Introduction of *Odontoglossum ringspot virus* Coat Protein Gene into *Cymbidium niveo-marginatum* mediated by *Agrobacterium tumefaciens* to Produce Transgenic Plants

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We cloned the *Odontoglossum ringspot virus* (ORSV) coat protein (CP) gene from ORSV-infected *Epidendrum* and constructed a pHPUNOJE-1 vector by ligating this CP gene, green fluorescence protein (GFP) gene, and hygromycin resistant (Hyg^r) gene into the plasmid pGreenII. This chimeric vector was mediated into *A. tumefaciens* LBA 4404. By infecting and co-culturing the rhizomes of *Cym. niveo-marginatum* with *A. tumefaciens* LBA 4404, ORSV CP gene transgenic shoots were obtained. GFP was observed in 64% of the 81 surviving rhizomes and in 39% of 215 regenerated shoots. The presence of ORSV CP, GFP, and Hyg^r genes was confirmed in the transgenic shoots by using polymerase chain reaction (PCR) analysis, whereas that of ORSV CP was confirmed by enzyme-linked immunosorbent assay (ELISA).

Key Words: *Agrobacterium*, cloning, *Cymbidium*, ORSV CP gene, transformation.

Introduction

In orchidaceous plants, *Odontoglossum ringspot virus* (ORSV) is especially prevalent and is distributed throughout the world (Erlinda and Benigno, 1983; Francki, 1966; Gibbs et al., 2000; Wisler et al., 1979; Zettler et al., 1990). ORSV was detected in 31 genera of orchid (Inouye, 2001). Inouye (1984) reported that even after soaking the 34 excised meristems (0.1–0.3 mm size) infected by ORSV in its antiserum, he only obtained 18 virus-free cultures. It is not possible to put this method to practical use because there is no effective means of preventing ORSV disease from infecting orchids.

Cross-protection with a mild strain of a given virus reduces the susceptibility of a plant to more virulent strain of the same virus (Zaitlin, 1976). This mechanism has been used to create virus-resistant plants by producing virus coat proteins (CP). Powell-Abel et al. (1986) obtained tobacco-mosaic-virus-resistant plants and Van Dun and John (1988) also obtained tobacco-rattle-virus-resistant plants by transforming their CP gene. Tumer et al. (1987) and Yoshioka et al. (1992) obtained transgenic tomato and melon plants with the CP genes of the alfalfa mosaic virus and cucumber mosaic virus, respectively. Resistance to each of these viruses has also been observed in the transgenic plants. Moreover, in a summary of 125 articles by Nishizawa et al. (1999), 52% of the plants that possess CP gene

provided virus tolerance to the transformed bodies.

The CP gene of ORSV has already been sequenced (Ajikuttira et al., 2002; Chng et al., 1996; Dubs and Van Regenmortel, 1990; Hilf and Dawson, 1993; Ikegami et al., 1995; Isomura et al., 1991; Ryu and Park, 1995). However, there has been no report on the CP-mediated transgenic orchidaceous plants. Here, we report the production of ORSV CP in the transgenic plants of *Cymbidium niveo-marginatum* for the first time.

Materials and Methods

Cloning of the ORSV CP gene

The total RNA of an ORSV-infected *Epidendrum* (*E. pink* Ball, known as J_E), which was cultured in the Hiroshima Mukaishima Orchid Center, was extracted and cleaned by using the RNeasy Plant Mini Kit, according to the manufacturer's instructions (QIAGEN, Germany). The DNA fragments of ORSV CP were obtained by RT-PCR (RT-PCR Kit: Rever Tra DashTM, TOYOBO, Japan) by using the total RNA with the reverse primer, 5'-CGC GAT ATC TTA GGA AGA GGT CCA AGT-3' and the forward primer, 5'-CCA GGA TCC ATG TCT TAC ACT ATT ACA G-3', which was designed to be based on the sequence reported by Isomura et al. (1991). These DNA fragments were inserted into the *Bam*H I and *Sma* I sites of a Glutathione S-transferase (GST) recombinant protein expression vector pGEX-6P-3 (Applied Biosystems, USA). This chimeric vector was named pHPUN001 and transformed into *Escherichia coli* (*E. coli*) DH 5α cells.

