Review

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Development and Establishment of Practical Tissue Culture Methods for Production of Virus-Free Garlic Seed Bulbs, a Novel Field Cultivation System and Convenient Methods for Detecting Garlic Infecting Viruses.

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Introduction

Garlic (*Allium sativum* L.) is an important plant widely used for culinary and medicinal purposes because of its ability to improve the taste of food and its biological activities that include antibiotic, antitumor, cholesterol lowering, and antithrombic effects on animal cells (Fujiwara and Natata, 1967). Garlic traditionally has been cultivated vegetatively because of its sexual sterility; therefore, viral disease is very serious problem (Mohamed and Young, 1981; Walkey and Antill, 1989; Walkey, 1990; Lot *et al.*, 1994). Most unselected commercial garlic cultivars have been shown to be infected with a complex of two or more viruses and many reports have described the causative viruses of garlic diseases (Cadilhac *et al.*, 1976; Bos *et al.*, 1978a, b; Delecolle and Lot, 1979; Lee *et al.*, 1979; Sako, 1976, 1978). However, identification of the viruses that infect garlic is complicated, and the pertinent literature is confusing (Walkey *et al.*, 1987; Van Dijk, 1991, 1993a, b; Conci *et al.*, 1992).

In Japan, two types of rod-shaped flexuous viruses have been detected in garlic plants (Lee *et al.*, 1979; Abiko *et al.*, 1980a, b). Each of these viruses has been classified into the potyvirus and carlavirus groups based on their morphological and cytopathological properties. The potyvirus, which is termed garlic mosaic virus (GMV), is thought to be the causative agent of garlic viral disease, whereas the carlavirus, termed garlic latent virus (GLV), can infect garlic systemically, but causes no apparent symptoms of disease (Lee *et al.*, 1979; Sako, 1989). However, GMV and GLV have not been biochemically or physically analyzed, and there is no information about their interrelationship. The relatively restricted host ranges and the lack of sensitive methods for detecting the individual viruses have led to confusion in differentiating garlic viruses.

Tissue culture is a useful technique for eliminating viruses from infected plantlets and for producing virus-free garlic seedlings. Although shoot-tip culture has been used for this purpose (Bhojwani, 1980; Walkey *et al.*, 1987), the propagation rate of virus-free plantlets is very low and it is a laborious, time-consuming process. Various tissue culture techniques have been reported to improve the efficiency of propagation (Havránek and Novák, 1973; Kehr and Schaeffer, 1976; Abo El-Nil, 1977; Nagakubo *et al.*, 1993), but all have inherent drawbacks as practical methods - the need for long-term cultivation, relatively low propagation rates, and the need for skilled techniques.

To overcome the problems of virus diseases in garlic cultivation, we first attempted to characterize the viruses infecting garlic plants based on molecular biology and then developed the methods for detecting the respective ones. In this approach, the viruses were isolated as cDNA clones and characterized them from their genomic information including genome sequence, predicted coat protein amino acid sequence and genomic organization, obtained by sequencing of the respective clones (Sumi *et al.*, 1993, 1999; Tsuneyoshi and Sumi, 1996; Tsuneyoshi *et al.*, 1997, 1998a, b). It should be noted that our strategy for cloning of viral cDNAs differs from others described previously, in that we cloned viral cDNA directly from diseased garlic plants, which probably were infected with a complex of viruses. This approach does not require the biological isolation of individual viruses in appropriate host plants, which is a major restraint in research progress on garlic viruses. Concurrently, we developed a novel tissue culture method for micropropagation of garlic plants with a much higher efficacy than previously reported, namely stem disc culture (Ayabe and Sumi, 1998). Furthermore, we established an effi-

cient tissue culture method, stem-disc dome culture (SD-Dome culture), for eliminating viruses from virus-infected garlic plants based on stem disc culture method (Ayabe and Sumi, 2001).

In this review, we describe the characterization, identification and detection of viruses infecting garlic plants as well as the development of novel and practical tissue culture methods, stem disc culture and SD-Dome culture, for producing virus-free garlic seedlings.

Characterization and identification of viruses infecting garlic plants

The experimental strategy employed in this study is schematically illustrated in **Fig. 1**. Our strategy for cloning viral cDNAs differs from previously described strategies in that we cloned viral cDNA directly from diseased garlic plants, which probably were infected with a complex of viruses, without isolating individual viruses in appropriate host plants. The isolation and characterization of viruses as their cDNAs makes it possible to prepare virus-specific antisera, primers, or probes to aid in identifying unknown viruses that are difficult to isolate by traditional methods. In addition, such molecular characteristics as the viral genome sequence, CP sequence and genome organization are useful for distinguishing virus strains and for determining the relationships between genera, species and subspecies of distinct viruses (Shukla and Ward, 1989a, b; Atreya, 1992; Brunt, 1992; Ward *et al.*, 1992; Sumi *et al.*, 1993; Tsuneyoshi *et al.*, 1998a, b).

Finally, we obtained eight independent viral cDNAs from the affected garlic plants, whose genomic organization of the 3' terminal region is illustrated in **Fig. 2**. From the similarities of the genomic organization and the putative amino acid sequences of the possible open reading frames (ORFs), they were divided into 3 groups and shown to be the cDNAs respectively from *Potyvirus*, leek yellow stripe virus (LYSV) and onion yellow streak virus (OYDV), *Carlavirus*, garlic common latent virus (GCLV) and shallot/garlic latent virus (SLV/GLV) and novel rod-shaped viruses unidentified so far (Sumi *et al.*, 1993; Tsuneyoshi and Sumi, 1996; Tsuneyoshi *et al.*, 1998a, b). The novel viruses, designated garlic virus A to D (GarV-A to -D), are clearly distinguished by their characteristic genomic organization of the 3' terminal region and the presence of an ORF that encodes a putative 40 kDa protein, which has no similarity to any of the other sequences in the DNA and protein sequence

