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Virus resistance in transgenic sweetpotato [*Ipomoea batatas* L. (Lam)] expressing the coat protein gene of sweet potato feathery mottle virus

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Abstract One of the most-serious diseases of sweet potato [*Ipomoea batatas* (L.) Lam] is russet crack disease caused by sweet potato feathery mottle virus (SPFMV). We constructed an expression vector carrying the coat protein (CP) and hygromycin phosphotransferase (hpt) genes driven by cauliflower mosaic virus 35 S promoters. Accordingly, we introduced the expression vector into sweet potato variety Chikei 682-11 by the electroporation method. Among 449 calli obtained after antibiotic selection, 19 plants from seven independent calli grew to form adventitious shoots. Three transgenic lines were obtained from independent calli, based on analysis of the CP and hpt genes. The transcription and translation of the CP gene were shown in these transgenic lines by Northern- and Western-blot analyses. To assay the virus resistance of the transgenic lines, each line was vegetatively propagated and then grafted with morning glory (*Ipomoea nil*) that had been infected with SPFMV-S. A PAS-ELISA assay with polyclonal antiserum of the CP demonstrated that virus accumulation 3 months after grafting with the infected morning glory was suppressed in the transgenic lines as compared with non-transgenic ones. These transgenic lines were shown to be highly resistant not only to primary but also to secondary infection by SPFMV-S. Thus we concluded that the three transgenic lines with the CP gene of SPFMV-S can be used for coat protein-mediated resistance to the virus.

Keywords Sweet potato feathery mottle virus · Coat protein gene · Electroporation · Virus resistance · Transgenic sweetpotato

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Introduction

Sweet potato is an important crop in tropical, subtropical and temperate areas, and also an efficient biomass-producing plant for starch. Russet crack disease, caused by an RNA virus named sweet potato feathery mottle virus (SPFMV), is one of the most serious constraints to the production and the quality of sweet potato. The russet crack (RC) and the severe (S) strains have been isolated in U.S.A. (Cali and Moyer 1981) and in Japan (Usugi et al. 1994), respectively. Because the virus is transmitted by aphids (*Myzus persicae*) and the infected tuberous roots are used for vegetative propagation, the disease spreads easily in the field and no effective pesticides are available. It is possible to produce virus-free clean seed stocks, since the virus is not transmitted to the seed. However, propagation by the seeds is neither economical nor common, owing to the possibility of segregation of the agricultural phenotypes.

Among the sweet potato genetic resources at the International Potato Center (CIP), some varieties were found to be resistant to the russet crack disease (Annual Report CIP 1989). The degree of the resistance, however, is inadequate and it takes a long time to introgress the resistant gene(s) into the hexaploid genome of sweet potato by conventional breeding.

Shoot-apex culture has been applied for sweetpotato to overcome the virus damage in Japan (Nagata 1984). This method is fairly effective for supplying virus-free young sweet potato, but it is costly, and the initially

virus-free sweet potato can be often become infected after cultivation. Therefore, it would be worthwhile to breed SPFMV resistant varieties using transgenic techniques, either with the resistant gene(s) of sweet potato or other gene(s) conditioning virus resistance.

Since the production of transgenic tobacco transformed with the tobacco mosaic virus coat-protein gene (Powel-Abel et al. 1986), coat-protein mediated resistance has been reported for a considerable number of plant viruses (Fitchen and Beachy 1993). We have sequenced the entire genome (10.8 kb) of SPFMV-S to predict the sequences coding for CP at the 3′-terminus (Mori et al. 1995; Sakai et al. 1997). The sequences of the SPFMV -RC (Cali and Moyer 1981) and SPFMV-S CP genes showed high homology to each other.

The regeneration method has been established for several sweet potato varieties (Murata et al. 1987, 1994). The efficiency of regeneration was extremely low, less than 10^{-8} (Okada et al. 1995). The combination of hygromycin and hpt genes was found to be more effective than the kanamycin/neomycin phosphotransferase II (NPT II) gene for screening the antibiotic-resistant calli after electroporation (Okada et al. 1995). The regeneration method was developed for protoplasts from mesophyll and cell suspension cultures of sweet potato varieties Chugoku No.25 and Chikei 682–11, which were bred for starch production and which are susceptible to SPFMV-S.

In this study, we constructed an expression vector haboring the CP gene of SPFMV-S and the hpt gene, obtained transgenic lines of sweet potato, var. Chikei 682–11 by electroporation, and showed that those were resistant to the virus.

Materials and methods

An expression vector carrying the CP and hpt genes driven by CaMV 35 S promoter

The cDNA clone, pVC-1 (1.8 kb), contained a portion of the 3′ terminal sequences of the nuclear inclusion protein-b (NIb) gene, full sequences (1.1 kb) for the CP gene and non-coding sequences including a polyA tail, was obtained from cDNA library from SPFMV RNA (Mori et al. 1995). Using this plasmid, Sonoda et al. (1999) constructed the expression-vector plasmid pMM4, which harbors the CP, 3′ non-coding region with poly A instead of the β-glucuronidase (GUS) gene in pBI 121 (Clontech). The CP expression cassete (*Sph*I and the *Sac*I fragment) from pMM4 was cloned into *Sph*I and *Sac*I sites of pREXH-1 (kindly provided by Dr. H. Hirochika: National Institute of Agrobiological Resources), to include the hpt gene driven by the CaMV 35 S promoter as a selectable marker. The resultant expression vector for CP and the hpt genes was referred to as pMMHA-4.

Plasmid DNA for fluorometric GUS activity

The plasmid GUS/NIa, used for fluorometric GUS assay, was obtained from Dr. Carrington (then at Texas A&M University; presently at Washington State University). The plasmid GUS/NIa contains the β-glucuronidase (GUS) gene fused to the nuclear inclusion protein-a (NIa) gene of tobacco etch virus between the CaMV 35 S promoter and the NOS terminator (Restropo et al. 1990). GUS activity was detected fluorometrically using 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG) as a substrate according to the method of Jefferson et al. (1987). Fluorescence emission at 455 nm was measured by fluorometer under an excitation wavelength of 365 nm.