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Expression of ORSV CP gene in *E. coli* DH 5 α cells by Western blotting analysis

The colonies of *E. coli* DH5 α cells that harbor the pHUN001 were cultured to produce GST-ORSV CP recombinant protein in the LB medium according to the manual of Amersham Pharmacia Biotech. The suspension cultures were vortexed gently with BugBuster Protein Extraction Reagent (Novagen, USA) for 40 min at room temperature to destroy the cell walls of the *E. coli* DH 5 α . The re-centrifuged supernatants were purified by a MicroSpin™ GST Purification Module (Amersham Pharmacia Biotech, USA). The recombinant protein was treated with PreScission Protease (Amersham Pharmacia Biotech) at 5°C for 16 h to cleave ORSV CP from the GST fusion proteins. The electrophoresis of the recombinant proteins was carried out by using the PAGEL-Compact (Atto Corp., Tokyo, Japan). The separated bands were transferred to the nylon membrane by using the TE 70 Series SemiPhor Semi-Dry Transfer Unit (Amersham Pharmacia Biotech). Western blotting analysis was carried out by reacting the ORSV antiserum with a conjugate IgG that originated from a rabbit (Japan Plant Protection Association Laboratory).

Sequencing of the ORSV CP gene

After being screened by Western blotting analysis, the pHUN001 DNA of 9 clones that reacted with the ORSV antiserum was extracted and purified by the polyethylene glycol precipitating method. Then, PCR of ORSV CP gene, by using puReTaq™ Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech), was carried out by utilizing the pUC/M13 forward and reverse primers (Promega, USA). The PCR products were sequenced with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

Structuring the plasmid vector pGreen II-CaMV 35S GFP-CaMV 35S ORSV CP-NOS Hyg^r (pHPUNOJ_E-1)

ORSV CP gene from the start codon to the stop codon was used for structuring the plasmid vector. All the other genes used for structuring the plasmid vector, such as nopaline synthase (NOS) promoter, pGreen II vector, green fluorescence protein (GFP), hygromycin resistance (Hyg^r), cauliflower mosaic virus (CaMV) 35S promoter, and CaMV 35S-GFP, NOS-Hyg^r were obtained from John Innes Centre (UK).

With the forward primer 5'(BamH I)-CCA GGA TCC

ATG TCT TAC ACT ATT ACA G-3' and the reverse primer 5'(EcoR V)-CGC GAT ATC TTA GGA AG GGT CCA AGT-3', the BamH I and EcoR V sites were added to both ends of ORSV CP gene by the PCR method, by using pHUN001 as the template. Then, the ORSV CP gene was ligated to the BamH I and Sma I sites of the CaMV35S promoter vector. The structured vector was named pHUN002.

Through ligating CaMV35S-GFP gene to the Hpa I site of the pGreen II vector, a new vector, named pHUN003 (pGreen II-CaMV 35S-GFP) was obtained. Then, by ligating CaMV 35S-ORSV CP gene to the Xho I and Apa I sites of pHUN003 vector, another vector named pHUN004 (pGreen II-CaMV 35S GFP-CaMV 35S ORSV CP) was obtained. At the end, by ligating the NOS-Hyg^r to the Stu I site of vector pHUN004, the plasmid vector pHPUNOJ_E-1 was obtained (Fig. 1).

Making of *Agrobacterium tumefaciens* LBA4404 (pAL4404, pSoup, pHPUNOJ_E-1)

Because the plasmid pGreen requires the function of the RepA gene from plasmid pSoup to replicate it successfully in *Agrobacterium* species (Hellens et al., 2000), the plasmid pSoup was transformed into *A. tumefaciens* LBA 4404 (pAL 4404) by the Triparental mating method (Ditta et al., 1980). Then, the pHPUNOJ_E-1 vector was transformed into the *A. tumefaciens* LBA 4404 (pAL 4404, pSoup) by the same method, and the *A. tumefaciens* LBA 4404 (pAL 4404, pSoup, pHPUNOJ_E-1) was obtained.