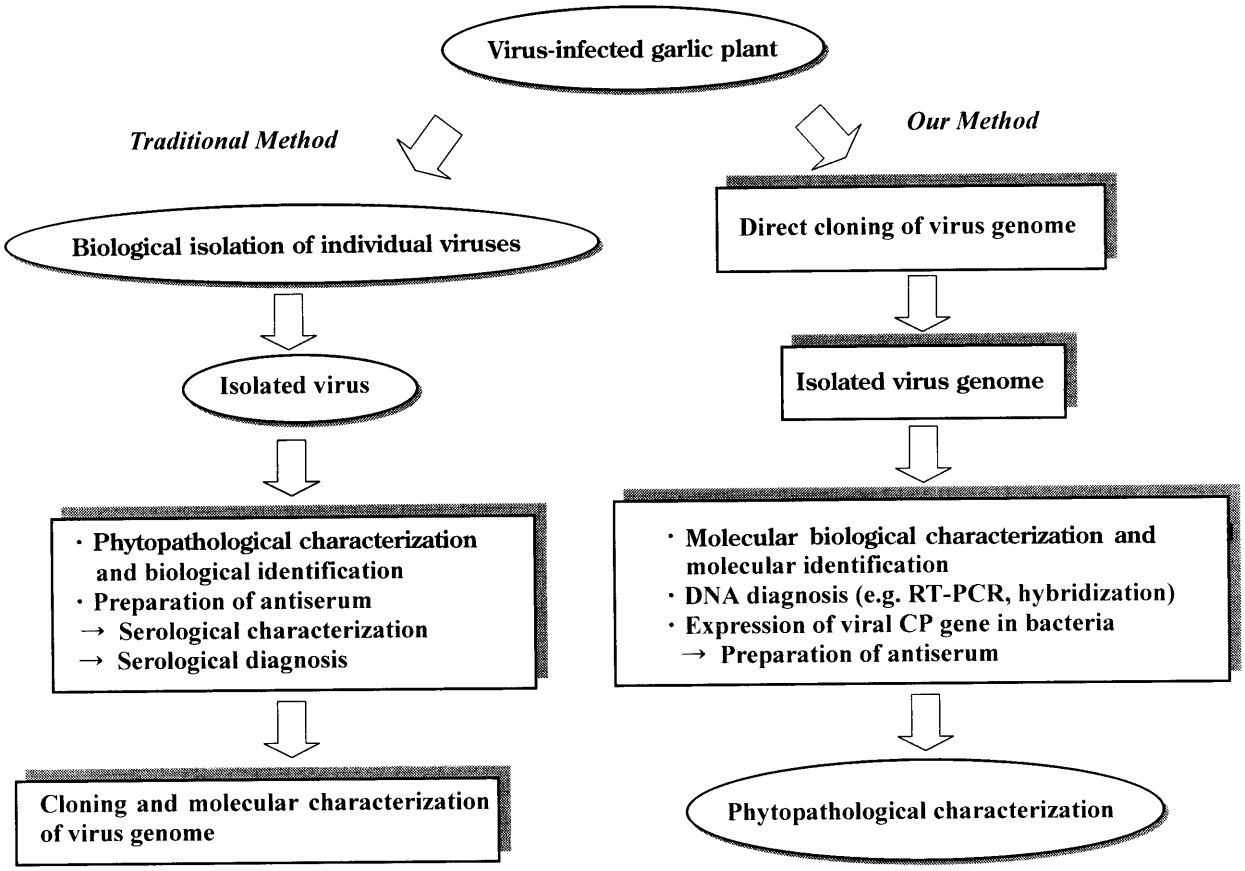


Fig. 1 Experimental strategy for characterization and identification of viruses infecting garlic. The novel strategy used in this work is schematically shown together with the traditional phytopathological method for comparison.

database (Sumi *et al.*, 1993). These viruses were classified in a new genus, *Allexivirus*, recently ratified by the International Committee on Taxonomy of Viruses (ICTV) (Prngle, 1999).

Development of diagnostic methods for garlic viruses

A total of eight viruses belonging to three distinct genera were identified from infected garlic plants based on partial cDNA cloning and sequencing of their genomes. From the sequence data of the respective viral cDNAs, it is possible to prepare virus-specific primers and antisera raised against the bacterially expressed viral CPs, for reverse transcription polymerase chain reaction (RT-PCR) and serological detection, respectively. The RT-PCR procedure is useful for detecting and identifying each virus with high sensitivity and accuracy, but unsuitable for large-scale indexing or for studies of plant virus epidemiology because it is laborious and time-consuming. In contrast, serological methods are not as sensitive as RT-PCR for detec-

Table 1. Primer sequence for RT-PCR detection of *Allium* viruses

Traget viruses	Sequence of primers	Correspnoding regions of the primer	Amplified size
GarV-A, B, C, D (common)	N-RT1: 5'-CCTGCTAAGCTATATGCTGA-3' N-RT2: 5'-GTAAGTTTAGCGATATCAAC-3'	3'-terminal ORF 3' non-coding	182-185bp
LYSV	P-RT3: 5'-AAGAGTCAACACTTGGTTTG-3' P-RT4: 5'-GGTCTCAATCCTAGCTAGTC-3'	3' non-coding 3' non-coding	191bp
OYDV garlic-type	OG-RT1: 5'-GAAGCGCACATGCAAATGAAG-3' OG-RT2: 5'-CGCCACAACTAGTGGTACAC-3'	CP-gene 3' non-coding	290bp
OYDV wakegi-type	ON-RT1: 5'-CACACACTGCTCGGTCTTCG-3' ON-RT2: 5'-CATGGTGCAAATAATGCTAGC-3'	CP-gene 3' non-coding	172bp
GLV/SLV	GS-RT1: 5'-TATGCTCGAGCTCGTAGAGC-3' GS-RT2: 5'-GGGTTTCACATTGTTACACC-3'	3'-terminal ORF 3'-terminal ORF	170bp
GCLV	GC-RT1: 5'-AATGGGTGTTCTAGGAGTAGC-3' GC-RT2: 5'-TTAAACCTTAGTCAAGCTATTC-3'	3'-terminal ORF 3' non-coding	306bp

