Plant Methods

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Production of genetically stable and *Odontoglossum* ringspot virus-free *Cymbidium* orchid 'New True' plants via meristem-derived protocorm-like body (PLB) subcultures

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Abstract

Background This study aimed to produce *Odontoglossum* ringspot virus (ORSV)-free *Cymbidium* orchid 'New True' plants from ORSV-infected mother plants by culturing their meristems and successively repeating subcultures of protocorm-like bodies (PLBs) derived from the meristems.

Results Initially, ORSV was confirmed as the causative agent of viral symptoms in orchid leaves via reverse transcription-polymerase chain reaction (RT-PCR) analysis. Meristems from infected plants were cultured to generate PLBs, which in sequence were repeatedly subcultured up to four times. RT-PCR and quantitative RT-PCR analyses revealed that while ORSV was undetectable in shoots derived from the first subculture, complete elimination of the virus required at least a second subculture. Genetic analysis using inter-simple sequence repeat markers indicated no somaclonal variation between regenerated plants and the mother plant, suggesting that genetic consistency was maintained.

Conclusion Overall, our findings demonstrate that subculturing PLBs for a second time is ideal for producing genetically stable, ORSV-free *Cymbidium* orchids, thus offering a practical means of generating genetically stable, virus-free plants and enhancing plant health and quality in the orchid industry.

Keywords *Cymbidium* orchid, Genetic stability, Meristem culture, ISSR marker, qRT-PCR analysis, Protocorm-like bodies (PLBs)

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Background

Odontoglossum ringspot virus (ORSV) and *Cymbidium* mosaic virus (CyMV) are widely recognized as the most prevalent viruses infecting orchids globally [1, 2]. Over the past decades, the incidence of these viruses in cultivated hybrids and native orchids has frequently been observed in Korea [3–6]. Hyun et al. [7] reported that among 1,148 individual orchid plants representing 42 species, CyMV and ORSV were detected in 10.4% and 27.0% of the samples, respectively, and Korean native Cymbidiums exhibited a 17.7% viral infection rate, indicating that ORSV is more prevalent than CyMV in orchids in Korea. ORSV manifests as chlorotic ringspots or necrosis on leaves and causes discoloration and deformation in flowers, diminishing both the plants' vigor and floral quality [8]. Studies have observed that certain ORSV-infected orchids experience significant declines in vegetative growth, flower size, and bloom quantity within 2 years of cultivation [1, 9, 10]. This degradation in floral quality not only fails to satisfy export standards but also leads to considerable economic losses. The most effective strategy to mitigate such losses involves replacing infected plants with virus-free stocks. The extensive application of plant tissue culture techniques has been instrumental in generating these virus-free stocks. For instance, Chen et al. [9] successfully eliminated CyMV and ORSV from *Phalaenopsis* hybrids using shoot-tip culture and protocorm-like body (PLB) selection. Similarly, Lim et al. [10] managed to eradicate these two viruses from an infected monopodial orchid (Mokara Char Kuan "Pink") via meristem culture and thin section culture techniques. Additionally, Retheesh and Bhat [11] reported the successful elimination of cucumber mosaic virus and CyMV from *Vanilla planifolia* via meristem culture.

In vitro propagation of orchids via PLB multiplication is a preferred methodology, primarily because of its rapid production of numerous PLBs and ability to facilitate complete plantlet regeneration, as demonstrated in studies by Liau et al. [12], Naing et al. [13], and Sreeramanan et al. [14]. The advantages of this technique include enhanced rates of multiplication, increased total yields, and consistent maturation into plantlets devoid of somaclonal variation, as described by Pornpienpakdee et al. [15] and Naing et al. [13]. In contrast, the induction of somaclonal variation in the PLBs of *Phalaenopsis* and *Dendrobium* orchids has been reported by Samarfard and Kadir [16] and Chin et al. [17]. These studies employed inter-simple sequence repeat (ISSR) markers, which are extensively utilized to ascertain genetic fidelity in various regenerated plants, including banana [18], *Camellia sinensis* [19], *Swertia chirayita* [20], and *Bletilla striata* [21]. Chen et al. [9] successfully eradicated ORSV and CyMV from *Phalaenopsis* orchids through repetitive PLB subculturing, a process known to potentially induce somaclonal variations, as indicated by Cote et al. [22]. However, somaclonal variation has not been assessed in PLB-derived virus-free plants using molecular markers.

In the present study, we aimed to investigate the efficacy of subculturing meristem-derived PLBs in producing ORSV-free plants from ORSV-infected *Cymbidium* orchid "New True" plants. Additionally, we determined the optimal number of subcultures required to achieve virus eradication. Moreover, we examined the occurrence of somaclonal variation in the PLB-derived virus-free plants using ISSR markers.