Protoplast isolation from the mesophyll of sweet potato leaves

Mesophyll protoplasts of sweet potato vars. Chugoku No. 25 and Chikei 682–11 were isolated from in vitro shoot tip cultures. Abaxial surfaces of the leaves were scratched with a razor blade. Mesophyll cells were plasmolysed for 1 h in 0.3 M sorbitol and 0.05 M $CaCl_2 \cdot 2H_2O$ at pH 5.6. Protoplasts were released using an enzyme solution [K3 medium (Nagy and Maliga 1976) containing 0.1% pectolyase Y-23, 2.0% cellulase Onozuka RS, 5 mM $CaCl_2 \cdot 2H_2O$, 0.5 M mannitol at pH 5.6]. The further preparation of protoplasts was performed as described previously (Murata et al. 1994, 1995).

Electroporation of protoplasts

Protoplasts (about 4×10^6) released by the enzyme treatments were rinsed with an equal amount of electroporation buffer containing 0.5 M mannitol at pH 5.6. For determination of protoplast viability, total and viable protoplast numbers were counted by phase-contrast microscopy using a FuchsRosenthal hemocytometer before and after staining with Evan's blue, respectively. The protoplast suspensions were adjusted to 5×10^5/ml. We compared three wave shapes of pulses from the two electroporation systems: the rectangular wave and the constant attenuated wave from HARIO Cell Fusion Provision (CFP-1), and the variable attenuated wave from Promega X-CELL 450. The results of electroporation without DNAs showed that 35–75% viability was obtained using a commercial cuvette (0.4-cm width, Bio-Rad), 10 ms of the pulse length, and 375–625 V/cm field strength for HARIO CFP-1 (data not shown). Twenty micrograms of plasmid DNA and 20 μg of carrier DNA (calf thymus) were added to 0.8 ml of the protoplasts (4×10^5 in 0.5 M mannitol solution) in a chilled cuvette. This mixture was incubated at 0°C for 5 min and mixed well by gentle inversion before electroporation. After the electric pulse, the solution containing the protoplasts was held on ice for 10 min to prolong the open-pore status.

Regeneration of transformed cultures

The electroporated protoplasts (4×10^5 cells) were diluted into an equal volume of 0.5 M mannitol, centrifuged, and re-suspended with 2 ml of 0.5 M mannitol. Two milliliters of 2×KM8P medium (Kao and Michailuk 1975) containing 0.1 mg/l of zeatin (ZEA), 0.1 mg/ of l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 M mannitol, and 10 g/l of sucrose at pH 5.6 were then added to the protoplast suspension (10^5/ml). The suspension was cultured on 1×KM8P medium plates (60×15 mm) or liquid medium. The protoplasts were incubated at 28°C in the dark for 3 days before the assay of GUS activity, and for 14 days before counting the plating efficiency. Thereafter, growing calli were transferred to a fresh medium every 2 weeks.

Sensitivities of sweet potato protoplasts to hygromycin and kanamycin

Protoplasts were spread on 1×KM8P plates (60×15 mm) at a concentration of 10^5/ml and cultured at 28°C in the dark for 84 days. By that time, the calli had grown to be 1–3 mm in diameter and were transferred to modified MS medium (Murashige and Skoog 1962) plates containing 800 mg/l of NH_4NO_3, 1.4 g/l of KNO_3, 0.5 mg/l of 2,4-D, 1.0 mg/l of abscisic acid (ABA), 0.5 mg/l of ZEA, 20 g/l sucrose, 2.0 g/l of Gellan Gum and different concen-

trations of antibiotics, kanamycin and hygromycin B (0, 5, 10, 20, 30, 40 and 50 mg/l). After the antibiotic treatment at 28°C in 16 h – daylight for 30 days, the length, width, and the color of the calli were observed to determine an optimal concentration of the antibiotic for antibiotic selection of the transformants.

Fluorometric GUS assay

Three days after electroporation, cells were collected by centrifugation at 750 rpm for 5 min and re-suspended in 400 µl of extraction buffer containing 50 mM NaH_2PO_4, 10 mM EDTA, 0.1% TritonX-100, 0.1% sodium lauryl sarcosine and 10 mM of β-mercaptoethanol. The GUS assay was carried out as reported (Jefferson et al. 1987).

In vitro selection of hygromycin-resistant calli and plant regeneration

Eighty four days after culture initiation, cell colonies were plated onto a modified MS medium containing hygromycin B (30 mg/l for Chugoku No. 25, 40 mg/l for Chikei 682-11). One month later, the micro-calli that developed in the presence or absence of hygromycin were transferred to an MS medium supplemented with 0.5 mg/l of ZEA, 30 g/l of sucrose and 2.0 g/l of Gellan Gum for regeneration. Regenerated shoots were individually transferred to hormone-free MS medium to induce rooting. Regenerated plants were transferred to soil and further maintained in a greenhouse.

PCR analysis of hygromycin-resistant calli

DNA was extracted from calli (100-mg fresh weight) using the SDS-potassium acetate method. The reaction mixture (50 µl) contained 100 ng of DNA, 200 µM of dNTPs, 1 µM of each primer, 0.5 units of *Taq* DNA polymerase and 1×Taq polymerase buffer. Samples were heated to 95°C for 2 min and then subjected to 35 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 60°C. PCR products were separated by 1.0% agarose-gel electrophoresis and visualized with ethidium bromide. The 24-mer forward primer (VCP-5) for the SPFMV-S CP gene was 5′-GTA CTG AAT TCA AAG ATG CGG GAG-3′, corresponding to nucleotides 9661–9684 of the SPFMV-S genome. The 23-mer reverse primer (VCP-7) of 5′-GCA CAC CCC TCA TTC CTA AGA GG-3′ was complementary to the nucleotide sequence 10571–10593. The 20-mer forward primer (HPT-1) for the hpt gene was 5′-AAG ACC TGC CTG AAA CCG AA-3′, corresponding to nucleotides 512–531. The 20-mer reverse primer (HPT-2) of 5′-AAG CTC TGA TAG AGT TGG TC-3′ was complementary to the nucleotide sequence 1024–1043 (Gritz and Davies 1983).