Transforming the ORSV CP gene to *Cym. niveo-marginatum*

Excised rhizome sections (136) of *Cym. niveo-marginatum* were cultured with *A. tumefaciens* LBA 4404 (pAL 4404, pSoup, pHPUNOJ_E-1) for two days in LB solid medium containing 25 mg·L⁻¹ streptomycin, 5 mg·L⁻¹ tetracycline, and 25 mg·L⁻¹ hygromycin (Hyg) at 28°C. Infection and co-cultivation were carried out according to Chen et al. (2002). Subsequently, the rhizome sections were cultured in MS solid medium with 5 mg·L⁻¹ Hyg and 2 mg·L⁻¹ 6-benzyladenine (BA) for one month to induce transformed shoots.

Confirming the expression of GFP

The regenerated explants were observed for GFP with an OLYMPUS BX50WI-Macro RFL fluorescent microscope (OLYMPUS Corp., Tokyo, Japan). The

Fig. 1. Schematic structure of the T-DNA region in the plasmid pHPUNOJ_E-1. LB, Left border; T19S, terminator of CaMV 19S; GFP, the coding region of GFP gene; P35S, CaMV 35S promoter; ORSV CP, the coding region of ORSV CP gene; TNOS, nos terminator; Hyg^r, the coding region of Hyg^r gene; PNOS, nos promoter; RB, right border.

Fig. 2. The GST recombinant protein region of pGEX-6P-3 vector (A) and fragment of ORSV CP gene (B). ORSV CP gene was inserted into *Bam*H I and *Sma* I sites of GST recombinant protein region.

wavelength of occurring light is 510 nm and that of filter excitation light is 460–490 nm.

Confirming the transformed genes

Six shoots were selected at random from the other transgenic shoots in which the expression of GFP was observed by fluorescent microscope, except the 2 shoots used for DAS-ELISA described as follows. The 6 shoots (total weight about 0.5 g) were macerated and the total DNA extracted for PCR. The forward primer 5'(*Bam*H I)-CCA GGA TCC ATG TCT TAC ACT ATT ACA G-3' with the reverse primer 5'(*Eco*R V)-CGC GAT ATC TTA GGA AG GGT CCA AGT-3', the forward primer 5'-GGA TCC AAG GAG ATA TAA CAA TGA-3' with the reverse primer 5'-GAG CTC TTA TTT GTA TAG TTC ATC-3', and the forward primer 5'-TCT GTC GAG AAG TTT CTG ATC GAA-3' with the reverse primer 5'-GTT CTC TCG CAT TTG CCT TGC TA-3 were tested for the presence of ORSV CP, GFP and Hyg^r, respectively.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

DAS-ELISA (Converse and Martin, 1990) was used to detect ORSV CP in 2 transgenic shoots in which GFP fluoresced most strongly. Samples of transgenic and non-transgenic shoots (100 mg) were homogenized with 1 mL of PBS-T buffer (pH 7.4) that included 0.85% NaCl, 0.02% NaN₃, and 0.05% Tween 20. The homogenate was centrifuged for 6 min at 3000 rpm (Inouye, 2001). The supernatants (200 µL) were added to each of 3 wells containing the ORSV CP antibody coating liquid (rabbit r-globulin, 0.05% NaN₃) and anti-rabbit IgG conjugate liquid (alkaline phosphatase rabbit r-globulin, 1% cow serum albumin, and 0.05% NaN₃). The test was carried out as described by Takahashi (1988). The absorbance was measured at 405 nm with a JASCO UBEST-30 UV/VIS Spectrophotometer (JASCO, Tokyo, Japan).

Results and Discussion

Cloning of ORSV CP gene

The double-stranded DNA fragments of the 730 bp

ORSV CP were obtained by using RT-PCR of the total RNA of an ORSV-infected *Epidendrum*. They were purified and digested by the restriction enzymes of *Bam*H I and *Eco*R V linked in both ends of the CP gene fragments, respectively. Then, by inserting the fragments into the *Bam*H I and *Sma* I sites of a GST recombinant protein expression vector-pGEX-6P-3, the vector pHPUN001 (Fig. 2) was obtained.

Fig. 3. Gel plates confirming the expression of GST-ORSV CP recombinant protein by SDS-PAGE analysis (A) and Western blotting analysis (B). M, 10–225 kDa Coomassie blue perfect protein markers (Novagen); 1–9, lysates of 9 colonies of *E. coli* DH 5α cells harbouring pGEX-6P-3-ORSV CP gene, all of them produced the 47 kDa GST-ORSV CP recombinant protein; 10, liquid of lysate of number 9 colony after treating by PreScission Protease (29 kDa, GST protein; 18 kDa, ORSV CP); C, control of lysate of *E. coli* DH 5α cells harbouring pGEX-6P-3 vector (29 kDa, GST protein).