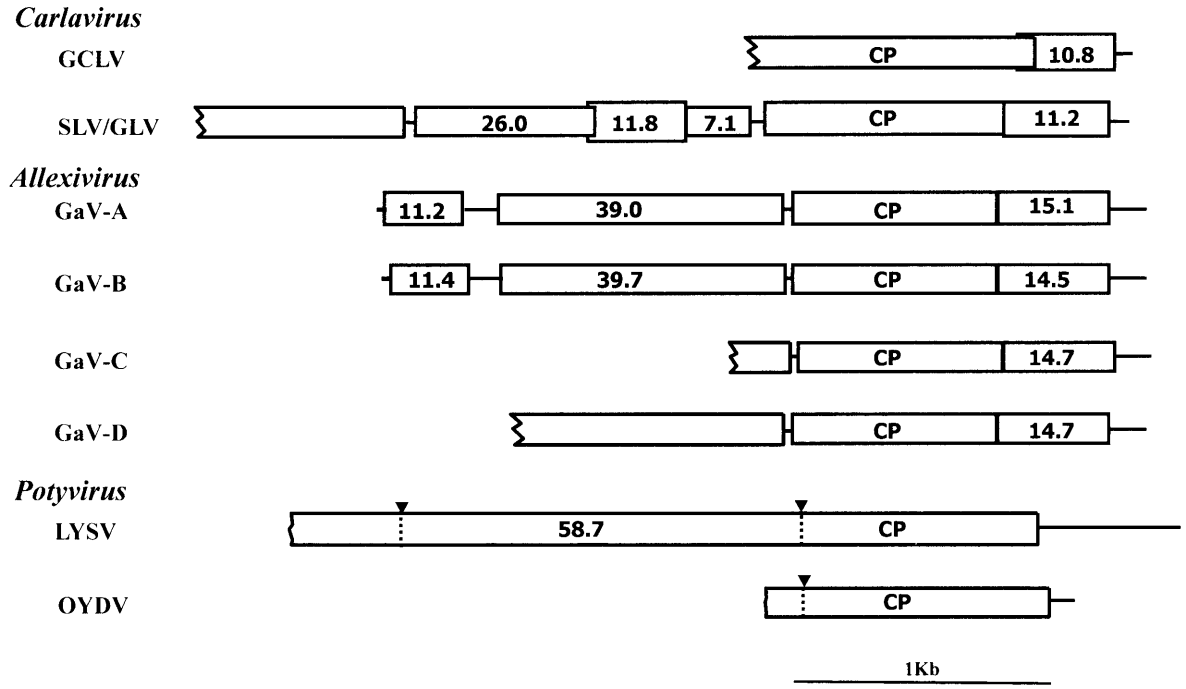
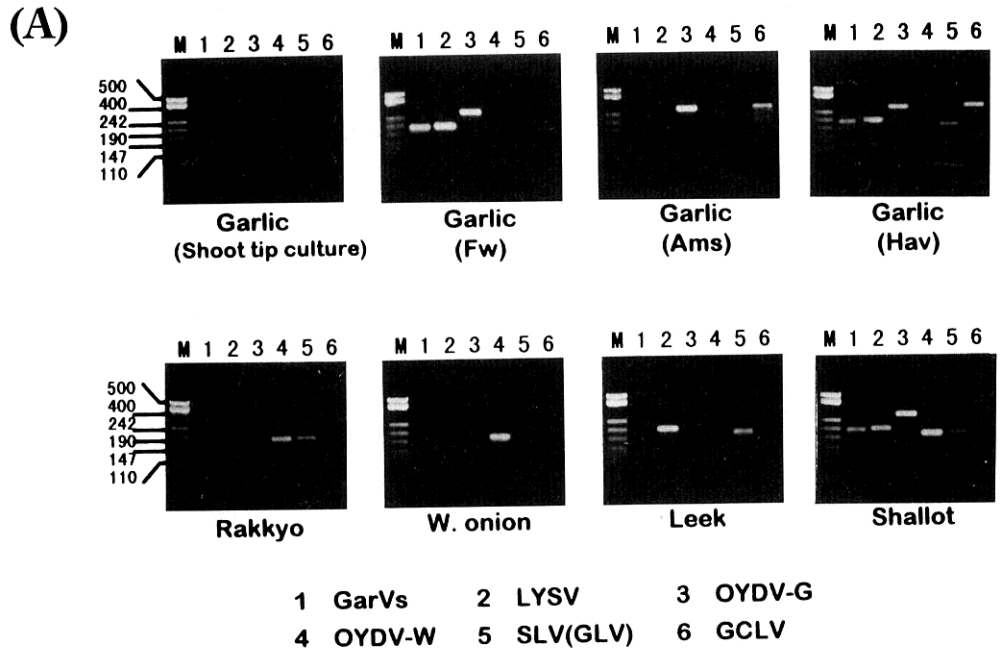


Fig. 2 The genomic organization of the 3' terminal regions of the viruses identified from garlic plants. The open reading frames are indicated as boxes with M_rs according to the putative translation products. Triangles indicate possible processing sites of single large translation products of the respective potyviruses.

tion, but much more convenient for the large-scale and the routine diagnosis of viral infection. We therefore developed diagnostic methods for garlic viruses based on both the RT-PCR procedure and a serological method, direct tissue blotting immunoassay (DTBIA) (Tsuneyoshi and Sumi, 1996).

The oligonucleotide primers used to detect viral infections in garlic plants by RT-PCR were designed based on differences in the 3' terminal nucleotide sequence among the viruses. The primer sequences are summarized in **Table 1**, along with their properties. In the case of GarVs, a highly conserved region in the 3' terminal sequence was selected as the common target of amplification. The unique recognition sites of the restriction enzymes *Rsa*I, *Ava*I, *Hin*fI, and *Mse*I make it possible to identify individual viruses in GarV-A, GarV-B, GarV-C and GarV-D by restriction analysis of the