PCR analysis and Southern hybridization of regenerated plants

DNA was extracted from the leaves of *in vitro* regenerated plants using the CTAB method (Murray and Thompson 1980). PCR analysis using primer (VCP-5 to VCP-7) was the same as that in hygromycin resistant calli. To amplify the DNA segment from the transgene, the 20-mer forward primer (35S-1) for the CaMV 35S promoter (5′-CCA TCG TTG AAG ATG CCT CT-3′), corresponding to nucleotides –211 to –192 of the CaMV 35 S promoter, was used in conjunction with the 23-mer reverse primer (VCP-6; 5′-CTC ATC ACC ATC CAT CAT AGT CC-3′), complementary to the nucleotide sequence 10186–10208 of the SPFMV-S genome.

For Southern blot analysis, 5 µg of DNA were digested with either *Eco*RI or *Xba*I and electrophoresed in an 0.8% agarose gel. The gel was blotted to a nylon membrane. The probe DNA of the 1.3-kb *Eco*RI fragment for the SPFMV-S CP or the 1.1-kb *Bam*HI fragment for the hpt in the pMMHA-4 was labeled by the random primer method with α-^{32}P-dCTP using a Megaprime labeling kit (Amersham) and hybridized to the membrane-bound DNA (Church and Gilbert 1984).

RT-PCR and Northern analysis of SPFMV-S CP gene in transgenic sweet potato

Total RNA was isolated from leaves of *in vitro* plants following the guanidinium isothiocyanate method (Strohman et al. 1977). RT-PCR was performed using the GeneAmp RNA PCR kit (Takara) according to the manufacturer's protocol. After first-strand-cDNA synthesis, the reaction mixture was used for DNA amplification. The following thermal cycling scheme was used for 35 cycles: initial denaturation at 95°C for 2 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and DNA synthesis at 60°C for 60 s. We used the same primers (VCP-5 to VCP-7) as for hygromycin-resistant calli. PCR products were separated by 1.0% agarose-gel electrophoresis and visualized with ethidium bromide.

For Northern-blot analysis, RNAs were separated using 20 µg of total RNA by electrophoresis in a 1.2% agarose/formaldehyde gel, and transferred to a nylon membrane. Probe and hybridization conditions were the same as those in Southern hybridization.

Western blot analysis

Total protein was extracted from leaves of *in vitro* grown plants. For protein extraction, leaf tissue (50 mg) from each transgenic and non-transgenic line was ground in 400 µl of cold 2×GMSB (30% Glycerin, 15% β-mercaptoethanol, 6.0% SDS, 0.01% BPB) and 400 µl of cold 0.1 M phosphate buffer. The homogenate was transferred into a microtube and boiled for 5 min. The extracts were centrifuged at 10,000 rpm for 5 min. A 15-µl aliquot of the extract (approximately 5 µg) was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The Western immunoblot analysis was performed according to the modified protocols (Ohnuki, unpublished) as follows. The blotted membrane was immersed in blocking solution containing 2.0% skim milk in PBST (0.02 M phosphate buffer, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) for 30 min at room temperature. The membrane was then washed twice with PBST and incubated for 90 min at room temperature with anti-SPFMV-S CP antiserum diluted 1/2000 with the sap of healthy morning glory in PBST. The membrane was then washed twice with PBST. A secondary antibody conjugated Protein A-alkaline phosphatase diluted 1/5000 with 1.0% ski m milk in PBST was added to the membrane and agitated gently for 90 min at room temperature. The membrane was then washed in PBST, followed by washing in distilled water. The presence of the coat protein was visualized on the filter using the chromogenic substrates 5-bromo-4-chloro-3-indyolyl phosphate (BCIP) or nitroblue terazolium (NBT) (Sambrook et al. 1989).

Graft-inoculation of sweet potato with SPFMV-S

The homogenate from SPFMV-S-infected morning glory (*Ipomoea nil*) leaves was inoculated onto cotyledons of morning glory plants using the carborundum method (Usugi et al. 1994) and the plants were grown for 3 weeks. In the infected morning glory, symptoms of vein clearing typical for SPFMV-S appeared on the first and the second true leaves. A total of five sweet potato clones were propagated by cuttings that were rooted and grown for 3 weeks. The five clones were inoculated by grafting with virus-infected morning glory and kept for 3 months in a greenhouse at 25°C. This inoculation period was sufficient to allow infection of the virus from the top of the morning glory to the bottom of the sweet potato through a graft (Usugi et al. 1994).

Three months after the graft inoculation, the leaves were homogenated with PBST and assayed for the presence of the virus by polyclonal antiserum for SPFMV-S CP, according to the modified protein-A sandwich ELISA (PAS-ELISA) method (Usugi et al. 1990). In the PAS-ELISA assay, protein A-alkaline phosphatase (ZYMED laboratories Co. Ltd.) was used after 1/500 dilution in 0.05 M Na_2CO_3 solution at pH 9.6.

Table 1 Frequency of stable transformation of electroporated protoplasts

Variety	Field strength (V/cm)	No. of treated protoplasts ($\times 10^5$)	No. of resistant calli[a]	Stable transformation frequency[b] ($\times 10^{-5}$)
Chugoku No. 25	450	72	58	0.8
	500	72	240	3.3
	550	72	192	2.7
	625	68	161	2.4
Chikei 682–11	375	52	130	2.5
	450	60	195	3.3
	500	76	209	2.8
	550	60	240	4.0
	625	48	152	3.2

[a] Number of calli obtained on modified MS medium supplemented with hygromycin B
[b] Stable transformation frequency was calculated as the number of resistant calli divided by the number of electroporated protoplasts