Materials and methods

ORSV and RSV detection in *Cymbidium* orchid

Symptoms of viral disease, showing chlorotic ringspots or necrosis, were observed on the leaves of *Cymbidium* orchid 'New True' plants grown in a greenhouse (Fig. 1a). Leaves exhibiting these symptoms were collected for RNA extraction and subsequent virus detection. RNA was extracted from the leaves using the RNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's instructions. An oligo (dT)20 primer was utilized for RNA reverse transcription into complementary DNA (cDNA) using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), as described by Naing et al. [23]. Subsequently, the presence of ORSV and *Cymbidium* ringspot virus (RSV) in the leaves was detected using reverse transcription-polymerase chain reaction (RT-PCR). The primers and PCR conditions used in this study are listed in Supplementary Table 1. The PCR products were separated on a 2% agarose gel and visualized using ethidium bromide staining. A 771-bp band was expected for ORSV, and a 504-bp band was expected for RSV.

Surface sterilization of ORSV-infected shoots

Shoots collected from ORSV-infected plants were subjected to surface sterilization prior to initiating meristem culture for PLB induction. The sterilization protocol involved sequential treatment commencing with 0.1% streptomycin for 30 s, followed by 70% ethanol for 1 min. This was succeeded by a series of mercuric chloride treatments at concentrations of 0.5%, 0.2%, and 0.1% for 10, 10, and 2 min, respectively. Thereafter, the shoots were extensively rinsed with sterilized double-distilled water to remove any residual sterilants. Under sterile conditions, young leaves were delicately removed from their shoots using a microscope to expose the meristem tip.

Meristem culture

Meristem tips (approximately 0.5 mm in size) were cultured on vitamin-enriched Murashige and Skoog (MS) medium containing 1.0 mg/L benzylaminopurine (BA),

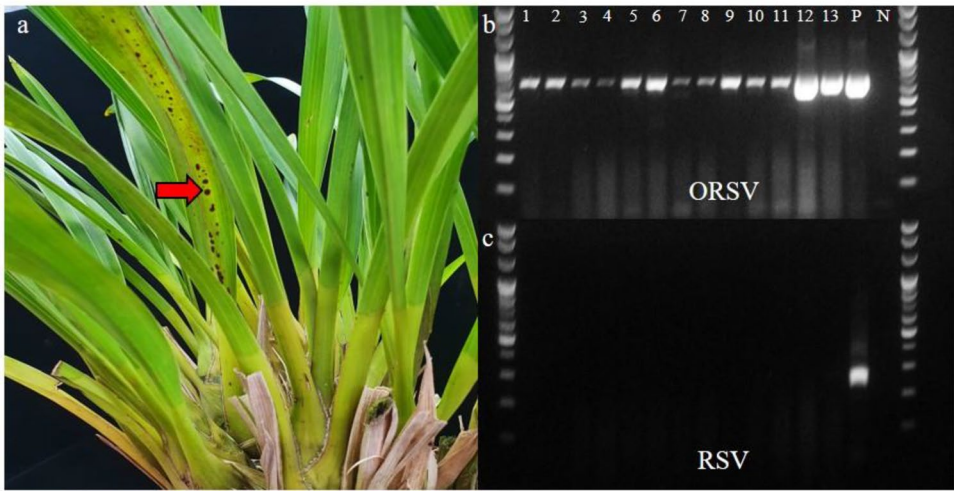

Fig. 1 Greenhouse-grown *Cymbidium* orchid 'New True' plants showing symptoms indicated by the arrow (**a**), and detection of ORSV (**b**) and RSV (**c**) using RT-PCR. Lanes 1–13 represent samples tested for ORSV (**b**) and RSV (**c**). P: Positive control for ORSV or RSV; N: Negative control (uninfected). Samples numbered 1–13 in panel (**b**) tested positive for ORSV but negative for RSV in panel (**c**)

Table 1 ORSV detection in shoots regenerated from the subsequent subculture of PLBs derived from ORSV-infected meristems

PLB subculture	No. of regenerated plantlets from PLBs	No. of plantlets sampled for RNA	Virus detection via RT-PCR	
			Negative (%)	Positive (%)
1	3	3	100	0
2	18	18	100	0
3	47	47	100	0
4	36	36	100	0

1.0 mg/L naphthaleneacetic acid (NAA), 30 g/L sucrose, and 7 g/L agar. The medium's pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. Culture plates were placed in a culture room with a temperature of 25 °C, photoperiod of 12 h, active photosynthetic radiation intensity of 70 μmol m^{-2} s^{-1}, and relative humidity of 70%. After 4 weeks of culture, we investigated the initiation of protocorm-like structure from the meristems.