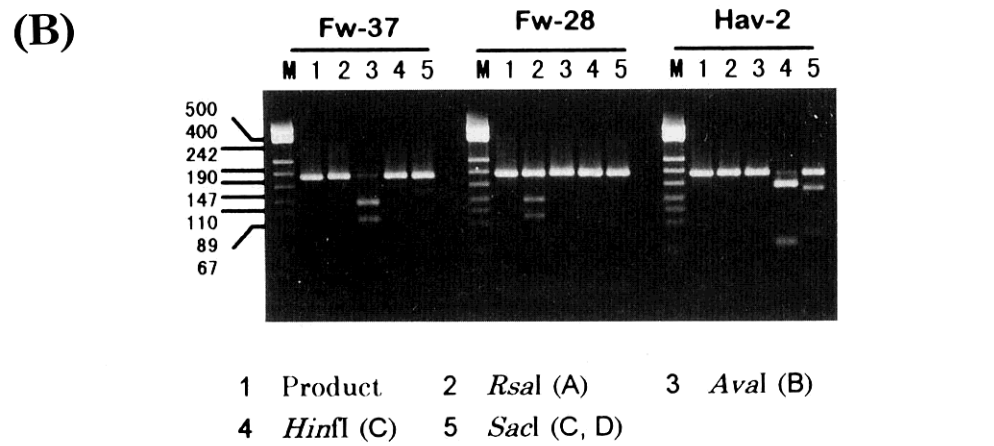


Fig. 3 Detection of *Allium* viruses based on RT-PCR procedures. (A) Amplification of DNA fragments from *Allium* samples by RT-PCR using the specific primers to each of six types of *Allium* viruses. Lane 1, 2, 3, 4, 5 and 6 are the specific primer sets to GarVs, LYSV, OYDV, WYDV, GLV/SLV and GCLV, respectively. Lane M is *Hpa*I digested pUC119 as size marker. (B) Identification of individual GarV by restriction analysis of amplified DNA fragments. Lanes 2, 3, 4 and 5 show digestion products with *Rsa*I, *Ava*I, *Hin*fI and *Sca*I, respectively. Lanes 1 and M show the PCR product and *Hae*III digest of pUC119, the size marker, respectively.

amplified DNA fragments (Tsuneyoshi and Sumi, 1996). Representative results of analyses of the PCR products amplified from garlic samples by 3% agarose gel electrophoresis are shown in **Fig. 3**. As shown in the figure, no DNA fragment was amplified in the virus-free garlic but specifically amplified fragments of expected size were found in the tested plants. In addition, the fact that more than two primer sets, a maximum of five out of the six primer sets, produced specific DNA fragments of expected size indicates that multiple infections generally occurred in garlic plants. The results of restriction analyses of the amplified DNA fragments using the common primer set for GarVs showed that RT-PCR combined with restriction analysis makes it possible to distinguish among the garlic viruses, GarV-A to -D (**Fig. 3B**). In conclusion, the RT-PCR procedure was confirmed to be useful for detection of garlic viruses with high sensitivity, accuracy and specificity.

Next, we prepared antisera against the respective garlic viruses using the bacterially expressed viral CPs as antigens. The reactivity and specificity of the resultant antisera were confirmed according to the trap decoration method by immunoelectron microscopy and enzyme-linked immunosorbent assay using purified viral particles of GLV and GarV-C (Tsuneyoshi and Sumi, 1996). We then developed a convenient and practical method of detecting garlic viruses based on the direct tissue blotting immunoassay (DTBIA). In this method, the blots on nitrocellulose membrane, prepared by briefly pressing the cut surface of a garlic leaf onto the membrane, were reacted with each of the rabbit antisera raised against bacterially expressed viral CPs of the respective garlic viruses. The resultant immunocomplex was detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies. A positive result was indicated by the development of purple coloring on the tissue blot as shown in **Fig. 4A**. The sensitivity of this method for detection was comparable to that of ELISA (Tsuneyoshi and Sumi, 1996). Finally, we constructed a garlic virus detection kit containing all of the reagents and apparatus required for detecting viruses by DTBIA (**Fig. 4B**). Using the test kit, a survey of viral infection is possible even in the field.

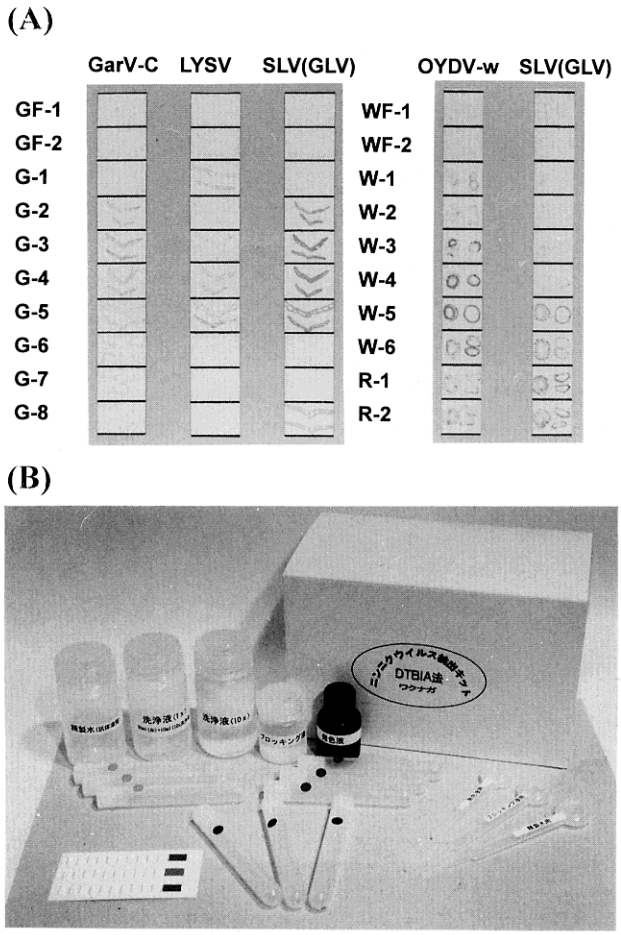


Fig. 4 Direct tissue blotting immunoassays of *Allium* viruses. (A) Immunochemical detection of *Allium* viruses, GarVs, LYSV, WYDV and GLV/SLV using direct tissue blotting immunoassay (DTBIA). GF-1 and -2, and WF-1 and -2 are virus-free garlic and *Allium wakegi* plants produced through shoot-tip culture. G-1 to -8 are garlic samples. W-1 to -6 and R-1 and -2 are *Allium wakegi* and *A. rakkyo* with apparent virus disease symptoms, respectively. (B) Garlic virus detection kit. All the reagents and apparatus required for detecting viruses by DTBIA are contained in the kit.