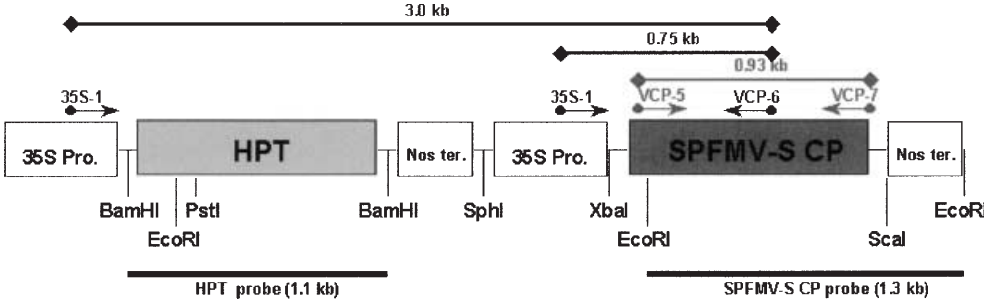

Fig. 1 Schematic representation of plasmid pMMHA-4. The restriction sites, probe (hpt, 1.1 kb; SPFMV-S CP, 1.3 kb) and primers (VCP-5, VCP-6, VCP-7 and 35S-1) are indicated. The *bold lines* above represent the PCR amplification fragments (VCP-5 to VCP-7, 0.93 kb; 35S-1 to VCP-6, 3.0 kb and 0.75 kb)

Results

Transient expression of the plasmid GUS/NIa was assayed by a fluorometric method to identify optimal electroporation conditions for Chugoku No.25 and Chikei 682–11. The viability of the protoplasts decreased with higher voltage in both varieties. Detectable levels of the GUS gene expression were obtained with the use of 450–625 V/cm in the electroporation of Chugoku No.25, and 375–625 V/cm for Chikei 682–11 (data not shown). Under these conditions, the plasmid pMMHA-4 (Fig. 1) was electroporated into protoplasts. A total of 651 and 926 hygromycin-resistant calli were obtained from 28.4×10^6 (Chugoku No.25) and 29.6×10^6 (Chikei 682–11) protoplasts, respectively. The frequency of transformation under a particular field strength was calculated as the number of hygromycin-resistant calli divided by the number of electroporated protoplasts. The highest frequency was obtained using electric fields of 500 V/cm for Chugoku No.25 and 550 V/cm for Chikei 682–11. In all treatments, colony formation was observed on the selection medium at a frequency of about 10^{-5} (Table 1; Fig. 2a).

For a subset of the hygromycin-resistant calli, PCR analysis was carried out to detect the SPFMV-S of CP gene. The frequency of detection of CP gene in the calli was 96% in Chugoku No.25 and 100% in Chikei 682–11 (data not shown). These calli were transferred to the regeneration medium. As the experimental control without electroporation, 430 and 420 calli of Chugoku No.25 and Chikei 682–11 were examined, respectively. Twenty four calli of the Chugoku No. 25 and 36 calli of the Chikei 682–11 formed adventitious roots, a few calli formed

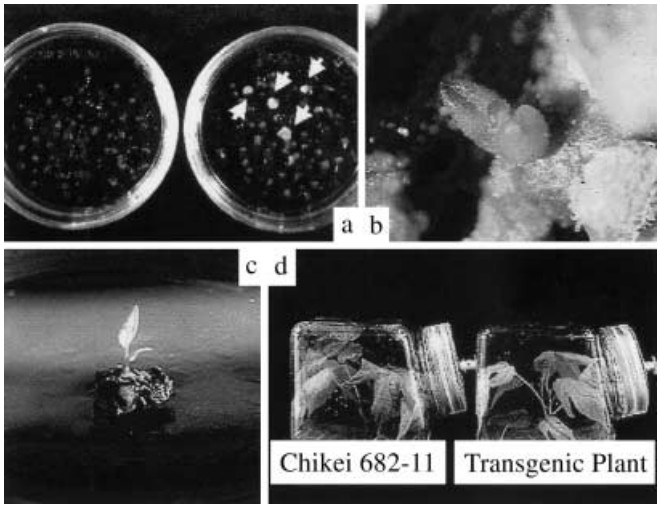

Fig. 2a–d Stages in the production of transgenic sweet potato plants. **a** Selection of hygromycin resistant calli on medium supplemented with hygromycin B (*left*: non-transformant, *right*: transformant). **b**, **c**, **d** Adventitious shoot regeneration from transgenic calli and growth of transgenic sweet potato plants

adventitious embryos in both varieties, and some of them regenerated shoots after 1 month.

The formation of adventitious roots, embryos and shoots were also observed in cultures derived from electroporated calli of both varieties in the presence of plasmid DNA. As a result, 19 plants were regenerated from Chikei 682–11 (Table 2; Fig. 2b, c and d).

We analyzed the presence of the CP gene in 19 regenerated plants by PCR analysis. Using the primers VCP-5 and VCP-7, a CP-positive single band (0.93 kb) was

Table 2 Frequency of organ formation from callus derived from electroporated protoplasts

Variety	Field strength (V/cm)	No. of calli cultured	No. of calli that formed adventitous roots	No. of calli that formed adventitous embryos	No. of calli that formed adventitious shoots
Chugoku	0	430	24	2 (3)	0
No. 25	450	58	11	0	0
	500	240	13	1 (0)	0
	550	192	15	1 (0)	0
	625	161	10	0	0
Chikei	0	420	36	3 (3)	0
682–11	375	130	0	0	0
	450	195	1	0	0
	500	209	0	0	4 (12)
	550	240	0	0	3 (7)
	625	152	0	0	0

