LETTER TO THE EDITOR

CLONING AND SEQUENCING OF COAT PROTEIN GENE OF AN INDIAN ODONTOGLOSSUM RINGSPOT VIRUS ISOLATE

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Orchids are of great commercial value due to attractive flowers and long vase life. In India, more than 1300 orchid species occur and most of them are commercially important. They are concentrated in north-eastern part of the country due to variable climatic condition, which favor their proper growth. Like other plants they are susceptible to diverse viruses. Namely Odontoglossum ringspot virus (ORSV) and Cymbidium mosaic virus (CymMV) cause considerable damage to orchid plants by affecting their flower quality, production and commercial value (*1–2*). CymMV but not ORSV has been reported to occur in India (*3*).

Here, we describe (i) screening of orchid plants for ORSV by double-antibody sandwich ELISA (DAS-ELISA) and RT-PCR, (ii) obtaining of an Indian ORSV isolate, and (iii) characterization of the coat protein (CP) gene of this isolate by nucleotide sequencing. To investigate the possible molecular heterogeneity of ORSV, (iv) we compared the CP gene sequence of the Indian isolate with the known sequences of the isolates obtained from different genera of orchids from geographically distinct regions of other countries (Singapore, Korea, Taiwan, Japan and Thailand).

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Abbreviations: CP = coat protein; CymMV = Cymbidium mosaic virus; DAS-ELISA = double-antibody sandwich ELISA; ORSV = Odontoglossum ringspot virus

Samples from orchid plants of different genera and species (*Cymbidium aloifolium, C. iridioides, Cymbidium* Great Flower (Hybrid)*, Dendrobium* spp*, Coelogyne* spp*, Aerides odoratum, Smitinandia micrantha, Rhynchostylis gigantea, R. retusa, Phaius* spp etc) from different regions of India (Sikkim, Kalimpong, Dehradun, Chandigarh and Palampur) were obtained and screened for the presence ORSV by DAS-ELISA and RT-PCR.

A standard two-day DAS-ELISA was performed according to the instructions of the manufacturer (Agdia, USA). A_{405} was read using a METASKAN immunoreader (Germany). The virus extraction buffer served as negative control. A sample was considered positive if its absorbance was at least five times higher than that of the negative control.

For the detection of ORSV by RT-PCR a fresh leaf tissue from different orchids (50 µg) was frozen in liquid nitrogen and ground to a fine powder. Total RNA was extracted using TRI-REAGENT Kit and resuspended in 50 µl of sterile water. The RT step was performed in an Eppendorf® thermocycler in a 50 µl volume containing about 2 µg of total RNA (10 µl), 200 ng of a down primer L1 (*4*), 40 mol/l dNTPs (2.5 µl), 5x RT buffer (10 µl) (Pharmacia), 28 U of RNase inhibitor (1 µl), and 200 U of MuMLV reverse transcriptase (1 µl). The reaction proceeded at 37°C for 75 mins followed by 70°C for 5 mins.

The PCR reaction mixture (50 µl) contained an aliquot of the cDNA product (5 µl), 200 ng of the primers U1 and

	Amino acid sequence homology of the Indian ORSV isolate AJ564563 with other ORSV isolates (%)															
	AJ564563	AF033848	AF141927	AF515606	AJ429091	AJ429092	AJ429093	AJ429094	X55295	NC-001728	X78966	U34586	X82130	AY360407	U89894	AY376394
AJ564563	X	100	100	100	96	99	99	100	99	96	96	100	96	100	100	100
AF033848	100	X	100	100	96	99	99	100	99	96	96	100	96	100	100	100
AF141927	100	100	X	100	96	99	99	100	99	96	96	100	96	100	100	100
AF515606	100	100	100	X	96	99	99	100	99	96	96	100	96	100	100	100
AJ429091	96	96	96	96	X	95	95	96	95	92	93	96	92	96	96	96
AJ429092	99	99	99	99	95	X	98	99	98	95	96	99	95	99	99	99
AJ429093	99	99	99	99	95	98	X	99	98	95	96	99	95	99	99	99
AJ429094	100	100	100	100	96	99	99	X	99	96	96	100	96	100	100	100
X55295	96	99	99	99	95	98	98	99	X	95	96	99	95	99	99	99
NC-001728	96	96	96	96	92	95	95	96	95	X	99	96	100	96	96	96
X78966	100	96	96	96	93	96	96	96	96	99	X	96	99	96	96	96
U34586	96	100	100	100	96	99	99	100	99	96	96	X	96	100	100	100
X82130	100	96	96	96	92	95	95	96	95	100	99	96	X	96	96	96
AY360407	100	100	100	100	96	99	99	100	99	96	96	100	96	X	100	100
U89894	100	100	100	100	96	99	99	100	99	96	96	100	96	100	X	100
AY376394	100	100	100	100	96	99	99	100	99	96	96	100	96	100	100	X

L1 (*4*), 10 mmol/l dNTPs (3 µl), 10x Taq reaction buffer (5 µl), and 5 U of Taq DNA polymerase (0.5 µl). The cycling conditions were as follows: 1 cycle of 94°C/5 mins and 34 cycles of 94°C/1 min, 55°C/1 min, 72°C/90 secs, and the last extension at 72°C for 10 mins. For amplification of the complete CP gene the primers Osu and Osd had to be employed. A specific PCR product of 477 bp was expected. The reaction mixture was the same, but the cycling conditions were a little changed: 1 cycle of 94°C/5 mins and 25 cycles of 94°C/1 min, 57.5°C/1 min, 72°C/90 secs and last extension at 72°C for 10 mins. PCR products were electrophoresed in 1% agarose gel in TAE buffer, stained with ethidium bromide and visualized under UV.

The amplified product was cloned in the pUC18 plasmid and the resulting recombinant was employed for transformation of *E. coli* strain DH5α. The recombinant plasmid was purified and sequenced by the dideoxy chain termination method (*5*) using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA) and the ABI PRISM® Big Dye™ Terminator (version 3.0) Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA).

The determined nucleotide sequence of CP gene of the Indian ORSV isolate (Acc. No AJ564563) was compared with corresponding sequences from other ORSV isolates available in the EMBL database using the BLAST (NCBI) program (*6*). The BLASTP program was used for searching the amino acid sequence database. The parities comparison was performed using the ALIGN-2 program (*7*) and multiple alignments were generated by the MULTALIN program (*8*).

By ELISA it was found that most of the orchids collected from Sikkim, Chail and Palampur regions were infected with ORSV indicating its widespread presence (data not shown).

RT-PCR revealed that the orchid samples found to be symptomless are also infected with the viruses. By using the primers U1 and L1 CymMV yielded a PCR product of about 534 bp, while ORSV gave an about 290 bp product. The ELISA-negative samples were positive in RT-PCR.

The complete CP sequence alignment revealed that the Indian isolate of ORSV shares 96–100% homology with other ORSV isolates at nucleotide (data not shown) as well as amino acid (Table) level.

Summing up, this is the first report of CP gene sequence for an Indian strain of ORSV. In general, the CP gene appears to be a good candidate for the development of transgenics by the pathogen-derived resistance strategy.

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