Production of Virus-free Bulblets by Meristematic Tip Culture with Antiviral Chemical in *Lilium brownii* var. *colchesteri*

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*Lilium brownii* var. *colchesteri* has unique and ornamental floral characteristics in graceful harmony with flower and anther colors, flower shape, elegant fragrance, and flower color change from yellowish cream to white during anthesis. There are, however, few accessions conserved in Japan up to the present, and they often show abnormally shaped flowers and mosaic leaves seemingly due to virus infection. Virus-free bulblets were established by combining meristematic tip culture and chemotherapy. At initial diagnosis with RT-PCR, *Cucumber mosaic virus* (CMV), *Lily mottle virus* (LMoV) and *Lily symptomless virus* (LSV) were detected from leaf tissues of the mother plants. All regenerated bulblets obtained by meristematic tip culture of the mother plants were still infected by at least one of the viruses. The meristematic tip of the bulblets infected with either LSV or LMoV was selected for subsequent culture with 2,4-dioxohexahydro-1,3,5-triazine (DHT), an antiviral chemical. LSV was eliminated successfully in mericlones from bulblets with LSV, whereas LMoV was not from those with LMoV. The virus-free bulblets were transferred to new medium without DHT, and multiplied by *in vitro* scaling. They were then acclimated in a phytotron glass room at 20°C. The plants were confirmed to be virus-free after 18-months’ acclimation. It was concluded that the combination of meristem tip culture and chemotherapy is practical for producing virus-free plants of *L. brownii* var. *colchesteri*.

**Key Words:** *Lilium brownii* var. *colchesteri*, meristematic tip culture with antiviral chemical, virus-free bulblets.

**Introduction**

*Lilium brownii* F. E. Brown var. *colchesteri* E. H. Wilson originated and grows naturally in central to south provinces of China (Liang, 1980; Shimizu, 1971). It has been cultivated for more than 400 years in Japan (Okubo, 2006) since it has unique and ornamental floral characteristics in graceful harmony with flower and anther colors, flower shape, elegant fragrance, and flower color change from yellowish cream to white during anthesis. There are, however, few accessions conserved in Japan up to the present. This may be due to increased morbidity of *Lilium*-specific viruses accompanied by clonal propagation from the limited number of resources introduced into Japan since current accessions in Japan are estimated to be clonally propagated (Saruwatari et al., 2007) and show strong self-incompatibility (Shimizu, 1971).

Most common virus diseases in *Lilium* species are *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), and *Lily mottle virus* (LMoV) (Asjes, 2000; Derks, 1995; Hagita et al., 1989; Niimi et al., 1999; Nishi et al., 2008). It has also been reported that *Lilium leichtlinii* var. *maximowiczii* was infected with *Lily virus X* (LVX) in Japan (Asjes, 2000; Hagita et al., 1995). LSV, a species of the genus *Carlavirus*, frequently co-infects with CMV or LMoV in cultivated lily plants, and lilies co-infected with these viruses become more seriously susceptible to other diseases (Asjes, 2000; Derks, 1995; Derks and Asjes, 1975). Virus infection...
consequently causes developmental abnormalities, such as growth reduction with severe leaf mosaic and dwarfing symptoms, smaller malformed flowers, and lower bulb yield in lily plants (Asjes, 2000; Derks, 1995). Similar symptoms, such as distorted and twisted growth of mosaic leaves (Fig. 1 left), abnormally shaped flowers (Fig. 1 right), color breaking in the flowers, and concentric brown-ring patterns on the bulbs, were observed in *L. brownii* var. *colchesteri*, probably causing difficulties in conserving plants of good quality.

Several tissue culture techniques have been used successfully to produce virus-free plants (Horst, 1988). The elimination of LSV was achieved in *Lilium × elegans* and *L. longiflorum* by tissue culture in early excision of shoots regenerated from scale pieces (Allen et al., 1980), meristematic tip culture (Allen and Anderson, 1980; Allen et al., 1980; Nesi et al., 2009), and meristematic tip (Nesi et al., 2009) or callus (Xu et al., 2000) culture in combination with thermotherapy. Meristematic tip culture was also used to eliminate *Tulip breaking virus* (TBV), which is genetically almost identical to LMoV, in *L. longiflorum* (Blom-Barnhoorn and van Aartrijk, 1985). Ozaki et al. (1996) and Xu et al. (2000) were successful in eradicating CMV in *L. longiflorum* through chemotherapy or in combination with callus culture.

In this study, we identified several viruses in clonally propagated *L. brownii* var. *colchesteri* by RT-PCR, and then successfully produced virus-free bulblets using meristem tip culture in combination with chemotherapy.