PLB subcultures and shoot regeneration

Meristem-initiated protocorm-like structures were cultured on PLB induction medium (vitamin-enriched MS medium containing 1.13 mg/L BA, 0.47 mg/L NAA, 30 g/L sucrose, and 7 g/L agar). After 4 weeks of culture, the initiated PLBs were subcultured on vitamin-enriched MS medium comprising 0.5 g/L activated charcoal (AC), 30 g/L sucrose, and 7 g/L agar for PLB proliferation and conversion to shoots; the obtained shoots were termed "first subculture-derived shoots." Subsequently, some PLBs from this subculture were re-subcultured on the same proliferation medium to establish a second subculture of PLBs and shoots. This procedure was repeated to generate third and fourth subcultures. Shoots derived from the third and fourth subcultures were termed "third subculture-derived shoots" and "fourth subculture-derived shoots," respectively. Shoots from each subculture were subsequently transferred to a shoot elongation and rooting medium (3 g/L Hyponex, 2 g/L peptone, 30 g/L sucrose, 0.5 g/L AC, and 8 g/L agar) for 4 weeks. The elongated shoots with roots were transferred to large bottles containing the same medium for further growth and development. The culture room conditions were maintained as previously described.

ORSV detection in PLB-derived plants

Leaves from the PLB-derived shoots were harvested for total RNA extraction and ORSV detection. In addition, total RNA was extracted from ORSV-infected mother plants that had been grown in a greenhouse to be used as a positive control. RNA extraction and reverse transcription of the RNA into cDNA were performed as described above. Subsequently, the presence of ORSV in the leaves of the shoots derived from the first, second, third, and fourth subcultures as well as the leaves of the ORSV-infected mother plants was detected using RT-PCR, as previously described. Specifically, 3, 18, 47, and 36 plants from the first, second, third, and fourth subcultures were subjected to ORSV detection, respectively.

Detection of ORSV expression levels in PLB-derived plants

ORSV expression levels in leaves from the PLB subculture-derived plants and those from the ORSV-infected mother plants were further detected using quantitative RT-PCR (qRT-PCR). Actin was used as the reference gene, and the quantitative comparative cycle threshold method (ΔΔCT) was employed to calculate relative gene

expression. Expression levels were assessed in triplicate in each of 3, 18, 47, and 36 plants from the first, second, third, and fourth subcultures, respectively. The primers and PCR conditions used in this analysis are described in Supplementary Table 2.

Detection of somaclonal variation in PLB-derived plants using ISSR markers

Leaves from the PLB subculture-derived shoots (1st to 4th subculture) and those from the greenhouse-grown mother plants were subjected to total genomic DNA isolation. DNA was isolated using the HiGene™ Genomic DNA Prep Kit (Biofact Biofactory). To detect somaclonal variation in PLB-derived shoots, ISSR analysis was conducted using nine different ISSR primers (UBC 812, UBC 834, UBC 840, UBC 842, UBC 864, T06, T05, 125, and 165) and PCR master mixes (Solg™ 2× Pfu PCR Pre-mix [SolGent Co., Ltd.]). The primers and PCR conditions used in the analysis are provided in Supplementary Table 3. Following PCR amplification, the PCR products were electrophoresed on a 2% (w/v) agarose gel stained with ethidium bromide and photographed under ultraviolet light. This analysis was performed on three biological replicates for each primer to verify the banding patterns.

Statistical analysis

Data were statistically analyzed using SPSS (version 11.09; IBM Corporation, Armonk, United States) and were expressed as the mean (of three replicates)±standard error. One-way analysis of variance with Tukey's post-hoc test was used to separate the mean values, and statistical significance was set at $p<0.05$.

Results

ORSV incidence in *Cymbidium* orchid 'New True 'plants

We observed viral disease symptoms in the leaves of certain greenhouse-grown *Cymbidium* orchid 'New True' plants (Fig. 1a). To identify the causative virus, we collected symptomatic leaves and performed RT-PCR analysis to detect two common orchid viruses: ORSV and RSV. The results indicated the presence of ORSV in the symptomatic leaves (Fig. 1b), while RSV was not detected in these samples (Fig. 1c). Although the possibility of other viral infections cannot be ruled out, our findings suggest that ORSV is the most likely cause of the observed symptoms in these plants.

Induction of PLBs and PLB-derived shoots from ORSV-infected plants

Shoots from the ORSV-infected plants were harvested to isolate meristems for PLB induction (Fig. 2a). Of total meristems cultured, 40 out of 64 meristems, which were cultured on vitamin-enriched MS medium containing a combination of 1.0 mg/L BA and 1.0 mg/L NAA, began to differentiate and form the initial protocorm-like structures (Fig. 2b). Four-week culturing of these protocorm-like structure tissues on PLB-induction medium elicited PLB development (Fig. 2c). The initiated PLBs induced

