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Biotechnological Approaches for Treating Viral Diseases in Orchids

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ABSTRACT

Viral diseases of orchids cause major losses in productivity and quality. Host plant resistance offers an effective means of controlling plant diseases caused by viruses. It minimizes the necessity for the application of pesticides. However, in most orchids no natural disease resistance is available. Genetic engineering allows the introduction of specific, in some instances broad spectrum, disease resistance derived from other species or even from the pathogen itself into plant genotypes that have been selected for desirable horticultural properties. This manuscript broadly provides an overview of these themes.

Keywords: genetic engineering, molecular breeding, virus resistance

INTRODUCTION

Orchids are an important group of ornamental plants; they have immense horticultural importance and also play a very useful role in the balance of forest ecosystems (Kaushik 1983). Orchidaceae constitutes two broad groups: terrestrial and epiphytic. Terrestrial orchids (e.g. *Cymbidium*, *Paphiopedilum*) grow on the ground, while epiphytes (e.g. *Vanda*, *Dendrobium*) having aerial roots, grow on trees. In India, orchids account for 9% of flora and about 1300 species are found in the Himalayas with others scattered in Eastern and Western Ghats. Some of the important orchid genera are *Arahnis*, *Phalaenopsis*, *Vanda*, and *Renanthera*. Intergeneric hybrids are also common as intergeneric crossing is possible and crosses involving two genera (biogeneric), three genera (trigeneric), four genera (tetrageneric) and more are available (Rajeevan *et al*. 2009). Orchids grow in a wide range of media *in vitro* and tissue culture is a potentially useful technique in *ex situ* multiplication and restoration in *ex situ* multiplication of rare or threatened taxa (Razdan 2003), generally achieved through seed cultures (Fay 1988). The most commonly used method of propagating orchids is through division (Rajeevan *et al*. 2009).

Orchids are attacked by a number of pests and diseases, including viruses. Strategies for the management of viral diseases normally include control of the vector population using insecticides, virus-free propagating material and resistant cultivars. Some of the important orchid virus diseases are briefly listed below with possible biotechnological methods for their control.

ORCHID VIRUS DISEASES

Orchids may be infected by a number of different plant viruses. The diseases they produce are difficult to tackle since viruses are not specific or reliable in the reactions they produce in different species e.g., *Cymbidium mosaic virus* (CymMV) produces mosaic mottle on leaves of *Cymbidium* whereas in *Cattelya* it produces necrotic spots (dead spot), which is entirely different in appearance from the mosaic mottle. The reverse situation may also occur in infected plants i.e., two different viruses producing two different diseases may induce the same lesions when they occur in the same plant (Thornberry and Phillippe 1964; Wisler *et al*. 1979). CymMV is a very common disease of orchids in Japan causing chlorotic areas and necrotic streaks in the leaves of *Cymbidium* and leaf necrosis in *Cattleya* (Inouye 1968). Another peculiarity of virus diseases is that, when the infection is mild, the virus may remain inside the plant for years without any visible symptoms (Burnet 1974) but is a dangerous source of fresh infections. The efficient control of orchid virus diseases depends on rapid and accurate diagnosis followed by the destruction of diseased plants. The symptoms produced by orchid viruses depend on the particular viruses and strains involved the hybrid, species and genera of orchids infected, the age of the leaves, the time of year and the environmental conditions. Abnormal nutrition and fungal infections also produce virus-like symptoms (Pataky 1990).

Cymbidium mosaic virus

CymMV is, without a doubt, the most common orchid virus in the world. *Cattleyas* and their hybrids are most often found infected, but the percentage is also high for *Phaius* and *Phalanenopsis* (Kado and Jenseon 1964). CymMV was reported for the first time by Jenson (1950, 1951) to induce mosaic or black streak in *Cymbidium*. CymMV has been recognized to be an infectious disease of many orchid genera (Hu *et al*. 1993).

1. Symptoms

On *Cymbidium* from which this disease was first described, the symptoms vary considerably in pattern and severity (Burnett 1985). They appear first on new growth about 6 weeks after infection as small, inconspicuous, elongate, chlorotic areas. Frequently these are confined initially to the leaf blade on one side of the midrib. After a few days, the spots and streaks become more sharply defined (Hu *et al*. 1993). Within 1 to 3 weeks, the affected area enlarges as a pale, chlorotic pattern approximately 1 cm long. Subsequent growth shows increasing contrast between light and dark green areas, and within 3 to 4 months sufficient symptom-

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Research Note

bearing tissue turns brown to make the disease conspicuous. Within 6 months after infection, black spots and streaks may appear on the lower surface of the older leaves. Necrosis in the youngest leaves usually appears only in very severe cases. Leaves having severe and extensive necrotic areas drop prematurely.

On *Cattleya*-type orchids, infection on the leaves is characterized by sunken, brown to purple-black patterns of various forms. Often they are elongated longitudinal streaks which give the leaf a roughened appearance. However, when tiny, sunken, necrotic areas are noted on a leaf, a virus can be suspected. In severe cases, the older leaves drop prematurely leaving a plant with 2 or 3 leaves and the balance of the plant becomes a leafless pseudobulb.