( ): no. of regenerated plants

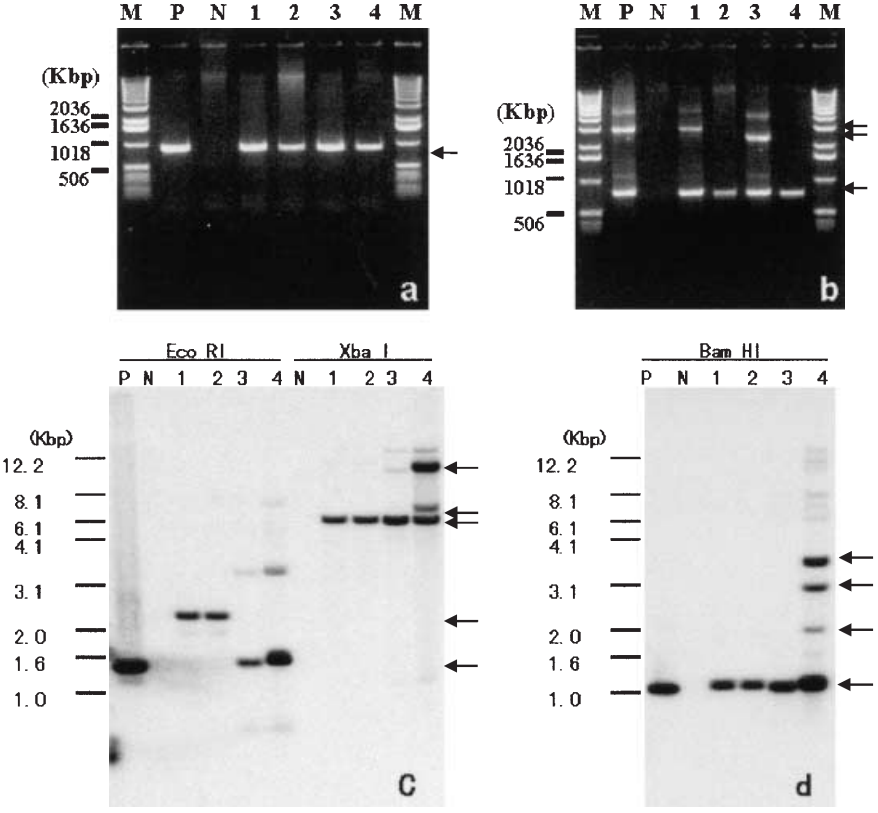

Fig. 3a–d PCR and Southern blot analysis of regenerated plants. *Lane M* 1-kb ladder; *P* digested pMMHA-4 for positive control; *N* non-transformant (original in vitro plants) for negative control; *1* EP200–1-1; *2* EP200–1-2; *3* EP220–1; *4* EP220–2. **a** Detection of the SPFMV-S CP gene in regenerated plants by PCR analysis. *The arrow* indicates the amplified DNA. **b** Detection of the 35S promoter to the SPFMV-S CP gene in regenerated plants by PCR analysis. *Arrows* indicate the amplified DNA. **c** Analysis of transgenic sweet potato plants by Southern blot analysis, hybridized with the SPFMV-S CP probe. *Arrows* indicate the CP fragments. **d** Analysis of transgenic sweet potato plants by Southern-blot analysis, hybridized with hpt probe. *Arrows* indicate the hpt fragments

obtained in four regenerated plants from three calli (Fig. 3a). Next, we used the 35S-1 and VCP-6 primers, which yielded different banding patterns in each plant (Fig. 3b). The amplified DNA fragments were 3 kb and 0.75 kb. The expected two fragments were observed in EP200–1-1 and EP220–1. Only a single band (a 0.75-kb fragment) was observed in EP200–1-2 and EP220–2.

The presence of the CP gene in the four transgenic plants was confirmed by Southern hybridization. The genomic DNA was digested with *Eco*RI (one site in the CP gene and one at the end of the NOS terminator, releasing a 1.3-kb fragment) or *Xba*I (one site between the promoter and the CP gene). DNA from EP220–1 and EP220–2 hybridized with a 1.3-kb fragment from the SPFMV-S CP gene (Fig. 3c, *Eco*RI lanes 3 and 4). In EP220–2, the intensity of the 1.3-kb band was stronger as compared with that of 1.3-kb bands in the other transgenic plants, suggesting that the vector might be integrated as a tandem repeat. Instead of the 1.3-kb band, a fragment of approximately 2.6 kb was present in the transformants EP 200–1-1 and EP 200–1-2 (Fig. 3c, *Eco*RI lanes 1 and 2), suggesting that plasmid recombination occurred during the process of transformation. The estimated copy number of CP genes in these transgenic plants was at least one (EP200–1-1, 1–2) to three (EP220–1) or four (EP220–2). The presence of the hpt gene was also confirmed by Southern hybridization. For EP200–1-1, EP200–1-2 and EP220–1, a 1.1-kb fragment hybridized with the hpt gene

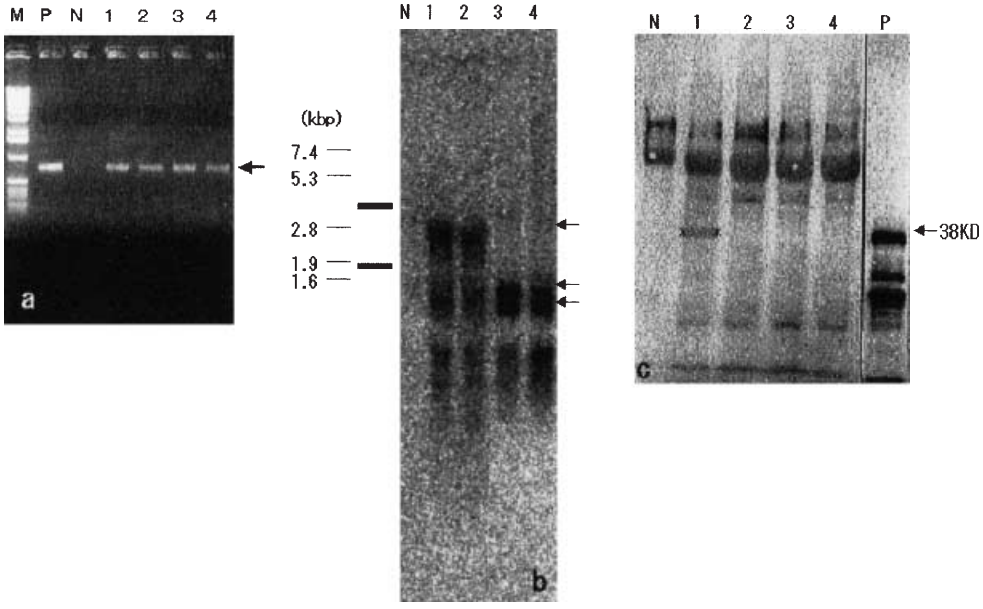

Fig. 4a–c Expression analysis of transgenic sweet potato plants. *P* virus infected morning glory for positive control; *N* non-transformant (original in vitro plants) for negative control; *1* EP200–1-1; *2* EP200–1-2; *3* EP220–1; *4* EP220–2. **a** Analysis of SPFMV-S CP gene expression in transgenic plants by RT-PCR. *The arrow* indicates the positions of amplified fragment of the CP sequence. **b** Analysis of SPFMV-S CP gene expression in transgenic plants by Northern blotting. *Bars* shows rRNA bands of 3.4 kb and 1.8 kb respectivery. **c** Detection of SPFMV-S CP expression in transgenic plants by Western blotting. *The arrow* indicates the positions of CP transcripts

(Fig. 3d, *Bam*HI lanes 1 to 3). For EP220–2, the 1.1-kb band was detected, with an unexpected high-molecular-weight fragment (Fig. 3d, *Bam*HI lane 4).

