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Virus-tested *Lycoris aurea* plants from apical meristems of adventitiously regenerated shoots

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ABSTRACT. Adventitious shoots were first regenerated in vitro from twin-scale sections of virus-infected *Lycoris aurea* Herb. cv. 'Golden spider lily'. Their apical meristems were then excised and cultured to redevelop shoots. Explants were not larger than 0.1 mm and were limited to the dome and subjacent stem, without leaf primordia. The redeveloped shoots were multiplied, rooted, hardened, and acclimatized, before transfer to an insect-free greenhouse. ELISA assays were performed for three lycoris potyviruses (lycoris potyvirus, LPV; lycoris mild mottle virus, LyMMV; and lycoris virus Taiwan, LVT) and for cucumber mosaic virus (CMV). Assays after one year and re-assays in the subsequent three years revealed no LyMMV, LPV, or CMV in the meristem-derived plants or in their clonal progeny. The virus-tested plants are now being increased as foundation stock.

Keywords: Cucumber mosaic virus; Lycoris mild mottle virus; Lycoris potyvirus; Lycoris virus Taiwan; *Lycoris*; Meristem culture; Virus tested plants.

INTRODUCTION

With clonally propagated crops, which include the bulbs, viruses are readily spread through plant propagation. The spread can be minimized by employing virus-free propagules. Commercial plantings of the indigenous *Lycoris aurea* Herb. cv. 'Golden Spider' in Taiwan are infected by three lycoris potyviruses—lycoris potyvirus (LPV), lycoris mild mottle virus (LyMMV), and lycoris virus Taiwan (LVT)—and by cucumber mosaic virus (CMV) (Chang et al., 2002). LyMMV induces mild symptoms. No symptoms have been observed in the rare instances of infection by LPV alone. This virus is usually found in mixed infections with LyMMV. The combination causes severe necrosis that can devastate plantings severely. CMV infection has also shown no characteristic symptoms. Currently, the four viruses do not cause a serious problem, but they are a potential threat to the budding *Lycoris* flower industry. Therefore, this investigation has pro-active intents: (1) to disclose a reliable method for recovering virus-tested plants from infected material and (2) to establish a foundation of uninfected stocks that can serve as a source of propagules to avoid the risk of an epidemic because the three lycoris potyviruses are easily spread through propagating by bulbs and are readily transmitted mechanically and by vector.

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MATERIALS AND METHODS

Regenerating adventitious shoots from bulb scale segments

Bulbs of four commercially grown plants that tested positively for LyMMV were potted in a 1:1:1 mixture of peat:perlite:vermiculite and cultivated in an insect-free greenhouse. These served as our infected controls and sources of adventitious shoots for meristem culture. Twin-scale segments were excised from the bulbs and cultured by the method described previously to obtain adventitious shoots (Huang and Liu, 1989).

Culturing meristems of regenerated shoots

When regenerated adventitious shoots were 5 cm in length, their apical meristems were excised. A dissecting microscope was employed to ensure that final explants were not longer than 0.1 mm and comprised only the dome and a portion of its subjacent stem without leaf primordia

(Figure 1). They were placed in a medium supplemented with 0.44 μM of BA (N^6-benzyladenine) and 0.54 μM NAA (α-naphthaleneacetic acid) to stimulate shoot emergence. As shoots developed up to 1 cm, they were removed and multiplied in another medium containing 44 μM BA and 16 μM NAA. When multiplied shoots were 5 cm tall, they were rooted, either individually or in clusters, in a third medium supplemented solely with 16 μM NAA. Bulbs also differentiated in this medium. Basal constituents of the three media were the same as those of the twin-scale culture medium. Rooted plants were grown in pots containing a 1:1:1 peat:perlite:vermiculite mixture; hardened and acclimatized in a growth chamber, the relative humidity of which was lowered in 5% increments at weekly intervals from 95 to 80%; then transferred to an insect-free greenhouse.