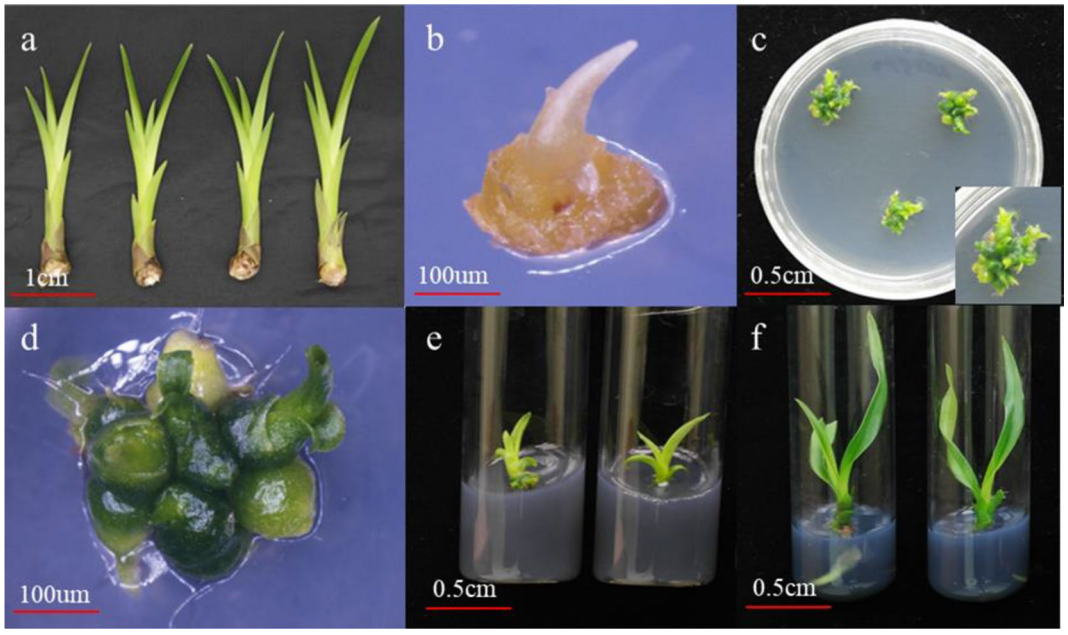

Fig. 2 PLB induction and shoot regeneration of meristems from ORSV-infected *Cymbidium* cv. 'New True' plants; (**a**) shoots harvested from ORSV-infected mother plants, (**b**) protocorm-like structure formation from meristems isolated from ORSV-infected shoots, (**c**) PLB induction from protocorm-like structures initiated from meristems, (**d**) PLB proliferation and conversion to shoots, (**e**) transfer of PLB-derived shoots to shoot-elongation medium, and (**f**) PLB-derived shoot elongation and rooting on shoot-elongation medium

new PLBs on the PLB-proliferation medium, and some of these newly induced PLBs converted to shoots (Fig. 2d), resulting in the first PLB and shoot culture. Repetitive subculturing (up to four times) of these newly induced PLBs on the same medium yielded additional PLBs and shoots. Once these shoots had been transferred to shoot-elongation medium (Fig. 2e), they grew well and produced roots after 4 weeks of culture (Fig. 2f). Further shoot and root growth was observed in the elongated shoots with roots after being transferred to large bottles containing the same medium for 4 weeks (Fig. 3).

ORSV detection in PLB-derived shoots from repetitive subcultures

RT-PCR analysis of ORSV in PLB-derived shoots from the various repetitive subcultures (1st to 4th subculture) revealed no ORSV bands in any of the tested samples. However, an ORSV band was clearly observed in the ORSV-infected mother plant (Fig. 4; Table 1). These results indicate that PLB induction and subculture can produce ORSV-free plants.

ORSV expression levels in PLB-derived shoots

As described above, ORSV bands were not detected in the shoots derived from repetitive PLB subcultures, including the first subculture; however, they were evident in the ORSV-infected mother plants. Therefore, the ORSV expression levels in the PLB subculture-derived shoots were analyzed using qRT-PCR, which demonstrated significantly high ORSV expression levels in the infected mother plants and undetectable ORSV expression levels in two out of three shoots derived from the first PLB subculture (Fig. 5). However, these expression levels were not detected at all in the shoots derived from the 2nd to the 4th PLB subcultures. These results suggest that at least a second PLB subculture is necessary to eliminate ORSV from PLB-derived shoots and produce ORSV-free plants.

Genetic variation analysis using ISSR markers

Nine different ISSR primers (UBC 812, UBC 834, UBC 840, UBC 842, UBC 864, T06, T05, 125, and 165) were utilized to detect potential somaclonal variation in samples derived from ORSV-infected meristems and regenerated via successive PLB subcultures (1st to 4th subculture) in comparison with that in samples from greenhouse-grown mother plants (M). Based on gel images (Fig. 6), no discernible genetic variation in all tested ISSR primers apparently existed between the mother plants and PLB subculture-regenerated shoots. All samples displayed consistent band patterns, suggesting that the regeneration process maintained the plants' genetic integrity across the different subcultures.