Establishment of a novel tissue culture method, stem-disc culture

The findings that at least eight distinct viruses infect garlic plants and that a complex infection is frequently observed in garlic cultivated in fields indicate the importance of virus-free garlic seed plants and the need for a practical tissue culture method with high efficiency.

The previous studies reported by Xeu *et al.* (1991) and Nagasawa and Finer (1988) suggested that the basal part of the clove, with or without the shoot apex, is a potent explant. We observed *in vitro* shoots developing directly from the basal part of the foliage leaf in a preliminary experiment. Taken together, these findings suggest that the basal part of the garlic clove is advantageous explant material for the micropropagation of garlic. We subsequently established a novel tissue culture method, namely stem-disc culture, for garlic (Ayabe and Sumi,

1998). This method is unique in that (1) the stem disc is used as an explant (**Fig. 5**); (2) multiple shoots differentiate directly from the surface of the explant within approximately 3 weeks (**Fig. 6**); and (3) shoot differentiation is independent of phytohormones. In addition, *in vitro* shoots produced from stem-discs formed *in vitro* bulblets on treatment of garlic bulbs at 4 ℃ for more than 4 weeks before preparing the stem-disc explants (**Fig. 7**). The gradual increase in shoot number paralleled the duration of the low-temperature pretreatment, with an average of 25 shoots differentiating from a single garlic clove stored for more than 8 weeks at 4 ℃ . The formation of *in vitro* bulblets was also enhanced, with more than 95% of the shoots forming bulblets after low-temperature pretreatment for more than 8 weeks. In contrast, garlic stored at room temperature showed neither enhancement of differentiation of *in vitro* shoots nor *in vitro* bulblet formation. The stem-disc culture method combined with the pretreatment of garlic at 4 ℃ for approximately 8 weeks consistently produced more than 100 *in vitro* bulblets from each bulb, in comparison to the bulb of the Japanese garlic cultivar "Fukuchi-howaito" which ordinarily has only 5-6 cloves. The *in vitro* bulblets could germinate and sprouting shoots planted in late August to early September produced garlic bulbs, late the next June, of comparable size and weight to those obtained by the usual clove cultivation method.

These findings indicate that the stem-disc culture method is of practical use for the micropropagation of garlic plants, in particular as virus-free seed plants produced by shoot-tip culture.

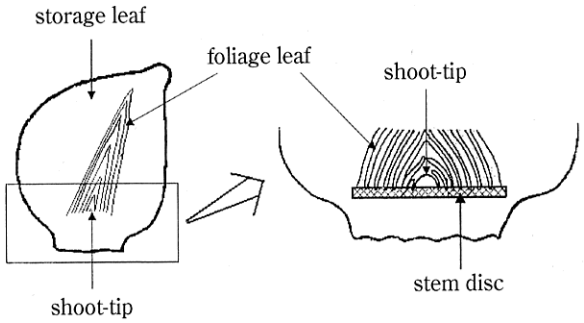


Fig. 5 Schematic depiction of garlic tissue designated the "stem disc". This is a restricted tissue (box) just under the basements of the immature foliage leaves approximately 1 mm thick.

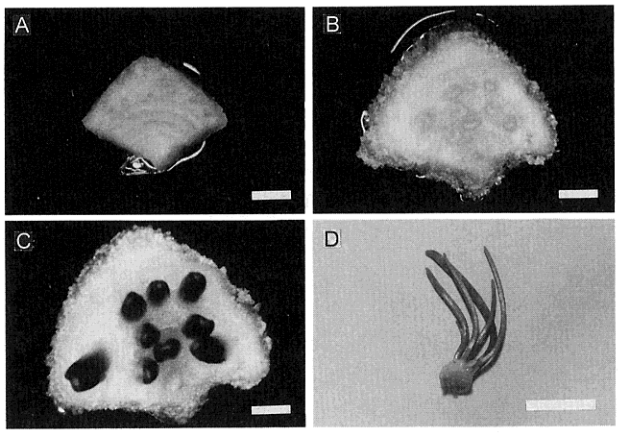


Fig. 6 Progressive development of *in vitro* shoots from a stem-disc explant. Morphological changes in one piece of a stem-disc explant cut into four pieces were observed by microscopy. (A) 1 day after culture, (B) dome-shaped structures after 1 week of culture, (C) green buds after 2 weeks of culture, (D) *in vitro* shoots after 3 weeks of culture. Bars in the figures show 1 mm, except 1 cm in (D).

Development of a practical tissue culture method, stem-disc dome culture, to produce virus-free seed plants

Microscopic observation of the developmental process of *in vitro* shoots from stem-disc explants showed that multiple, dome-shaped structures first appeared on the surface of the stem-disc explant approximately 1 week after the initiation of culture. These structures appeared concentrically on the explant and developed rapidly to produce green buds after approximately 2 weeks of culture (**Fig. 6**). Scanning electron microscopy (SEM) of stem discs sampled serially 0-5 days after culture showed that development of the *in vitro* shoots was restricted to regions surrounded by the basal parts of foliage leaves and that the morphological changes apparently occurred within the first 3 days of culture (**Fig. 8**). Histological observations showed that both the internal cell organization and formation process of the dome-shaped structures were

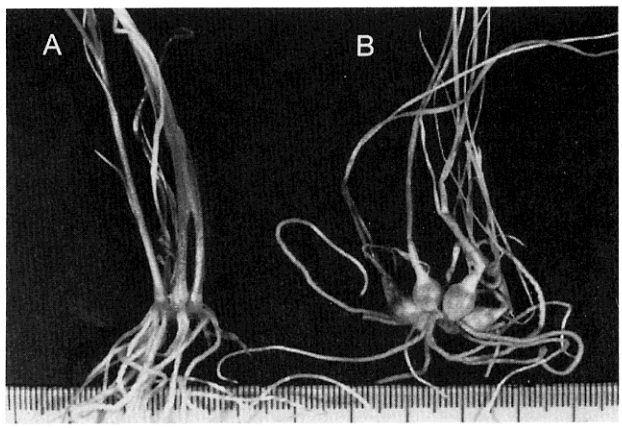


Fig. 7 Representative *in vitro* shoots produced from stem discs cultured for 2 months. (A) and (B) show *in vitro* shoots from explants stored at room temperature and at 4 ℃ , respectively.