Virus assays

Initial virus assays were performed a year after meristem-derived plants were transferred to the greenhouse. They were re-assayed another year later and again after the third year. The re-assays included their clonal progeny. LPV, LyMMV, LVT and CMV contents were determined by ELISA, following the procedure for the detection tuberose potyviruses (Chen and Chang, 1998) using the specific rabbit antisera for the corresponding viral coat proteins. Samples of ca. 0.2 g leaf tips were ground in 0.05 M phosphate buffer (pH 7.5), and a duplicate of 200-μl aliquots of juice supernatants were coated onto ELISA plates by incubating at 37ºC overnight. Plates were washed, and individual rabbit antisera were applied, followed by alkaline phosphatase-conjugated goat antibody. Nitrophenylphosphate substrate was added and OD_{405} measured. Positive virus controls were conducted with 1 mg ml^{-1} each of LPV and LyMMV coat proteins (Chang et al., 2002). LVT and CMV were determined by infected tissues.

Figure 1. Excised shoot apical meristem of *L. aurea* cv. 'Golden spider' Feulgen stained and viewed with dissecting microscope. Note absence of leaf primordia. The 0.1 mm tall tissue is shown at 500-fold magnification.

RESULTS

Previous observations with the presence the symptom in Table 1 are the results of ELISA assays performed the plants in vitro as well as on plants transplanted to the greenhouse and grown in the field. The sample of a healthy clone, previously tested as being free of the four viruses, is indicated by H. Only values above the double value of H (2 H) were considered evidence of virus presence. LyMMV, LPV, LVT, and CMV denote positive controls from the coat proteins of diseased plants. The leaf samples of 17 original bulbs were analyzed for four viruses. As in Table 1 the rate of infected plants was detected by either single infection or mixed infection with a reading above 2 H on OD_{405}. Readings of 96 plants from their meristem cultures and established in vitro were monitored first in 2001 and then again in 2004. They proved to be free of the four viruses, Subsequently, each time with random selection from 320 greenhouse plants in 2002 and 45 field grown plants each time in 2002 and 2005 were conducted thrice as seen in Table 1 indicated no virus presence. Virus exclusion was thus confirmed when re-assays with ELISA in were performed in the subsequent five years, which included examinations of clonal progeny.

DISCUSSION AND CONCLUSIONS

Lycoris aurea cv. 'Golden spider lily' is a very precious indigenous plant in Taiwan with a high economic value. The recent identification of viruses in commercial plantings of *Lycoris aurea* serves as a wake up call to the still emerging and promising component of Taiwan's flower industry. A combined infection by the lycoris potyviruses LyMMV and LPV can devastate diseased fields (Chang et al., 2002). Recently, another strain LVT was discovered specifically in Taiwan field plants (Dr. Chin-An Chang, personal communication). Epidemics are possible since these viruses are spread through propagules, by mechanical means, and insect vectors. Crop losses can be minimized by employing virus-certified plants as sources of propagules, as has been done successfully for citrus, strawberry, potato, carnation, and a number of other clonally propagated crops (Murashige, 1974). Their virus-certified plants were obtained by culturing shoot apical meristems or by in vitro grafting of shoot apices excised directly from infected plants (Murashige, 1974). Applicability of the meristem culture method, albeit modified, has now been demonstrated for *Lycoris* viruses. To maximize exclusion of infected cells, the isolated tissue was not larger than 0.1 mm and comprised only the meristem dome and its immediately subjacent stem without leaf primordia. Also, the meristems were isolated from adventitious shoots that developed in tissue cultures and not directly from greenhouse or field grown plants. Use of these adventitious shoots made disinfestation unnecessary. It also provided a large supply of meristems, whereas only one explant was obtainable per field-grown shoot. More importantly, the in vitro adventitious shoots

Table 1. Results of ELISA of *Lycoris aurea* cv. 'Golden spider lily' plants obtained by apical meristem cultures of adventitious shoots that regenerated from bulb-scale segment cultures.