Discussion

Orchids occupy a significant place in Korean culture, supporting their market despite economic and regulatory challenges [24]. In 2007, the production of orchids in Korea was notable, with 9.7 million pots generating a wholesale value of 1 billion US dollars, highlighting their enduring significance in South Korea's flower industry [25]. However, the incidence of ORSV and CyMV in

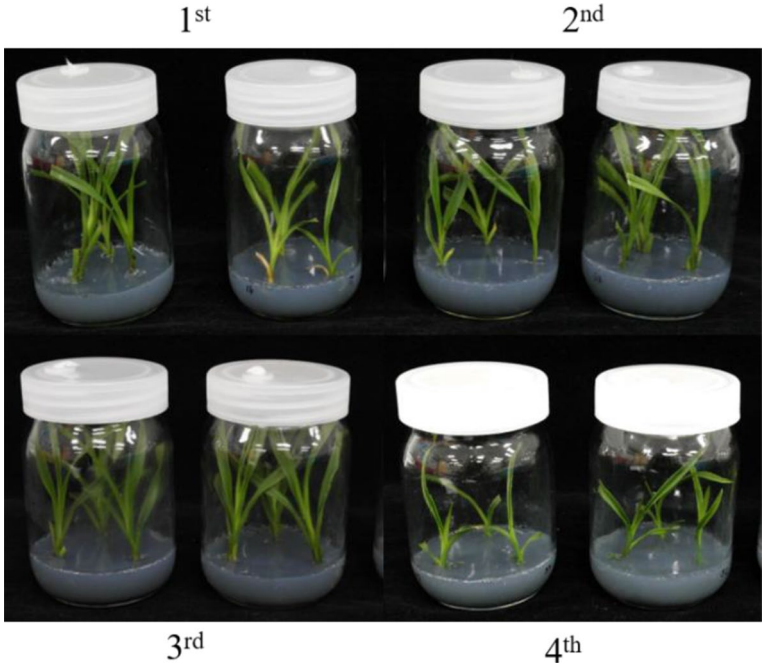

Fig. 3 Growth and development of shoots derived from repetitive PLB subcultures on shoot-elongation medium after 4 weeks of culture