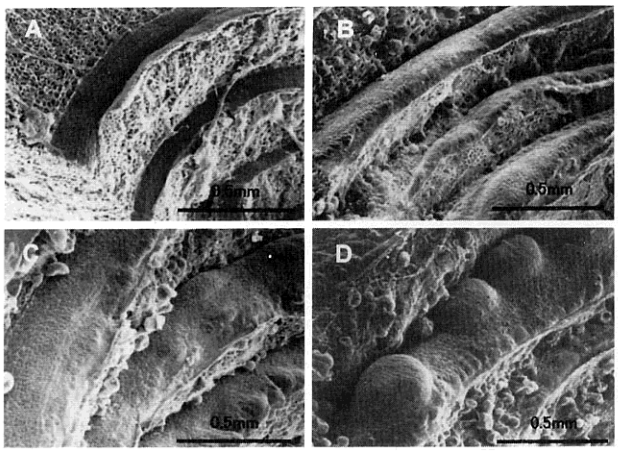


Fig. 8 SEM observations of the early formative stages of the dome-shaped structures on a stem-disc explant. (A) The stem-disc explant just after preparation, (B) the explant after 3 days of culture, (C) the explant after 4 days of culture, (D) the explant after 5 days of culture. Bars show 1 mm.

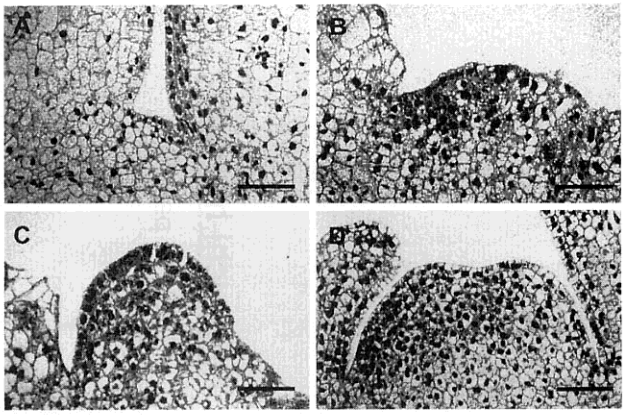


Fig. 9 Histological observations of the dome-shaped structures during formation. (A) Thin section from a stem disc before culture, (B) the same section after 3 days of culture, (C) the same sections after 5 days of culture, (D) the garlic shoot apex. Bars show 0.1 mm.

similar to those of the meristem (**Fig. 9**). In addition, the events leading to the formation of these structures appeared to be initiated by vigorous cell division in the epidermis of concentric tissue zones surrounded by the basal parts of immature foliage leaves. These findings implied a possibility that the respective dome-shaped structures are equivalent to meristem in properties including a virus-free character.

To test this hypothesis, we first determined whether the dome-shaped structures could be cultured when separated from the stem disc explant. Dome-shaped structures that had been excised and placed on LS media without phytohormones grew vigorously and developed into *in vitro* shoots approximately 1 cm long after 2 weeks, just as observed in the stem disc culture. This was evidence that an isolated dome-shaped structure can be used as an explant for *in vitro* culture. We have tentatively named this *in vitro* culture method, "stem disc-dome (SD-Dome) culture".

To determine whether the viruses were eliminated from the affected parent garlic plant through *in vitro* SD-Dome culture, we conducted DTBIA and RT-PCR on garlic plants differentiated from the separated dome-shaped structures. The results confirmed that viruses were eliminated by the SD-Dome culture, whereas garlic plants produced by the usual stem disc culture were infected with the same viruses as their parents (**Fig. 10**). We continued viral surveillance through three generations of progeny obtained by SD-Dome culture and detected no evidence of viral infection. These findings provide evidence that the viruses were completely eliminated by the SD-Dome culture. Moreover, the dome-shaped structures remained free from viruses, with separation from the stem-disc explants being crucial for the elimination of virus. Indeed, viral infections were detected in the shoots grown from the immature shoots separated at 2 or 3 weeks after the onset of the stem disc culture, but not in the shoots from shoot-buds at 10-days culture (**Fig. 11**). Microscopic observation of the internal configurations of the dome-shaped structure, shoot-bud, and shoot showed that the first 2 tissues consisted of small cells, with no organized internal structure (**Fig. 12A, B**). In contrast, large cells with well developing vacuoles were present in the internal portion of a section prepared from a shoot after 2 weeks of culture. In addition, some well-stained cell clusters were present in the section from the shoot (**Fig. 12C**). These clusters probably were procambia which differentiate into vascular bundle systems. Viruses are reported to spread throughout the plant body by the mechanisms of cell-to-cell and long-distance movement (Maule, 1991; Leisner and Turgeon, 1993; Lucas and Gilbertson, 1994; Gilbertson and Lucas, 1996; Oparka *et al.*, 1996). In the cell-to-cell movement, viruses move into surrounding cells through the plasmodesmata. Vascular bundle systems function in long-distance movement. These findings suggest that neither the plasmodesmata nor vascular bundle systems are fully differentiated in the early developmental stage of the dome-shaped structures, and that this is the reason why viruses from neighboring infected cells can not invade them. Once such connecting systems are formed, however, viruses immediately appear to initiate invasion. The process by which viruses are completely eliminated during the formation of dome