Samples	Experiment Date	Total No. examined plants	Positive detection rate and ELISA reading at OD_{405}			
			LPV[1]	LyMMV	LVT	CMV
Original mother plants	2000.03.20	7	0/7[3,4]	4/7 (0.49±0.18)[3,4]	–[5]	0/7
Original mother plants	2000.05.17	6	4/6 (0.32±0.05)	6/6 (0.43±0.23)	–	–
Original mother plants	2004.05.12	4	0/4	2/4 (0.96±0.28)	3/4 (0.34±0.09)	0/4
Tissue-culture plants in vitro	2001.01.11	80	0/80	0/80	0/80	0/80
Tissue-culture plants in vitro	2004.04.23	16	0/16	0/16	0/16	0/16
Tissue-culture plants in green house	2002.05.03	320	0/320	0/320	0/320	0/320
Tissue-culture plants in field	2002.06.12	45	0/45	0/45	0/45	0/45
Tissue-culture plants in field	2005.03.15	45	0/45	0/45	0/45	0/45
2 H. CK[2]	2000-2005	8	0.22±0.02	0.22±0.06	0.22±0.03	0.22±0.05
D. CK[6]	2000-2005	8	2.75±0.72	2.83±0.34	0.98±0.20	2.7±0.34

[1]LPV: Lycoris potyvirus; LyMMV: Lycoris mild mottle virus; LVT: Lycoris virus T; CMV: Cucumber mosaic virus.
[2]2 H: Twice performed ELISA readings of healthy control antigens, which serve as threshold for determining positive virus detection.
[3]Virus detection rate (No. of samples with positive detection/total samples indexed).
[4]0: ELISA readings less than 0.22, which is the threshold of healthy control plants.
[5]–: Not tested. [6]D: ELISA readings of extracted virus coat protein from diseased plants employed as positive control.

Figure 2. Foundation stock of virus-tested *L. aurea* cv. 'Golden spider' plants initiated from apical meristems of adventitious shoots that regenerated in bulb scale segments in vitro.

were perhaps less likely to harbor viruses than field grown shoots, thus, increasing the probability of virus free meristems.

Smith and Murashige (1970) demonstrated that isolated shoot apical meristems, without leaf primordia, were capable of developing into whole plants when provided suitable nutrient media. Hence as expected, a small but sufficient number of our tiny isolates re-developed into shoots very slowly and eventually grew into plant clones of all four bulbs. All clones tested free of the viruses that are currently detected in commercial plantings (Chang, Chen and Hsu, in press). They are being increased as foundation stock in insect-free greenhouses (Figure 2).

Lycoris plants that test free of LyMMV, LPV, LVT, and CMV exist among commercial plantings and can also be employed for establishing foundation stocks, but they may contain other unidentified viruses. By confining plant initials to small numbers, as was done in our meristem culture method, removal of all infecting viruses is virtually assured. In all instances the virus elimination must be verified by specific tests, as were performed in this investigation. We are also continuing to monitor our foundation stock for any evidence of somaclonal variants that sometimes occur among plants recovered through tissue culture.

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金花石蒜試管內之不定芽再生莖其生長點培養建立無病毒健康苗

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金花石蒜又名金色蜘蛛百合為台灣原生花卉，本研究先以試管內雙鱗片培養建立試管再生系統，誘導不定芽大量分化和繁殖，不定芽長大成苗後再切取其小於 0.1 毫米高之微小莖頂生長點（不帶有葉原體），促使其發育抽長成莖、及繁殖、發根、馴化後，移植管外於隔離溫室繼續生長茁壯。以酵素抗体分析法（ELISA）鑑定種植一年後及連續四年共五年追蹤四種金花石蒜 potyviruses 病毒（LPV、LyMMV 和 LVT）及胡瓜嵌紋病毒（CMV），確定無上述四種病毒，即無 LPV、LyMMV、LVT、CMV 之毒素病存在。自莖頂生長點培養獲得之健康無性系，現已成為原原種（Fundation stock）建立大量田間種植及繁殖。

關鍵詞：胡瓜嵌紋病毒；石蒜微斑紋病毒；石蒜 Y 病毒；石蒜 T 病毒；生長點培養；無病毒驗證植株。