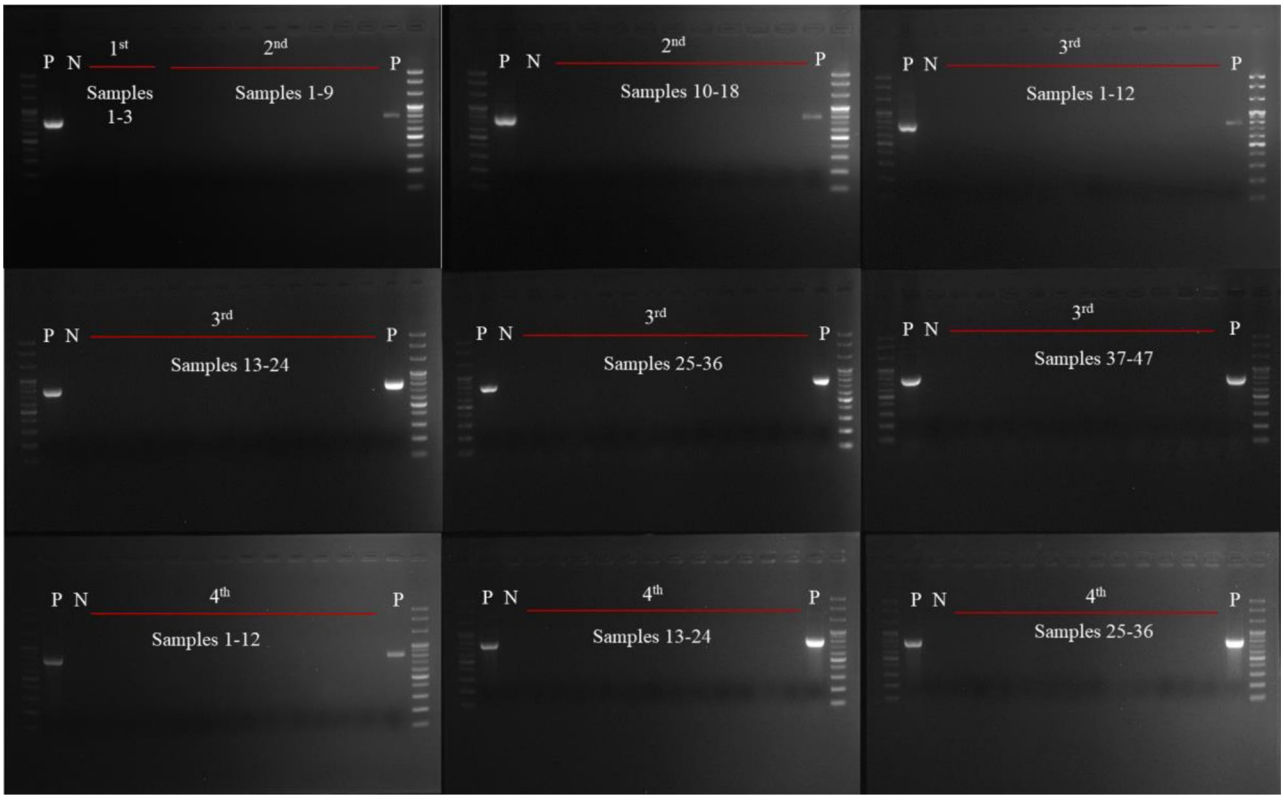

Fig. 4 RT-PCR detection of ORSV in mother plants and shoots derived from the subsequent subculture of meristem-derived PLBs. P: samples (positive control) from ORSV-infected mother plants, 1st : shoot samples derived from the first PLB subculture, 2nd : shoot samples derived from the second PLB subculture, 3rd : shoot samples derived from the third PLB subculture, 4th : shoot samples derived from the fourth PLB subculture, N: samples (negative control; uninfected plants)

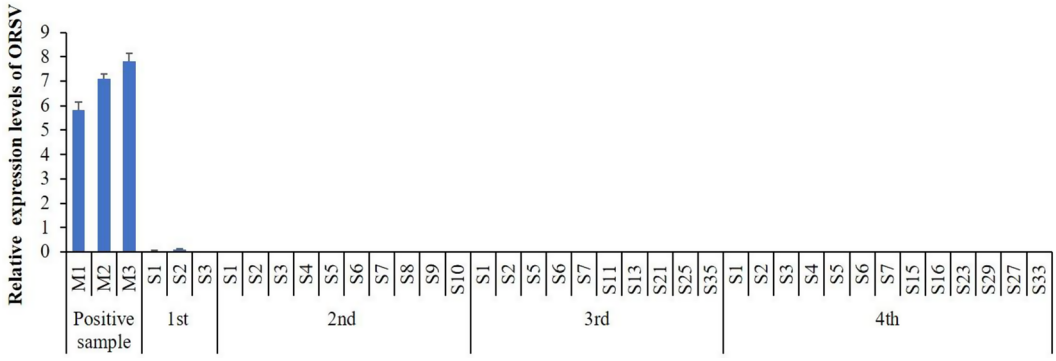

Fig. 5 ORSV expression levels in mother plants (positive samples) and shoots derived from the subsequent subculture of meristem-derived PLBs based on qRT-PCR analysis. 1st : shoot samples derived from the first PLB subculture, 2nd : shoot samples derived from the second PLB subculture, 3rd : shoot samples derived from the third PLB subculture, 4th : shoot samples derived from the fourth PLB subcultures

cultivated hybrids and native orchids has frequently been observed in Korea [3–6], with ORSV being more prevalent than CyMV [7]. ORSV causes leaf discoloration and flower deformation, thus not only failing to satisfy export standards but also leading to considerable economic losses [1, 8, 26, 27]. The most effective strategy toward mitigating such losses involves producing virus-free stock to replace infected plants. In this study, we successfully produced ORSV-free orchid plants from ORSV-infected *Cymbidium* orchid "New True" plants via repetitive PLB subculturing.

Chien et al. [28] successfully eliminated CyMV and ORSV from *Phalaenopsis* hybrids using subcultures of PLBs derived from shoot-tip cultures. In their study, 68.75% and 18.18% of the shoots derived from the first and second PLB subcultures exhibited viral infection, respectively; however, all shoots derived from the third subculture were virus-free. In contrast, our RT-PCR