Expression of CP gene in *E. coli* DH 5 α cells by Western blotting analysis

To confirm the expression of CP gene by Western blotting analysis, the pHUN001 were transformed into *E. coli* DH 5 α cells. The 9 colonies of *E. coli* DH 5 α cells were transformed by the pHUN001 also expressed the 47 kDa recombinant proteins (Fig. 3A, lanes 1–9). When the recombinant proteins were treated with PreScission Protease at 5°C for 16 h, the 18 kDa ORSV CPs were cleaved from the GST fusion proteins (Fig. 3A, lane 10). All of the GST-ORSV CP recombinant proteins of the 9 colonies and ORSV CP reacted with the ORSV antiserum (Fig. 3B), indicating that the pHUN001 was transformed into the 9 colonies of *E. coli* DH 5 α containing the ORSV CP gene. Therefore, the ORSV CP genes from all 9 colonies were used for sequencing.

Sequence of ORSV CP gene

The sequences of the 730 bp ORSV CP genes derived from two colonies were in 100% correspondence. Those of the other 7 colonies attained 98–99% correspondence

with each other. Furthermore the sequences of the 477 bp from the start to stop codons of all 9 colonies were identical. In this range, the correspondences were 99.58%, 99.37%, 99.37%, 99.79%, and 99.58% with the sequences isolated in France (Type-Strain, called it as F_{TS}, Dubs and Van Regenmortel, 1990), Japan (Cy-1, J_{Cy-1}, Isomura et al., 1991), the U.S. (TMV-O, A_{TMV-O}, Hilf and Dawson, 1993), Korea (Cy, K_{Cy}, Ryu et al., 1995), and Singapore (S₁, Chng et al., 1996), respectively (Fig. 4A). Their deduced amino acid sequences were in 100% correspondence (Fig. 4B), showing that the rate of ORSV mutation is universally low. Thus, the transformed orchid with the CP gene of the one strain of ORSV, which was obtained by gene transformation, seemed to be resistant to all other strains of ORSV.

Survival rate of transformed explants selected by Hyg

When the infected rhizome sections were cultured for a month on MS solid medium, containing 5 mg·L⁻¹ Hyg and 2 mg·L⁻¹ BA, 81 rhizome sections out of a total of 136 (59.7%) survived. From the 81 rhizome sections, 227 shoots were induced of which 215 (94.6%) survived.

Fig. 4. Comparison of the nucleotide sequence of ORSV-J_E coat protein from start codon to stop codon with those of other five strains (A) and deduced amino acid sequence of ORSV CP gene (B). J_E, analyzed by us in this time isolated from an ORSV-infected *Epidendrum* in Japan; J_{Cy-1}, reported by Isomura et al (1991) isolated from Cy-1 strain in Japan; S₁, reported by Chng et al (1996) isolated from a Singapore strain; K_{Cy}, reported by Ryu et al (1995) isolated from Cy strain in Korea; F_{TS}, reported by Dubs and Van Regenmortel (1990) isolated from Type-Strain; A_{TMV-O}, reported by Hilf and Dawson (1993) isolated from TMV-O strain.

Confirming the expression of GFP by using a fluorescent microscope

The GFP was observed in 52 of 81 (64%) surviving rhizomes, whereas it was observed in 84 of 215 (39%) of the regenerated shoots. The expression of GFP appeared strong in 5 rhizome sections and shoots that were induced from these rhizomes. However, in the remaining 47 sections, the expression of GFP appeared only in some parts of the rhizome or induced shoots.