The expression of the CP gene in the four transgenic plants was analysed by RT-PCR. Amplified DNA fragments of expected size were obtained from all transgenic plants (Fig. 4a). We further analyzed all the lines by Northern-blot analysis. Figure 4b shows a Northern-blot analysis of the transgenic plants EP200–1-1, EP200–1-2, EP220–1 and EP220–2. The isolated RNAs without Oligo-dT purification exhibited clear and intact ribosomal RNA bands of 3.4 kb and 1.8 kb. We observed the positive profiles as smears in EP200–1-1 and EP200–1-2, while two distinct bands could be distinguished in the smears in EP220–1 and EP220–2. The sizes of the two bands were estimated to be 1.3 kb and 1.5 kb. On the other hand, smeared bands from 1.3 kb to 2.8 kb in length observed in EP200–1-1 and EP200–1-2 showed heterologous lengths of the CP mRNA, perhaps due to heterogeneity of the polyA tailing. These results indicated that the CP gene was transcribed in the four transgenic plants.

We carried out a dot-immunobinding assay (DIBA) using polyclonal antiserum for the CP of the SPFMV-S, to confirm whether the transcripts detected by Northern analysis would be translated in the transgenic plants. The result showed that specific spots were found in all transgenic plants using 1×10^{-3} diluted antiserum, compared to control plants (data not shown). Additionally, Western-blot analysis was performed to detect the putative specific protein band of 38 kDa corresponding to the CP. The specific signal of SPFMV-S CP was found as a faint band only in EP200–1-1; this band was not detected in the transgenic plants of EP200–1-2, EP220–1 and EP220–2 (Fig. 4c).

Resistance in transgenic plants was tested using Protein A sandwich ELISA analysis after grafting with virus-infected morning glory. Approximately 3 months after virus infection by grafting, ELISA values in young leaves taken from the inoculated non-transformants (positive control) were 1.19±0.31, while those in the virus-free plants made by shoot-apex culture method (negative control) were 0.16±0.05. Values of five clones from an each transgenic line were measured at 0.07±0.05, 0.11±0.05, 0.16±0.09 and 0.29±0.18 in EP200–1-1, EP200–1-2, EP220–1-1 and EP220–2-1, respectively (Fig. 5). These results showed significant levels of virus resistance in the transgenic lines according to the statistical analysis (Table 3).

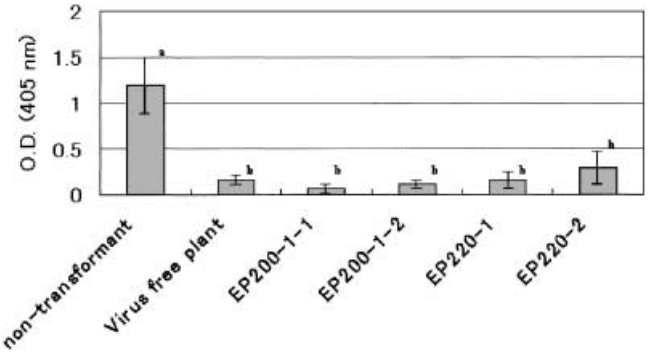

Fig. 5 Analysis of virus detection after graft-inoculation with SPFMV-S by ELISA. The different letters above each column indicate significant differences at P=0.001 (LSD test)

Table 3 ANOVA table for analysis of virus detection by ELISA

Factor	df	MS	*F* value	*P* value
Total	17			
Replication	2	0.099	4.269	>0.0456
Line	5	0.548	23.592	>0.000309
Error	10	0.023		

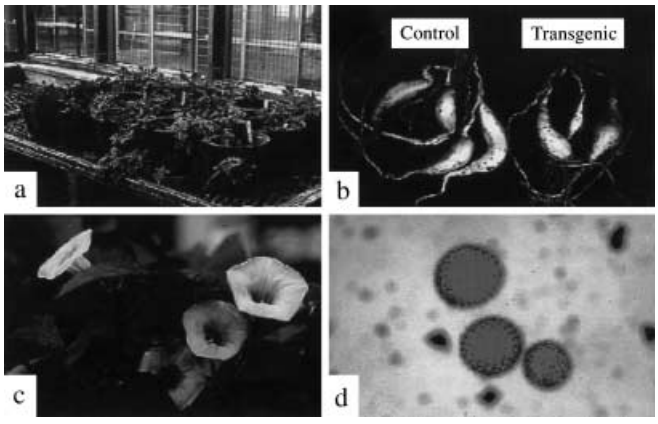

Fig. 6a–d Characters of transgenic sweet potato plants. **a** Produced transgenic sweet potato plants. **b** Production of transgenic tuberous roots (*left*, non-transformant; *right*, transformant). **c** Flowering of transgenic sweet potato plants. **d** Fertile pollen of transgenic plants

The adventitious shoots grew and rooted well, and these plantlets were transferred to pots in the greenhouse. The stems of these clones were grafted onto dwarf-type morning glory for flowering and pollen fertility. The characteristics examined were the following morphological types: pole climing, internode length, nodal root, leaf shape, apical leaf color, leaf color, flower color, flower crown length, flower crown width and pollen fertility (Fig. 6). There were no significant differences between transformants and non-transformants in the characteristics tested (data not shown).

