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Establishment of Virus-Free Taro (*Colocasia esulenta* cv. Fenghuayunaitou) by Meristem-Tip Culture Combined with Thermotherapy

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Abstract: Virus-free taro (*Colocasia esculenta*) was obtained by elimination of dasheen mosaic virus (DsMV) through thermotherapy and meristem-tip culture. Shoot tips as explants of field taro (cv. Fenghuayunaitou) were cultured on MS basal salts medium suppled with 2.0 mg l^{-1} benzylamiopurinne (BA) and tips were proliferated and proliferated for production of new buds. When the rootless seedlings transferred on MS basal salts medium, plantlets with roots were obtained within 20 days. For detection of DsMV from tube-seedlings, a molecular probe of the genomic RNA of DsMV HZ isolate (DsMV-HZ) was constructed by RT-PCR, for the 3' end partial sequence and used for dot blotting hybridization with ^{32}P probe. Analysis of nucleotides and deduced amino acid sequence between DsMV-HZ isolate with documented references presented 80-95% similarity among the coat protein region and 3' end untranslated region. About 10% seedlings obtained by meristem-tip culture were identified as DsMV-free seedlings in this experiment.

Key words: *Colocasia esculenta*, shoot tips, virus-free culture, dasheen mosaic virus

Introduction

Cultivated taro (*Colocasia esculenta*) is a widely distributed food crop in the humid tropics and subtropics (Xu, 2001). Dasheen mosaic virus (DsMV) has been found as the most important viral pathogen of both cultivated and wild *Araceae* plants worldwide (Zettler and Hartman, 1987; Shimoyama, 1992). As vegetatively propagated aroids, taro plants are commonly infected with DsMV and other pathogens. Previously, a total infection of field samples of several *Araceae* species from Zhejiang and some other parts of China was detected. This virus induces conspicuous mosaic, malformation, dwarfing or feathering on leaves in taro. As the results of infection, it reduces the quality and yield of taro production greatly. This virus is thus considered as a major limiting factor in the production of taro in China (Zettler, 1987; Cao, 1990).

As the same situation of other crop viral diseases, no effective chemicals are available for controlling viruses from infected plants. *In vitro* tissue culture has contributed significantly to crop improvement by overcoming certain limitations, while meristem-culture has been proved as one of the most effective methods for the diminution of viral disease. But sometimes, meristem culture fail to result in virus-free seedlings, even combined with chemotherapy or thermotherapy as antiviral treatments. For this reason, plants derived from meristems should be detected for pathogenic viruses before field releasing for cultivation. Many methods have been developed for virus detection at meristems stage and/or in planta. Nucleic acid hybridization and RT-PCR detection are more sensitive methods for the detection of virus, in compare with immunological assays (Seal and Coater, 1998; James, 1999). Up to now, only immunological assays, electron microscopic detection and western blots were used for the detection of DsMV on taro and related *Araceae* species (Li, 1999; Bi, 1998).

As the only principal virus infecting taro, the genome of DsMV was outlined by us presently (Chen, 2001). It consists of 9991 nucleotides for its single-stranded positive-sense RNA coding ten conserved proteins.

As the most famous monocorm cultivar in China, Fenghuayunaitou was found commonly infected by DsMV and plant quality declining has been a key problem in taro production. Virus elimination by thermotherapy and meristem-culture was used for obtaining virus-free plants, while a sensitive dot hybridization method was used for detection of DsMV.

Materials and Methods

Plant material: Taro cultivar Fenghuayunaitou was used in these experiments and the plant materials were obtained from Fenghua, Zhejiang Province, East China. The infection of Fenghuayunaitou with DsMV in Fenghua was found to be 100% as estimated by dot blotting hybridization (data not showed). After harvest, corms were air-dried and stored for a month before use. Buds were shot from corms under 25°C, 24 h light photoperiod and 70% relative humidity and used for meristem-culture after treating for 30 days at 38°C, until 2-3 cm high.

Establishment of DsMV-free meristem-cultures: Buds were excised from taro corms, washed with sterilized distilled-water (sd-H_2O) for 30 min, sterilized in saturated in Ca-hypochlorite solution for 15 min, dipped in 70% ethanol and one drop of tween-80 for 60 sec, and rinsed with sd-H_2O by four times. Shoot tips were then excised for use as explants, and maintained by serial subculture at three weeks intervals on MS solid medium (0.7% agar) with sucrose (3%) as the carbon source, BA (2.0 mg l^{-1}) as plant growth regulator. All of the components were added to the medium before autoclaving at 121°C and 1.1 kg cm^{-2} for 15 min, and the pH was adjusted to 5.8 with 1 mol l^{-1} NaOH before sterilization.