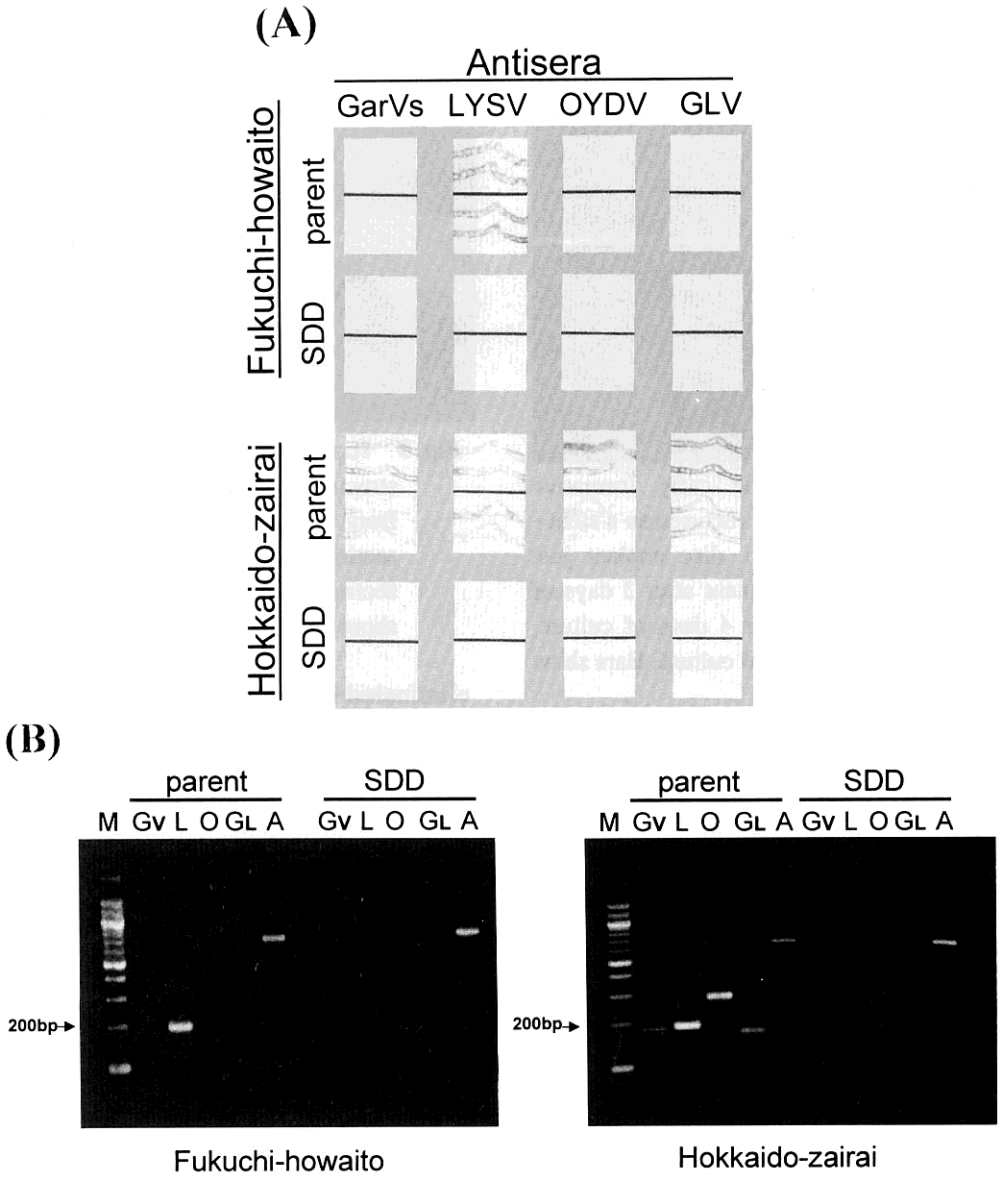


Fig. 10 Elimination of viruses through SD-Dome culture of garlic plants infected with a complex of viruses. The resultant plants produced after SD-Dome culture (SDD) and their parental garlic (parent) were examined for infection with each of four garlic viruses, GarVs, LYSV, OYDV and GLV/SLV using DTBIA (A) and RT-PCR (B). Gv, L, O, GL and A above each lane in (B) indicate the set of primers for GarVs, LYSV, OYDV, GLV and alliin lyase cDNA sequence, respectively. Lane M shows 100 bp ladder as size marker.

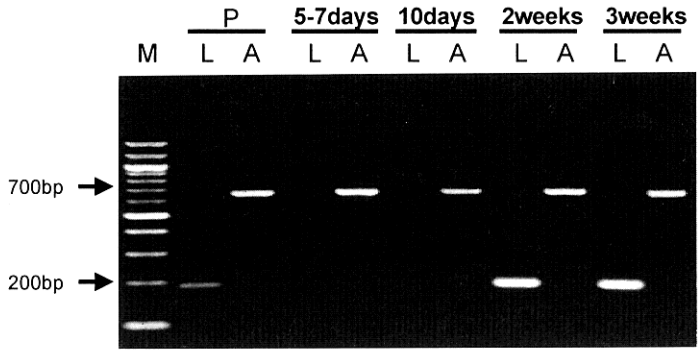
-shaped structures, however, is not yet clear.

In summary, we have developed a novel and practical culture method, SD-Dome culture, for producing virus-free garlic, that has much higher efficiency than the usual shoot-tip culture. In this method, 15-25 virus-free plants are consistently obtained from a single clove in the same period as in shoot-tip culture.

Conclusion

We clarified the identity of garlic viruses in complicated taxonomic situations using a new approach, combining molecular biology and quantitative taxonomic criteria. In this approach, the viruses were isolated as cDNAs to characterize at the nucleotide sequence level. The strategy for the cloning of viral cDNAs used here differs from previous ones in that viral cDNAs were cloned directly from diseased garlic plants without the biological isolation of individual viruses in appropriate host plants. This is unusual but useful for analyzing viruses such as garlic viruses, the host ranges of which are too strictly restricted and closely overlapped to isolate them from each other employing traditional tools of plant virology (Bos, 1983; Walkey, 1990). The isolation of the viruses as cDNAs and analysis at the nucleotide sequence

A



B

Culture period	5-7 days	10 days	2 weeks	3 weeks
Developmental stage	Dome-shaped structure 0.5mm	Shoot-bud 1mm	Shoot 1cm	Shoot >2cm
Detection of viruses	—	—	+	+

Fig. 11 Influence of the developmental stages of the dome-shaped structure on virus elimination. (A) Amplification of DNA fragments by RT-PCR using the specific primers to LYSV. P indicates parental garlic plant. Lanes L and A indicate the specific primer sets to LYSV and alliin lyase cDNA, respectively. Lane M is the size marker (100 bp ladder). (B) Timing to excise dome-shaped structures from stem disc explant for virus elimination.

