtained after heat treatment were subcultured to fresh medium more than eight times every month, while Yamaga conducted ELISA after only four subcultures. Thus, the elimination of virus may become easy after repeated subcultures. As virus inactivation may be affected by the cell metabolism, further studies should be carried out to verify this assumption.

The ASGV-free pear plants which were obtained in the three cultivars are expected to be also free from other viruses causing pear necrotic spot and pear vein yellows, because they were obtained by meristem culture in combination with heat treatment. If the ASGV-free plants were also free from other viruses, they may be utilized as mother trees for the propagation of virus free trees of these three cultivars.

V Summary

Meristem culture of pear ('Kosui', 'Hosui' and 'La France') in combination with heat treatment was carried out to obtain ASGV-free plants.

There was no consistent relation between the shoot elongation and the duration of the heat treatment (Table 1). Current shoots of 'Hosui' and 'Kosui' tended to elongate more during a heat treatment of 10 and 15 weeks whereas those of 'La France' hardly elongated during the heat treatment.

Green wood grafting of cultured shoots was found to be a useful method for establishing plantlets of Japanese pear such as 'Hosui' and 'Kosui'. In the case of 'La France', the induction of *in vitro* rooting was easy, although most of the rooted shoots were damaged in the process of acclimatization.

As a result, 15 plants were obtained through heat treatment and meristem culture (Table 2).

There were clear differences in the absorbance values in spectrophotometry in the ELISA test between healthy and ASGV infected plants (Fig. 1). Visual assessment was consistent with the spectrophotometric absorbance results. ASGV-free plants of 'Hosui', 'Kosui' and 'La France' were obtained in this study.

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Apple Stem Grooving Virus (ASGV)

無毒のナシ植物体の作出

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摘　　要

熱処理(昼間38℃，夜間33℃)と茎頂培養を併用して，ASGV 無毒のナシ植物体（'豊水'，'幸水'，'ラ・フランス'）の作出を試みた．

1．熱処理期間の長さと新梢の伸びの関係をみると，'豊水'，'幸水'では10週間以上の熱処理期間でも新梢の伸びがみられるのに対し，'ラ・フランス'では15週間処理しても伸びの悪い個体が多かった（Table 1）．

2．'豊水'，'幸水'の培養した茎葉を直接ナシの実生に接ぎ木し鉢上げを試みた．一方，'ラ・フランス'は組織培養で容易に発根したが，多くの個体が鉢上げ馴化の過程で糸状菌の感染，その他の原因のために枯死し，結果的に'豊水'，'幸水'及び'ラ・フランス'合わせて15個体が得られた（Table 2）．

3．ASGV の検定を間接 ELISA により行った．無毒化処理で得られた個体中，供試材料の十分取れる5個体と，ASGV に感染している植物あるいは無毒の材料（実生苗および健全キノア）を含めて，検定に供した．無毒化処理で得られた個体の吸光度は，既知の無毒の材料のそれと近い値となり，ASGV 無毒であると判定された（Fig. 1）．

無毒化の困難とされる ASGV を無毒にした植物体は他のウイルスについても無毒である可能性が高い．今後，他のウイルスも無毒であることと品種特性に変異のないことを確かめれば，本植物体は重要な3品種の増殖母樹としての活用が期待できる．