Multiple shoots were developed in a growth chamber at 25°C with a 16 hr photoperiod, and a photosynthetic photon flux of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Multiple shoots were sub-cultured in MS medium supplemented with BA (2.0 mg l^{-1}) every 3 weeks and were rooted in MS basal medium for 3 weeks. Regenerated plantlets were transferred to soil in pots and raised in greenhouse. For the first three weeks, the plants were watered with 1/10-strength MS macronutrients.

Cloning and sequencing of the 3' terminal sequence of DsMV: DsMV-HZ was isolated from an *Araceae* plant of *Z. aethiopica* grown in Hangzhou ornamental Nursery Garden, Zhejiang, China in 1993, and maintained in insect-proof greenhouse. The virus was firstly identified by electron microscopic examination for filamentous particles and serological tests (Chen, 1993).

DsMV RNA was extracted from 10 mg leaves with obvious signs of disease. Leaf-tissue with vein removed was added to 500 μl PBST buffer (5 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4, containing 0.8% NaCl, 0.02% KCl and 0.04% Tween-20) and homogenized completely. The solution was then transferred to a fresh Eppendorf tube and incubated at 25°C for 20 min. The tube was then washed for three times with PBST buffer after removing the homogenized leaf-sap solution. The RNA was dried and re-suspended in 10 μl DEPC-treated, autoclaved water.

First-strand cDNA was synthesized by using Expand Reverse Transcriptase kit (Roche), according to the manufacturer's instructions and NOT(T) containing EcoRI (underlined) and Not I (italic) restriction sites as the 3' reverse primer (5'-**GAATTC***GCGGCCGC*TTTTTTTTTTTT-TTTTT). We designed 5' primer (DMVF: 5'-CC**AAGCTT**ATGAYGARGTTGTRTTGC-3') containing a Hind III restriction site (underlined) and 3' primer (DMVR: 5'-AA**GGATCC**GTCYG-ATGTAGGTGCAG-3') containing a

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BamH I restriction site (underlined) for the subsequent PCR primers. We designed 3' primer DMVR to produce a cDNA fragment without its poly-A tail as template DNA for the subsequent hybridization. cDNA was amplified by polymerase chain reaction (PCR) using DMVF and NOT(T) primers. PCR reaction was carried out with Taq polymerase system, using the following amplification procedure: 94°C for 3 min, followed by 35 cycles of 30s at 94°C, 45s at 58°C, 1 min 30s at 72°C and final 10 min elongation at 72°C. PCR products were examined through 1.0% agarose electrophoresis. Target DNA fragment was harvested with a PCR Fragment Recovery Kit (TaKaRa) following the manufacturers' protocols. PCR fragments were cloned into pGEM-T easy vector (Promega) following the manufacturers' protocols. Clones containing insertion fragments were then sequenced. The nucleotide sequence and the predicted amino acid sequences of the cloned DNA was compared and aligned with documented sequences from Genbank database by using Clustalw software (http://www.clustalw.genome.ad.jp).

Preparation of ^{32}P probe and hybridization: Total RNA was extracted by trizol solution from 200 mg seedling-tissue of meristem-tip cultures, followed by chloroform extraction, isopropyl alcohol precipitation. RNA samples were air dried and diluted in denatured buffer (40 µl DEPC-treated water, 80 µl Fermamide, 28 µl HCHO, 8 µl 20×SSC). RNA solution was then incubated at 68°C for 15 min and quenched on ice immediately. The denatured samples were dotted onto a Hybond nylon membrane (Amersham). Pre-hybridization and hybridization were performed as described by hybridization guide (Hybaid) at 42°C overnight. ^{32}P-labelled radioprobe were produced with Random Primer DNA Labeling Kit (TaKaRa) following the manufacturers' protocols. The membranes were washed twice with low stringency solution (2×SSC, 0.1%SDS) at 42°C, once with high stringency solution (1×SSC, 0.1%SDS) at 42°C and once with higher stringency solution (0.1×SSC, 0.1%SDS) at 42°C. The hybridization signals were detected under Typhoon (Amersham).