Discussion

Since extremely low efficiency ($<10^{-8}$) of regeneration from the protoplasts in sweet potato leaves was reported (Murata et al. 1995), there seemed to be little possibility of transformation as an effective strategy for molecular breeding. In this study, however, we introduced an expression vector DNA carrying the hpt gene and the SPFMV-S CP gene into protoplasts from leaves of sweet potato by electroporation, and obtained nearly 900 hygromycin-resistant calli. Plants were regenerated from seven independent calli. These results suggest encouraging the possibilities for the transformation of valuable genes for sweet potato improvement.

There have been several reports on the transformation of sweet potato (Carelli et al. 1991; Uehara et al. 1991; Prakash and Varadarajian 1992; Otani et al. 1993; Newell et al. 1995; Gama et al. 1996; Otani et al. 1998). Among these studies, however, very few transgenic sweet potatoes with normal root production were obtained using *Agrobacterium tumefaciens*-mediated transformation and kanamycin selection (Newell et al. 1995; Gama et al. 1996). Although *A. tumefaciens*-mediated transformation is available for sweet potato, kanamycin selection was not very effective, so that the final efficiencies of transformation were excessively low.

Some reports have been published on plant regeneration from protoplasts in sweet potato (Murata et al. 1987; Sihachahr and Duenrever 1987; Murata et al. 1994), although protoplast culture technique has not yet been fully established for the direct gene transfer in sweet potato. This study is the first report of transgenic sweet potato plants with hpt and SPFMV-S CP genes using electroporation. We used two genotypes of sweet potato, Chugoku No.25 and Chikei 682–11. However, regenerated transgenic plants could be produced only from Chikei 682–11, even though regenerated plants were obtained from non-electroporated protoplasts of Chugoku No.25.

The presence of CP gene in the four transgenic plants was confirmed by PCR and Southern hybridization (Fig. 3). The results suggested that transformation eventsranged from simple integration to complex rearrangements. Two of the four transgenic plants, EP200–1-1 and EP200–1-2, were calliclones. Thus three plants EP200–1, EP220–1 and EP220–2 were independent transformants.

We analyzed expression of the CP gene in the four transgenic plants by RT-PCR analysis. Amplified DNA fragments of expected size were obtained from all transgenic plants. Northern-blot analysis showed, unexpectedly, two lengths of transcripts, one of which was the expected size of 1.3 kb and the other was 1.5 kb. If the 2.15-kb fragment from the *Sph*I site to the *Eco*RI site in the expression vector (including the 35S promoter, the CP gene and the NOS terminator in the expression vector of pMMHA-4) was transformed into the genome in an intact form, the CP gene should be transcribed from the initiation site in the promoter followed by polyA tailing from the polyA site in the NOS terminator with the same lengths.

Using the same construct, transgenic tobacco plants were obtained, and two types of transcripts were also observed (Sonoda et al. 1999). Thus, the unexpected transcription may be characteristic of the construct. Larger transcripts than these two were found in EP200–1-1 and EP200–1-2. Because they have multiple copies, transcription might occur differently on each site.

Western-blot analysis showed that only one transformant (EP200–1-1) had the signal of SPFMV-S CP. Considering the similar levels of transcripts among four plants, it would be reasonable to expect that positive CP bands in all the transformed lines were observed with DIBA. It remains to understand this discrepancy. It should be noted that the antiserum was made from the purified SPFMV-S in morning glory and included antibodies mostly for the 38-kDa coat protein, as well as for degraded products and contaminants encountered during virus purification from the infected morning glory. However, the latter did not cause a background as shown in the positive control (the virus-infected morning glory), because the anti-SPFMV-S CP antiserum was diluted 1/2000 with a healthy morning glory homogenate extracted in PBST. High background in the negative control (non-transformed) indicated that the antibody for the contaminants was not absorbed enough by the healthy morning glory homogenate extract.

Transgenic sweet potato plants expressing the SPFMV-S CP gene were challenged by graft-inoculation with SPFMV-S, because direct manual inoculation was difficult in sweet potato (data not shown). All of the transgenic sweet potato plants were highly protected against SPFMV-S. After further growth, these plants produced normal and ELISA-negative tuberous roots in the pots. Thus, we concluded that the four transgenic plants (T_0) obtained a significant resistance to SPFMV-S. It is important that the virus resistance in T_0 plants can be inherited in further progenies in the breeding program. We have obtained the seed sets after crossing, and the analysis for the progenies are underway.

Sweet potato is a vegetatively propagated plant. Herbaceous cutting progeny of sweet potato are designated as V_n. We further confirmed the resistance for V_1 by herbaceous cutting (data not shown). SPFMV is transmitted by aphids under natural conditions. Generally graft-inoculation is more efficient than aphid inoculation. If aphids are used, higher resistance could be obtained. Further experiments in the fields are planned.

SPFMV resistance would be important not only in Japan but also in the other sweet potato-producing countries that have suffered from the SPFMV-S and the closely related strains of RC, C, and so on. In Africa, SPFMV alone is not important in terms of symptoms, while the mixed infection with SPFMV and a whitefly transmitted Sweet Potato Chlorotic Stund Virus (SPCSV) causes severe symptoms called the sweet potato virus disease symptom (Gibson et al. 1998). Most of the sweet potatoes with different virus symptoms reacted to a SPFMV antiserum, whereas there was little serological variation. This is consistent with our recent finding that the amino-acid and DNA sequences of the CP genes were highly homologous among SPFMV-S, RC and C (Mori et al. 1995). Transgenic sweet potato containing the SPFMV-S CP gene might have a potential to confer general SPFMV resistance.

Recently it was reported that virus resistance in transgenic plants with viral sequences was based on the same phenomenon as post-transcriptional gene-silencing (Baulcombe 1995). Although our data showed high resistance to the virus using graft-inoculation, we think that this mechanism of resistance is CP-mediated. First, Northern analysis showed rather strong bands. In gene-silenced transgenic plants, the level of mRNA was very low or below the detection level (Smith et al. 1994). Second ly, the Western-blot analysis showed the production of the CP in the transgenic plants (EP200–1-1) and the DIBA showed that all four plants were positive. Sonoda et al. (1999) observed that in transgenic tobacco plants with the SPFMV-S CP gene most of the resistant lines exhibited the relatively low steady state accumulation of the CP gene mRNA and little or no protein products. Their results suggest that the resistance was manifested by a post-transcriptional gene-silencing mechanism. Additional features of the resistance mechanism reported here need to be elucidated in other experiments, including aphid inoculation.