Size confirmation of the transformed genes by PCR

The sizes of all transformed genes were confirmed by PCR (Fig. 5) that for GFP was about 750 bp, whereas that of ORSV CP was about 500 bp from the start codon to stop codon and adding restriction enzymes sites of *Bam*H I and *Eco*R V, while that of *Hyg*^r was about 1000 bp.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

The liquid absorbance value of transgenic plant I (TI) was 0.526 ± 0.099 , that of transgenic plant II (TII) was 0.682 ± 0.108 , and that of untransformed control plant (C) was 0.182 ± 0.037 , so, $2C < TI < 3C < TII$. Thus, the absorbance values of TI and TII were 2.89 and 3.47 times that of the control, respectively. Takahashi (1988) examined 81 research theses, in which ELISA was used, found that if the absorbance value of the transformation body was twice or more than that of the non-transformed body, the protein appeared to possess the transformed gene. According to this, the ORSV CP gene obtained in this research was transformed.

It is known that ORSV reduces the economic value of orchidaceous plants. There is no effective means of preventing ORSV disease, but since the late 1980s many virus-resistant plants have been created by producing and expressing CP by transforming the CP gene. In orchidaceous plants, Anzai et al. (1996), Belarmino and Mii (2000), Chia et al. (1994), Kuehnle and Sugii (1992),

Yang et al. (1999), and Yu et al. (1999) isolated the β -glucuronidase (GUS) or firefly luciferase gene transformants of *Phalaenopsis*, *Cymbidium* or *Dendrobium*, whereas Knapp et al. (2000) produced the bar gene transformants of *Brassia*, *Cattleya* and *Doritaenopsis*. More recently, Chen et al. (2002), Niimi et al. (2001a, b) developed the GUS transformants of *Neofinetia falcata* Hu and *Cym. niveo-marginatum* Makino, while Yu et al. (2001) did the same with *Dendrobium* with the class 1 *Knox* gene *DOH1*. Liao et al. (2003) also obtained the sweet pepper ferredoxin-like protein (*pflp*) transformants of *Oncidium*, that conferred resistance against soft rot disease. Liao et al. (2004) was successful in obtaining transgenic *Phalaenopsis* possessing the CP of *Cymbidium* mosaic virus. Currently, however, very few reports deal with transgenic engineering in orchidaceous plants, one of the larger families of plants. In this research, we tried to create ORSV-resistance orchidaceous plants by transforming the ORSV CP gene. It seems that we confirmed the presence of the ORSV CP gene and ORSV CP in the transformed shoots of *Cym. niveo-marginatum* by PCR and ELISA methods, respectively. We hope to continue this research on the resistance to ORSV and its inheritance by new hybrid progenies.

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Fig. 5. A gel plate confirming the transformed genes by PCR. M1 and M2, size marker gene; 1, transgenic shoots genome DNA; 2, transgenic shoots genome DNA after treating by *Apa* I and *Xho* I; 3, PCR products of transgenic shoots genome DNA with GFP primers; 4, PCR products of transgenic shoots genome DNA with ORSV CP gene primers; 5, PCR products of transgenic shoots genome DNA with *Hyg*^r gene primers.

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Agrobacterium tumefaciens による *Odontoglossum ringspot virus* 外皮タンパク質遺伝子導入玉花ランの作出陳 莉¹・河合仁美¹・奥 尚¹・高橋智佐子²・新美善行¹¹広島県立大学生物資源学部 727-0023 庄原市七塚町²向島洋ランセンター 722-8510 広島県御調郡向島町

Odontoglossum ringspot virus (ORSV) 外皮タンパク質 (CP) 遺伝子をウイルスに感染した *Epidendrum* からクローニングし, green fluorescence protein (GFP) 遺伝子およびハイグロマイシン抵抗性 (Hyg^r) 遺伝子をプラスミドベクター pGreenII に組み込み, 植物発現ベクター pHPUNOJ_E-1 を構築した. プラスミド pHPUNOJ_E-1 ベクターを *Agrobacterium tumefaciens* LBA 4404 に導入し, 玉花ラン (*Cymbidium niveo-marginatum*) ライゾームと共存培養した. その結果, ハイグロマイシンセレクション 136

個のライゾーム中 81 個の生存を確認した. 生存したライゾームから 227 個のシュートが発生し, その中で 215 個の生存を確認した. 64%の生存したライゾームおよび 39%の生存したシュートから GFP 蛍光が観察された. GFP 蛍光が観察されたシュートから polymerase chain reaction (PCR) 法で ORSV CP, GFP, Hyg^r 遺伝子を検出した. ORSV CP は enzyme-linked immunosorbent assay (ELISA) で確認した.