**Materials and Methods**

**Meristematic tip culture, multiplication in vitro, acclimation, and cultivation in a phytotron**

Bulbs of *L. brownii* var. *colchesteri* collected in Korea were maintained in an experimental field of Kyushu University. Genotyping based on amplified fragment length polymorphism (AFLP) analysis indicated that the bulbs had been vegetatively propagated and were genetically identical to those conserved in Japan (Saruwatari et al., 2007). One bulb was cleaned under running tap water for 1 h after harvest, and damaged outer scales were removed. Clean inner scales were excised and surface-sterilized sequentially, first in 70% (v/v) ethanol for 30 s, then 1% sodium hypochlorite solution for 30 min, followed by three washes with sterilized distilled water. The scale pieces were then used for bulblet multiplication on MS medium (Murashige and Skoog, 1962) with 2.2 μM benzyladenine (BA), 2.9 μM indole-3-acetic acid (IAA), 30 g·L⁻¹ sucrose, and 3 g·L⁻¹ gellan gum (pH 5.8 before autoclaving). The meristems excised from the bulblets were cultured on MS medium with the same composition as above. MS medium containing 50 μM 2,4-dioxohexa-hydro-1,3,5-triazine (DHT), an antiviral chemical for reduction of virus concentration (Schuster et al., 1979), was used for the second meristematic tip culture, as described by Xu et al. (2000).

The obtained meristem-tip-derived bulblets were subcultured on 1/2 MS medium with 0.5 μM naphthaleneacetic acid (NAA), 30 g·L⁻¹ sucrose, and 3 g·L⁻¹ gellan gum (pH 5.8 before autoclaving). All explants and bulblets were placed at 25°C under continuous light (approximately 40 μmol·m⁻²·s⁻¹ PPFD) provided by fluorescent lamps for plant growth (Toshiba Lighting and Technology Co., Japan). The bulblets judged to be virus-free were multiplied *in vitro* and treated at 4°C for 6 weeks for dormancy break (Tsuchiya et al., 2006). They were transferred subsequently to a 20°C phytotron glass room of the Biotron Institute, Kyushu University for acclimatization and further growth. The course from breaking dormancy to cultivation was repeated three times (18 months have passed since the first dormancy break treatment started).

**Detection of viruses**

Leaves were sampled from the plants grown in the experimental field, in flasks during the first and second meristematic tip culture, and in the phytotron after acclimation. Total RNA was extracted from the leaves using an RNeasy Plant mini Kit (Qiagen, Japan), and cDNA was synthesized from total RNA with an RNA PCR kit (AMV Ver. 3.0, Takara Bio Inc., Japan) or a PrimeScript RT-PCR kit (Takara Bio Inc.). Reverse transcriptase was added for a positive control or not added for a negative control. The cDNA was used as a template for PCR with each specific primer, designed on the basis of the sequences of CMV, LMoV, LSV, and LVX, as described by Suehiro et al. (2005) and Tojo et al. (2007). Sequences of the primer pairs used in this study are listed in Table 1. PCR was conducted using the temperature shift program of one cycle of 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C using Ex Taq (Takara Bio Inc.). PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

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**Fig. 1.** Abnormal flower shape and distorted and twisted mosaic leaf development suspected of virus infection in a clonally propagated *L. brownii* var. *colchesteri.*
Results and Discussion

First meristematic tip culture

Amplification products of 1008 bp, 1649 bp, and 660 bp corresponding to the target regions of CMV, LMoV, and LSV genomic RNAs, respectively, were obtained by RT-PCR in the mother plants, but the 507 bp band corresponding to LVX was not detected (Fig. 2). No specific bands corresponding to CMV, LMoV, LSV, and LVX were visible in the negative control (data not shown). These results indicate that the mother plants were infected with three viruses, CMV, LMoV, and LSV.

The result of RT-PCR after the first meristematic tip culture indicated that all the regenerated bulblets still carried three (CMV, LMoV, and LSV), two (LMoV and LSV), one (LMoV) or one (LSV) viruses (Fig. 3, Table 2). The eradication rate of CMV, LMoV, and LSV after the first meristematic tip culture was 73.3% (bulblets with both LMoV and LSV and with either LMoV or LSV), 26.7% (bulblets with LSV only), and 13.3% (bulblets with LMoV only), respectively. These results are in agreement with the result in the previous report in which the virus elimination rate was higher in the order of CMV, TBV-L (LMoV), and LSV after shoot regeneration from callus in *L. longiflorum* (Xu et al., 2000).

Completely virus-free bulblets were not obtained by meristematic tip culture only. A more efficient method using the combination of chemotherapy with antiviral chemicals was therefore carried out to completely eradicate the viruses from the bulblets in the next experiment.

Second meristematic tip culture

The second meristematic tip culture was conducted using bulblets infected with LMoV or LSV on MS medium supplemented with DHT. No RT-PCR products were detected in 71.4% of regenerated bulblets from LSV-infected bulblets, whereas they were detected in all regenerated bulblets through culture from LMoV-infected bulblets (Fig. 4, Table 2). It was thus shown that DHT was effective for LSV elimination, but not for LMoV elimination in agreement with the effect of Virazole (ribavirin), another antiviral chemical, on LMoV and LSV elimination in *Lilium × parkmanii* hybrids and *L. longiflorum* (Cohen, 1985; Xu and Niimi, 1999).