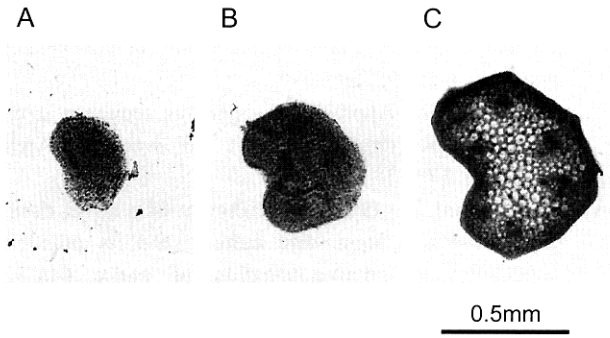


Fig. 12 Observation of transverse sections of the dome-shaped structures at different developing stages. (A) The dome-shaped structure after 7 days of culture. (B) The shoot-bud after 10 days of culture. (C) The shoot after 2 weeks of culture.

level make it possible to differentiate among the viruses. Besides, the sequence data enable us to prepare virus-specific antisera, primers, or probe to aid in identifying unknown viruses, or in differentiating among variants, that are difficult to isolate by traditional methods.

In conclusion, we revealed that at least 8 distinct viruses, LYSV, OYDV, SLV (GLV), GCLV, and GarV-A to -D, belonging to *Potyvirus, Carlavirus* and the newly ratified *Allexivirus* infect garlic plants (Sumi *et al.*, 1993, 1999; Tsuneyoshi *et al.*, 1998a, b; Tsuneyoshi and Sumi, 1996). In addition, we developed the methods for detecting the respective viruses based on RT-PCR and DTBIA using the virus-type specific primers and the antisera raised against bacterially expressed viral CPs, respectively (Tsuneyoshi and Sumi, 1996). RT-PCR combined with restriction analysis could identify each of the viruses with high sensitivity and specificity. DTBIA was less sensitive but much more convenient and simple than the RT-PCR procedure, and as sensitive as ELISA. Therefore, our garlic virus detection kit, which contains all of the reagents and apparatus required for detecting viruses by DTBIA, is suitable for large-scale and routine diagnosis of garlic viruses.

A survey of virus incidence by DTBIA and RT-PCR revealed and reconfirmed that garlic plants are very frequently infected with a complex of viruses and the development of a practical tissue culture method for producing virus-free seed plants is important. We found that a restricted part of the undeveloped stem of the garlic clove, called the "stem disc," which is just under the basement of the immature foliage leaves, was a very potent explant for the micropropagation of garlic (Ayabe and

Sumi, 1998). Twenty to thirty tissue-cultured shoots were consistently differentiated from a single clove during one month of culture on phytohormone-free Linsmaier and Skoog medium. In addition, more than 90% of the shoots formed bulblets *in vitro* during an additional one month of culture by pretreatment of the garlic bulbs at 4℃ before culturing. Shoot development in this type of *in vitro* culture, stem-disc culture, apparently is divided into four stages; expansion of tissue zones surrounded by the basal parts of the immature foliage leaves, formation of dome-shaped structures, bud differentiation directly from each dome, and development into shoots and bulblets. The dome-shaped structures appeared within five days of the onset of the culture and had developed independently into shoots approximately 1 cm high three weeks later. Histological observations showed that both the internal cell organization and formation process of the dome-shaped structures were similar to those in the meristem. Indeed, we showed that the dome-shaped structures are virus-free and they, even though separated from the stem disc explant, independently grew into shoots more than 5-cm high and rooted after 8 weeks, which were successfully transplanted in soil. We designated this culture method "stem-disc dome (SD-Dome) culture." Leaves of garlic plants produced by the SD-Dome culture showed no viral symptoms and were deep green, whereas leaves of the parent garlic infected with viruses showed severe mosaic and yellow streak symptoms. Viral surveillance, conducted by DTBIA and RT-PCR analyses, on 3 generations of progeny obtained by SD-Dome culture detected no evidence of viral infection, confirming that viruses were completely eliminated during culturing. Microscopic observation of transverse sections of tissues in different developmental stages during stem disc culture; dome-shaped structures, shoot-buds, and shoots, suggested that the elimination of viruses is correlated with the stages of development and organization of vascular bundle structures (**Fig. 12**). These findings indicate that the SD-Dome culture method, as well as stem-disc culture, should be useful for producing virus-free garlic seeds, and it should provide useful experimental tool to study the mechanism by which the meristem is protected against invasion by virus.

Furthermore, we developed a novel, epoch-making field cultivation system for garlic, in which seedlings, instead of the usual cloves, produced from *in vitro* bulblets are used for propagation (**Fig. 13**). We expect that this culture system will be generally employed for the practical cultivation of garlic, in particular large-scale cultivation, because seedlings are much more easily planted by machinery than cloves.

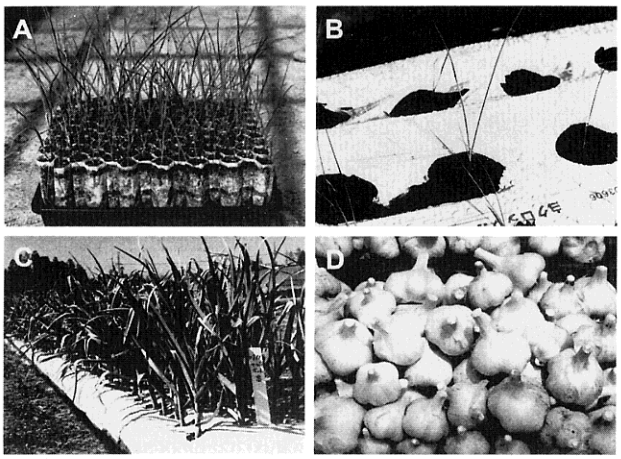


Fig. 13 A novel and epoch-making field cultivation system of garlic using seedlings from *in vitro* bulblets instead of the usual cloves.

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