Results

Establishment of meristem-tip cultures: Meristem-tip cultures were established from taro Fenghuayunaitou, the most famous and important cultivar in China. Corms harvested from the field were found commonly contaminated with fungi and bacteria. It was so necessary to remove the hairs from corms and to dry for over one mouth in order to reduce the contamination before tube-culturing. Shoot tips were excised from buds, which had been pretreated by thermotherapy and cultured into MS medium supplemented with BA (2.0 mg l^{-1}). Tip tissues were found about 90% free of contamination when excised buds sterilized according to above described methods. For the first two weeks, the tips grew normally on all media. In later stages, some tips proved detrimental and caused death of tip tissues. After two months, a higher percentage (about 62%) of the meristems died due to the small size of the explant and only about 28% tip tissues developed into shoots. Seven to ten shoots formed from one meristem-tip without callus formation (Fig. 1B). Protocorm-like bodies are proliferated from buds (Fig.1A). When the protocorms were cut up and sub-cultured in MS medium containing 2.0 mg l^{-1} BA, the adventitious protocorm continued formation. Multiple buds (Fig. 1B) proliferated from these shoot-tip-derived buds and were subcultured on medium containing 2.0 mg l^{-1} BA. Complete plantlets (Fig. 1C) were regenerated from multiple shoots on MS medium. The regenerated plants were transferred to sand and soil for further growth when plantlet height reached 2-3 cm (Fig. 1D). Under a greenhouse condition of 25°C with 70% relative humidity, new taro plants were grown up.

Sequence of DsMV coat protein and 3' end untranslated region: The sequences of the 3' terminal part of DsMV-HZ include a coding region for coat protein of 948 nucleotides, coding 316 amino acids, followed by the 3' end untranslated region of 248 nucleotides (Fig. 2). The coat protein and 3'-UTR sequences of DsMV-HZ were compared with those of published DsMV isolates (Pappu, 1994; Li, 1998; Chen, 1999). Size of the coat protein and

Fig. 1: Meristem-tip tissue culture of *Colocasia esculenta*
(A) Protocorm formation and shoots derived from shoots
(B) Proliferated multiple shoots derived from *in vitro* meristem tip-culture
(C) Regenerated plants with well developed roots,
(D) Potted regenerant in greenhouse

1	ATGATGAAGT	TGTGTTGCAA	GCTGACGATA	CAGTTGATGC	AGGAAATAAT
51	GATAATAAGA	CAAAAACAAC	TGAAACAAAA	ACTCCTGCAG	CAGGTGGTGG
101	GAACAACACA	AATAATACTC	CACCACCGCC	AGCAGACAAC	ACAACAAACA
151	ACAATCCTCC	ACCGCCACCA	CCGGCGGTAC	CAAAGGCGAC	AGAGACACCA
201	ACCAACACAC	AAGTAGTCCC	ACCAGCAAGC	GAGAAAGGTA	AAGAGATTGT
251	CAAAGATGTT	AACGCTGGCA	CCAGTGGAAC	TTATTCTGTA	CCTCGATTAA
301	ACAAAATCAC	AAATAAGATG	AACTTACCTT	TGGTTAAAGG	TAAGTGCATC
351	TTAAATTTGA	ATCACTTGAT	CGAGTACAAA	CCTGAACAGC	GTGACATTTT
401	CAATACCAGA	GCCACCCACA	CACAGTTTGA	GGTCTGGTAC	AATGCAGTCA
451	AGAGGGAATA	TGAGCTAGAA	GACGAGCAGA	TGCACATCGT	AATGAATGGT
501	TTCATGGTCT	GGTGCATCGA	TAATGGGACA	TCACCTGATA	TTAACGGGGC
551	TTGGGTGATG	ATGGACGGAA	ATGAACAAAT	TGAATACCCG	TTAAAGCCTA
601	TTGTGGAGAA	TGCAAAACCC	ACCTTGCGTC	AGATAATGCA	TCACTTTTCT
651	GACGCAGCAG	AGGCATACAT	TGAACTGAGG	AATGCGGAGA	AACCGTACAT
701	GCCTAGGTAC	GGTCTTATTC	GCAACTTACG	TGATGCAAGT	CTCGCTCGGT
751	ACGCTTTTGA	CTTCTACGAG	GTCAATTCTA	AAACACCGGT	GCGAGCAAGA
801	GAGGCAGTTG	CGCAGATGAA	GGCCGCTGCA	CTCTCCAACG	TTACCACTAG
851	GTTGTTCGGT	TTGGATGGTA	ACGTTTCAAC	TTCAAGCGAG	AACACTGAAA
901	GGCACACTGC	AAAGGACGTC	ACACCAAATA	TGCATACATT	GCTCGGTGTG
951	TCTCCTCCAC	AGTAAAGGGC	TGGTAAACAG	TTCACAGCTA	TTATCTCGCT
1001	ATCTGTAGTT	TTATATATAT	AAAGTACTGT	TTGTGTTCGA	ATAGTGTTAT
1051	TTGGATATAA	GCTACAGAGT	GGTTTTCCAC	CGATGTGGAG	AGGTGCTGTG
1101	CATCCTGTTA	TCCACGTCCT	TTATATATTA	GAAAACTACT	GAACTACTGC
1151	ACCTACATCA	GACCGTAAGT	GCGCCATAGG	CGCGGTAGGC	GAGATGCTTC
1201	GTGCACGGTG	TTCA			