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References

Annual Report CIP (1989) pp 61–63

Baulcombe DC (1995) Mechanism of pathogen-derived resistance to viruses in transgenic plants. Plant Cell 8:1833–1844

Cali BB, Moyer JW (1981) Purification, serology and particle morphology of two russet crack strains of sweet potato feathery mottle virus. Phytopathology 71:302–305

Carelli ML, Skirvin RM, Harry DA (1991) Transformation and regeneration studies with ”Jewel” sweet potato. Proc Int Symp Sweet Potato Technology for the 21st Century, Tuskegee University, Tuskegee, Alabama, June 2–6, 1991, p 8 (Abstract)

Church G, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Fitchen JH, Beachy RN (1993) Genetically engineered protection against viruses in transgenic plants. Annu Rev Microbiol 47:739–763

Gama MICS, Leite Jr. RP, Cordeiro AR, Cantliffe DJ (1996) Transgenic sweet potato plants obtained by *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Tissue Org Cult 46:237–244

Gibson RW, Mpembe I, Alicai T, Carey EE, Mwanga RO, Seal SE Vetten HJ (1998) Symptoms, aetiology and serological analysis of sweet potato virus disease in Uganda. Plant Pathol 47:95–102

Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Esherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188

Jefferson RA, Kavanagh TA, Bevan M W (1987) Gus fusion: β-glucronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kao KN, Michayluk MR (1975) Nutrional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta 126:105–110

Mori M, Sakai J, Kimura T, Usugi T, Hayashi T, Hanada K, Nishiguchi M (1995) Nucleotide sequence analysis of two nuclear inclusion body and coat protein genes of a sweet potato feathery mottle virus severe strain (SPFMV-S) genomic RNA. Arch Virol 140:1473–1482

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

Murata T, Hoshino K, Miyaji Y (1987) Callus formation and plant regeneration from petiole protoplast of sweet potato, *Ipomoea batatas* (L.) Lam. Japan J Breed 37:291–298

Murata T, Fukuoka H, Kishimoto M (1994) Plant regeneration from mesophyll and cell suspension of sweet potato, *Ipomoea batatas* (L.) Lam. Breed Sci 44:35–40

Murata T, Okada Y, Fukuoka H, Saito A, Kimura T, Mori M, Nishiguchi M (1995) Genetic transformation of sweet potato, *Ipomoea batatas* (L.) Lam. Proc 1st Chinese-Japanese Symposium on Sweet Potato and Potato, pp 369–374

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Nagata R (1984) Transmission of russet crack-like symptom by grafting to sweet potato grown from apical meristem tips (in Japanese). Proc Assoc Pl Protein. Kyushu 30:33–35

Nagy JJ, Maliga P (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z Pflanzenphysiol 78:453–455

Newel l CA, Lowe JM, Merryweather A, Rooke LM, Hamilton WDO (1995) Transformation of sweet potato [*Ipomoea batatas* (L.] Lam) with *Agrobacterium tumefaciens* and regeneration of plants expressing cowpea trypsin inhibitor and snowdrop lectin. Plant Sci 107:215–227

Okada Y, Kimura T, Mori M, Nishiguchi M, Saito A, Murata T, Fukuoka H (1995) Introduction of sweet potato feathery mottle virus severe strain (SPFMV-S) coat protein gene to sweet potato and regeneration of transgenic plants by electroporation. Japan J Breed 45 (suppl 1):116

Otani M, Mii M, Handa T, Kamada H, Shimada T (1993) Transformation of sweet potato [*Ipomoea batatas* (L.) Lam] plants by *Agrobacterium rhizogenes*. Plant Sci 94:151–159

Otani M, Shimada T, Kimura T, Saito A (1998) Transgenic plant production from embryogenic callus of sweet potato [*Ipomoea batatas* (L.) Lam.] using *Agrobacterium tumefeciens*. Plant Biotech 15:11–16

Powell-Abel P, Nelson RS, De B, Hoffman N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat-protein gene. Science 232:738–743

Prakash CS, Varadarajian U (1992) Genetic transformation of sweet potato by particle bombardment. Plant Cell Rep 11:53–57

Restropo MA, Freed DD, Carrington JC (1990) Nuclear transport of plant potyvirus proteins. Plant Cell 2:987–998

Sakai J, Mori M, Morishita T, Tanaka M, Hanada K, Usugi T, Nishiguchi M (1997) Complete nucleotide sequence and genome organization of sweet potato feathery mottle virus (S strain) genomic RNA: the large coding region of the P1 gene. ArchVirol 142:1553–1562

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sihachahr D, Duenrevr G (1987) Plant regeneration from protoplast culture of sweet potato (*Ipomoea batatas* Lam.). Plant Cell Rep 6:326–328

Smith HA, Swaney SL, Parks TD, Wernsman EA, Dougherty WG (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. Plant Cell 6:1441–1453

Sonoda S, Mori M, Nishiguchi M (1999) Homology dependent virus resistance in transgenic plants with the coat protein gene of sweet potato feathery mottle potyvirus; target specificity and transgene methylation. Phytopathology 89:385–391

Strohman RC, Moss PS, Eastwood JM, Spector D, Przybyla A, Paterson B (1977) Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. Cell 10:265

Uehara Y, Komaki K, Nishiguchi M (1991) Gene transfer into sweet potato protoplasts by electroporation. Kyushu Agric Res 54:21

Usugi T, Onuki M, Hayashi T (1990) Detection of some viruses by protein A sandwich ELISA (PAS-ELISA). Kyushu Agric Res 53:106

Usugi T, Nakano M, Onuki M, Maoka T, Hayashi T (1994) A new strain of sweet potato feathery mottle virus that causes russet crack on fleshy roots of some Japanese cultivars of sweet potato. Ann Phytopathol Soc Jpn 60:545–554