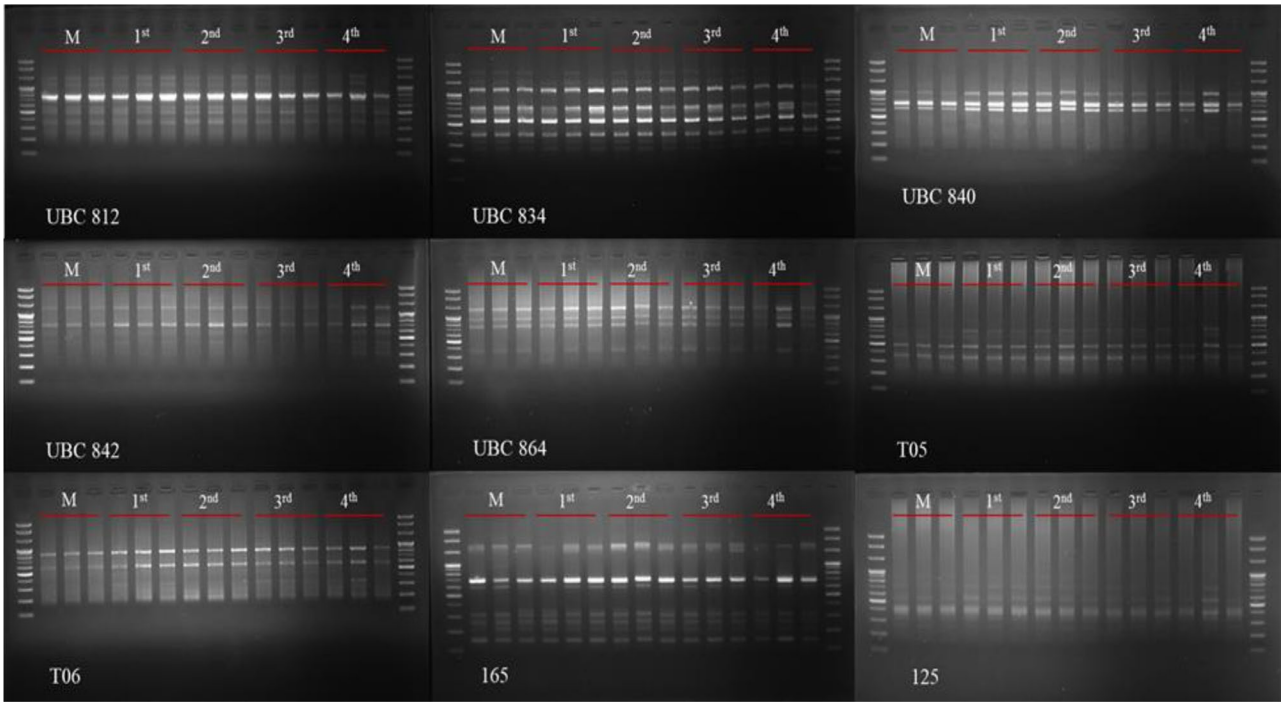

Fig. 6 ISSR-banding pattern in mother plants and shoots derived from the subsequent subculture of meristem-derived PLBs. Nine different ISSR primers were used to illustrate the banding patterns. M: mother plant samples, 1st : shoots derived from the first PLB subculture, 2nd : shoots derived from the second PLB subculture, 3rd : shoots derived from the third PLB subculture, 4th : shoots derived from the fourth PLB subculture

analysis revealed the absence of ORSV in shoots derived from the first and subsequent PLB subcultures of ORSV-infected meristems. However, extremely low ORSV expression levels were detected in shoots from the first PLB subculture, while none were detected in shoots from subsequent PLB subcultures. The observed qRT-PCR results, showing extremely low ORSV expression levels in the first PLB subculture and none in subsequent subcultures, may be explained by the fact that repeated subculturing of PLBs likely diluted and eventually eliminated the virus from the Cymbidium orchid samples. Continuous cell division required for the formation of new PLBs during subculture may produce cells that harbor relatively low levels of the virus, contributing to the production of virus-free plantlets. Our results indicate that at least a second PLB subculture is necessary to produce completely virus-free plants, while the findings of Chien et al. [28] suggest that a third PLB subculture is required to yield the same. This discrepancy between our findings and those of Chien et al. [28] potentially emanates from the use of different initial sources of plant tissues (meristems versus shoot tips). Meristem cultures have widely been used in various plant species to produce virus-free plants, including several orchids. For example, Lim et al. [10] employed meristem cultures to successfully eradicate ORSV and CyMV from an infected monopodial orchid (Mokara Char Kuan 'Pink'). Similarly, Retheesh and Bhat [11] observed the successful elimination of cucumber mosaic virus and CyMV from *Vanilla planifolia* via meristem culture. Additionally, Shen and Hsu [29] reported virus elimination in orchids using meristem culture. RT-PCR and real-time PCR (qRT-PCR) analysis were employed to detect the presence of the virus in orchids [9, 30–33]. Our results suggest that ORSV detection in shoots using RT-PCR alone is insufficient, as RT-PCR analysis may fail to detect minute amounts of the virus in plants. Therefore, we recommend employing both RT-PCR and qRT-PCR analyses to detect the presence of the virus in regenerated shoots to achieve virus-free production.

Samarfard and Kadir [16] reported that the combined presence of thidiazuron and chitosan in the medium induced somaclonal variation in the PLBs of *Phalaenopsis*. Similarly, Chin et al. [17] observed the influence of plant growth regulators and activated charcoal on somaclonal variation in the PLBs of the *Dendrobium* orchid. These studies employed ISSR markers, which are extensively use to ascertain genetic fidelity in diverse regenerated plants. Chien et al. [28] successfully eradicated ORSV and CyMV from *Phalaenopsis* orchids via repetitive PLB subculturing, a process known to potentially induce somaclonal variations, as indicated by Cote et al. [22]. However, they did not assess somaclonal variation in PLB-derived virus-free plants using molecular markers. In the present study, we examined the occurrence of somaclonal variation in PLB-derived virus-free