Fig. 2: Nucleotide sequence of DsMV-HZ coat protein gene and 3' end untranslated region

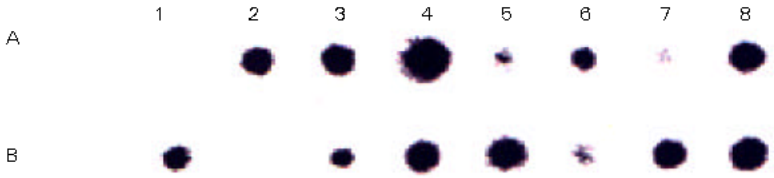

Fig. 3: Results for detection of DsMV from taro seedings

Table 1: Percent sequence similarity of the CP nucleotides and amino acids and UTR of DsMV-HZ to other isolates

DsMV isolates	Accession number of genbank	Percent similarity with DsMV-HZ CP(nt)	CP(aa)	UTR(nt)
DsMV-LA	DMU00122	80	85	86
DsMV-Ten	DMU08124	89	95	88
DsMV-CH	AF048981	88	93	85
DsMV-TW	AF169832	81	88	88

3'-UTR of DsMV ranged from 939 to 987nts and 247 to 250nts, respectively. Multiple alignments of these sequences showed that coat protein amino acid and 3'-UTR nucleotide sequence identity varied from 85-95 and 85-88%, respectively (Table 1). This part was selected as cDNA probe for detection of DsMV from plant tissue.

Detection of DsMV from meristem-tip cultures: These tip-derived regenerates and plants propagated via multiple-shoot protocol were then tested for contamination of DsMV by dot blotting hybridization with ^{32}P-labelled radio-probe. The ^{32}P-labelled DsMV probe presented detection results in total RNA with high specificity and efficiency. No signal was detected for sample B2 (Fig. 3), just as negative control, while other samples presented hybridization signals as positive control (Fig. 3). In these experiments, ^{32}P-labelled DsMV probe detected the presence of DsMV infection in 30 seedlings, but three others were free of this virus. When more multiple shoots were induced from stock, and seedlings in which no DsMV was detected were subsequently sub-cultured. A second diagnosis by hybridization was performed, resulting that all plants re-generated from DsMV-free mother seedlings exhibited no infection of this virus.

Discussion

In recent years, the development of gene probes has offered considerable promise as diagnostic tools for plant viruses as one of the most sensitive and accurate methods, especially for detecting specific viruses in tissue culture operations. In this study, DsMV-free taro seedlings were obtained by meristem-tip culture combined with thermotherapy and detection of DsMV by RNA dot blotting hybridization. Although serological methods based on the protein components of viral replication have been commonly used in plant virus detection, nucleic acid hybridization has proved to be a very reliable and more sensitive technique in plant virus disease diagnosis (Matthews, 1991; Hull and Al-hakim 1993; Van Regenmortel and Dubs, 1993). Our previous results showed that the presence of DsMV infection could be identified by serological methods (Chen, 1993, 1996), with the results of a quite high ratio of DsMV infection of the field taro. Hsu *et al.* (2000) reported the limitation of virus as little as 490 pg of bamboo mosaic virus (BaMV) from infected plants could be detected by using nucleic acid hybridization. The sensitivity of detection of BaMV using hybridization was at least 500 times higher than that of using ELISA.

As in the case of BaMV, Hsu *et al.* (2000) reported that immunoelectron microcopy (IEM) and ELISA could only detect BaMV infection in 50 and 75% of the infected plants respectively, whereas RNA hybridization with ^{32}P-labelled probe identified BaMV infection in 100% of the plants. Previous evidence indicated that

treatment of virus-infected plants with chemotherapy or thermotherapy could not completely eliminate the virus (Koruza and Jelaska, 1993). Therefore meristem-culture combined with virus detection remains one of the most suitable methods for the elimination of DsMV from taro.

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