Meristematic tip culture was effective to eradicate LSV and/or LMoV in *L. longiflorum* and *L. × elegans* (Allen and Anderson, 1980; Allen et al., 1980; Blom-Barbhoom and van Aartrijk, 1985; Nesi et al., 2009). It was also confirmed that meristematic tip culture could eliminate CMV in our current study in *L. brownii* var. *colchesteri*. The effect of DHT on a relative wide spectrum of plant viruses has been reported extensively in many plant species, such as cherry, potato, and strawberry, and it is known that treatment reduced the concentration of viruses (Bittner et al., 1987; Bogusch et al., 1985; Borissenko et al., 1985; Kondakova and Schuster, 1991; Schuster et al., 1979). Xu et al. (2000) demonstrated that the application of DHT or Virazole reduces the CMV and LSV infection rate in *L. longiflorum*, and the eradicable effect of DHT on CMV in *L. longiflorum* (Ozaki et al., 1996). It

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Table 1. Specific primer pairs for detection of CMV, LMoV, LSV, and LVX by RT-PCR.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>CMV</td>
<td>CMV-F</td>
<td>GAACGATCCACAACAGITCG</td>
</tr>
<tr>
<td></td>
<td>CMV-R</td>
<td>GGCAATCTCTTCAGTCTACG</td>
</tr>
<tr>
<td>LMoV</td>
<td>LMoV-F</td>
<td>GCTACAGAATCTGTIAACTG</td>
</tr>
<tr>
<td></td>
<td>LMoV-R</td>
<td>ATGGCCGTTCATGTAACC</td>
</tr>
<tr>
<td>LSV</td>
<td>LSV-F</td>
<td>CTGTACACCTTCACAGCAC</td>
</tr>
<tr>
<td></td>
<td>LSV-R</td>
<td>GIAATTCGTGCCTCACCTAC</td>
</tr>
<tr>
<td>LVX</td>
<td>LVX-F</td>
<td>AGGAGCTACAATCCATGCGC</td>
</tr>
<tr>
<td></td>
<td>LVX-R</td>
<td>ATTCAGACCTCCACCTCC</td>
</tr>
</tbody>
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Fig. 2. Agarose gel electrophoresis of RT-PCR products from leaves of mother plants to detect presence or absence of CMV, LMoV, LSV, and LVX. Lane M: *λ* phage marker.
was shown that DHT could not eradicate LMoV in our experiments, in agreement with previous experiments of Virazole effects on *L. longiflorum* (Xu et al., 2000). Although thermotherapy has been applied to eliminate LSV effectively in *L. × elegans* (Nesi et al., 2009), there has been no report on the successful elimination of CMV and LMoV using thermotherapy; therefore, it seems that meristematic tip culture and either meristematic tip culture or chemotherapy should be used for LMoV and CMV elimination, respectively, and meristematic tip culture, chemotherapy or thermotherapy can eradicate LSV.

**Table 2.** Efficiency of virus elimination in bulblets regenerated by first and second meristematic tip cultures.

<table>
<thead>
<tr>
<th>First meristematic tip culture from bulblets with CMV, LMoV, and LSV infection</th>
<th>Second meristematic tip culture with DHT from bulblets with LMoV or LSV infection</th>
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</thead>
<tbody>
<tr>
<td>Number of bulblets used</td>
<td>Infected viruses</td>
</tr>
<tr>
<td>30</td>
<td>CMV, LMoV, and LSV</td>
</tr>
<tr>
<td></td>
<td>LMoV and LSV</td>
</tr>
<tr>
<td></td>
<td>LMoV</td>
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<td></td>
<td>LSV</td>
</tr>
</tbody>
</table>

— not examined.
Multiplication in vitro, acclimation, and cultivation in a phytotron

It has been reported that the virus infection rate increased in acclimated plantlets in many bulbous plants (Phillips, 1990; Vcelar et al., 1992; Xu and Niimi, 1999; Xu et al., 2000). In this study, to check for virus propagation after acclimation, virus-free bulblets produced by meristematic tip culture with DHT were grown in the phytotron and examined by RT-PCR virus detection methods. The leaves of fifteen acclimated plantlets grown in the phytotron did not exhibit any symptom of distorted and twisted growth or mosaic (Fig. 5 left). RT-PCR products corresponding to CMV, LMoV, LSV, and LVX were not detected in any plantlets (Fig. 5 right). Many researchers have assessed virus infection before acclimation using enzyme-linked immunosorbent assay (ELISA), in which detection sensitivity is very limited with very low virus concentration (Niimi

Fig. 4. Agarose gel electrophoresis of RT-PCR products from leaves in regenerated bulblets after second meristematic tip culture with DHT to detect the presence or absence of CMV, LMoV, LSV, and LVX. Lane M: λ styI marker. a and b: Bulblets regenerated from LSV-infected bulblets, c: Bulblets regenerated from LMoV-infected bulblets.

Fig. 5. Leaves without virus-infected symptoms and agarose gel electrophoresis of RT-PCR products from leaves after acclimation. Lane M: λ styI marker.
et al., 2003). Alternatively, our results using a sensitive virus detection method, RT-PCR, confirmed complete virus eradication in the bulblets after meristematic tip culture. In conclusion, meristematic tip culture in combination with chemotherapy is significantly effective to obtain virus-free bulblets of *L. brownii* var. *colchecteri*.

**Literature Cited**