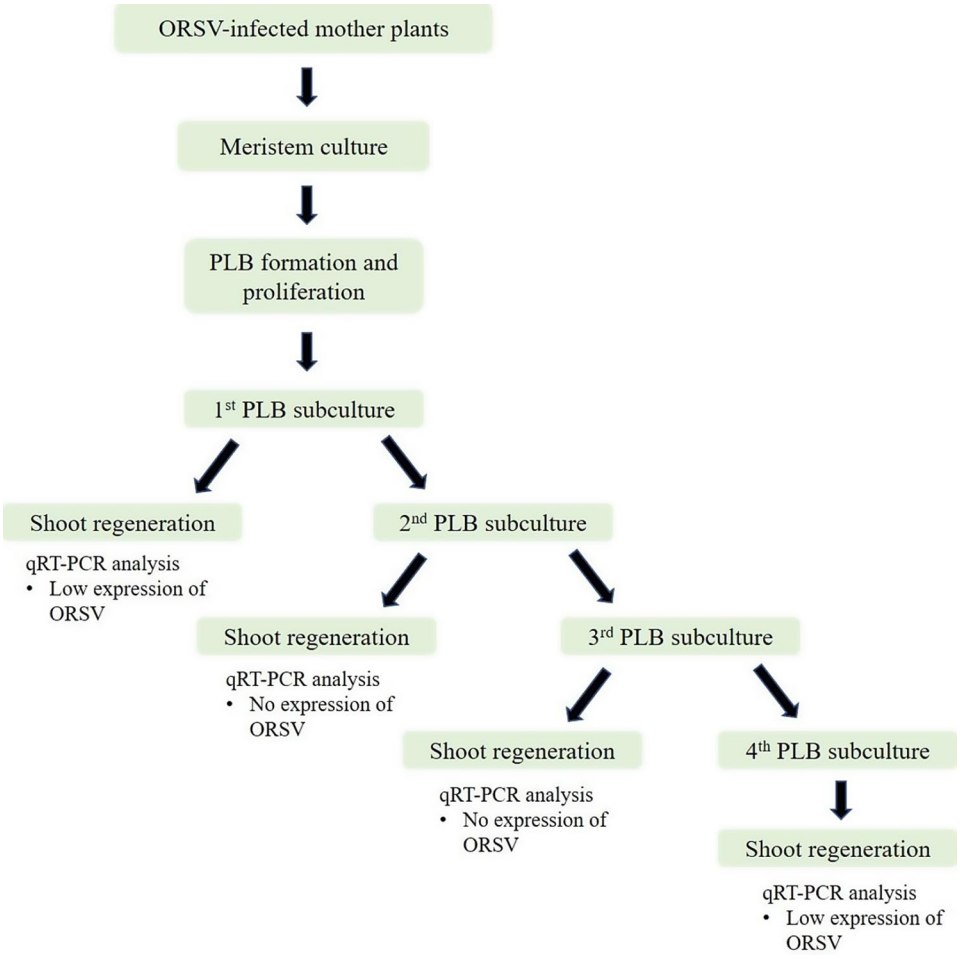

Fig. 7 Schematic representation of the production of ORSV-free plants through repetitive subculturing of PLBs originating from ORSV-infected mother plants

plants using ISSR primers. The banding patterns exhibited remarkable consistency across the mother plant and repetitive PLB subculture-regenerated shoots, indicating no apparent genetic variation. These results suggest that the regeneration process used to produce ORSV-free plants did not induce detectable genetic variability and can be employed to produce virus-free plants in this orchid species. The discrepancies observed between previous studies [16, 17] and our present work may arise from differences in the plant growth regulators and plant genotypes used, which play crucial roles in causing somaclonal variation in orchids.

Conclusion

In this study, we successfully produced ORSV-free *Cymbidium* orchid "New True" plants from ORSV-infected mother plants using repetitive PLB subcultures derived from ORSV-infected meristems (Fig. 7). RT-PCR and qRT-PCR analyses demonstrated that at least a second PLB subculture is necessary to eliminate ORSV, with subsequent subcultures displaying no viral presence. ISSR marker analysis revealed no somaclonal variation between regenerated plants and the mother plant, indicating maintained genetic integrity. Our findings suggest that subculturing PLBs for a second time is ideal for producing genetically stable, ORSV-free *Cymbidium* orchids, providing a practical means of combating viral infections and ensuring genetic stability in orchids.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-024-01269-1.

Supplementary Material 1

Author contributions

AHN and CKK designed the experiment. JRC conducted the experiment. HMA helped PLB subcultures. SBC, HK, and MYC assisted RNA extraction and PCR analysis. JRC collected data, took photos, and did data analysis. AHN and JRC wrote the manuscript. AHN revised the manuscript. CKK supervised the whole experiment.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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