CymMV belongs to potex virus genus having a +sense SSRNA genome within the range of 5.9-7.0 kb. Particles are flexible filaments with a helical symmetry about 475 nm long and 13 nm wide as observed in electron microscopy (Brandes and Berks 1965).

Cattleya blossom brown necrotic streak

Cattleya orchid plants infected with CymMV sometimes produce symptoms on flowers. This part of the disease is called *Cattleya* blossom brown necrotic streak or flower necrosis (Hu *et al*. 1993). On *Cattleya*-type flowers, brown spots and streaks develop from 5 to 21 days after the flower opens. Brown necrosis usually appears first on the midrib or larger veins of the sepals and petals as elongated spots that usually enlarge to form streaks. Lesions on some blossoms may increase in number and size to involve essentially the entire blossom, while lesions on other blossoms may remain as isolated brown spots or streaks without perceptible enlargement. The delay of flower symptoms 5 days longer after opening creates a problem for orchid growers who sell their blooms. Infected symptomless flowers may produce necrotic symptoms within 3 to 5 days after they are cut from the plant (Burnett 1985).

Cattleya flower-break

Tobacco mosaic virus "o" strain (TMV-O) is now the commonly accepted name for *Odontoglossum ringspot virus* (*Cattleya color-break* or *flower break virus*) in *Cattleya*-type flowers (Kado 1970). Strains of TMV-O have some properties similar to common TMV but are distinctly different. TMV infecting orchids can infect only a few species outside the orchid family.

TMV-O belongs to the genus *Tobamo*. This virus has particles which are rigid rods, having a normal length of < 3 nm and a width of < 18 nm. It contains 6600 nucleotides organized in four open reading frames (ORFs) that indicate a sequence of bases that could potentially encode a protein (Ikegami *et al*. 1995; Chng *et al*. 1996).

1. Symptoms

The most conspicuous symptom is characterized by variegation in the flowers of lavender *Cattleya*-type orchids. The normal pigment of the sepals and petals is replaced with irregular blotches of pigment which are more intense in color than that of normal flowers. There is no set pattern for breaking and develops at random in petals and sepals. Individual flowers on the same plant may show different degrees of color-breaking from one flowering season to the next. Flowers that show strong or mild symptoms during one flowering season may show stronger or milder symptoms in the succeeding flowering periods. TMV-O may produce a variety of symptoms in *Cattleya*-type leaves, including chlorotic and necrotic spots, streak and rings. The rings may be distinguished as ringspots with a distinct pinpoint spot surrounded by a circle of chlorotic tissue. Symptoms produced by both CymMV and TMV-O may differ greatly from one variety to another of the same genus and among different genera infected with the same virus although a virus-diseased orchid plant may produce normal foliage (McMillan *et al*. 2005).

BIOTECHNOLOGICAL APPROACHES FOR ORCHID VIRUSES

Many crops are susceptible to multiple viruses, each of which may cause serious economic losses. In addition, infected plant material may not be acceptable for export (Loebenstein *et al*. 1995). Control of viral diseases of floral crops usually focuses on use of clean propagation materials that have been indexed and shown to be free of known pathogens. However, the use of virus-free propagation material is not in itself adequate, as many viruses can also be transmitted by an insect vector such as aphids, whiteflies or thrips. Biotechnology offers a new means of improving ornamental crops by the addition of desirable traits to existing horticulturally adopted cultivars. Traditional plant breeding involves the exchange of many hundreds of thousands of genes between related species to create novel combinations, and improved genotypes must be selected from thousands of segregating progeny over multiple cycles of selection. Introgression of a single desirable now character such as disease resistance from an unadopted relative of a crop species may require multiple backcross generations to regain the horticultural or ornamental qualities of the original cultivar, often resulting in reduced intensity of expression of the back crossed character. Strategies for the management of viral diseases normally include control of vector population using insecticides, use of virus-free propagating material and use of resistant cultivars. Rapid advances in the techniques of molecular biology have resulted in the cloning and sequence analysis of the genomic components of a number of plant viruses. Plant tissue culture and genetic transformation of a number of crop plants have opened up the possibility of an entirely new approach of genetic engineering towards controlling plant virus diseases (see Teixeira da Silva 2006).

Meristem tip culture

The meristem tip refers in the region of shoot apex lying distal to the youngest leaf primodia, whereas the shoot apex includes meristem tips plus a few subadjacent leaf primordia. Although meristem culture has been extensively used for vegetative propagation of many crops, in orchids it is mainly employed to obtain virus-free plants since in infected plants the apical meristems are generally either free or carry a low concentration of viruses (Quak 1977). In the meristematic region competition between cell production and virus multiplication occurs. The first virus resistant plant obtained by Morel in 1960 against CymMV.

Kushnir and Cherevchenko (1984) obtained virus-free propagated plants from meristems of *Cattelya*, *Cymbidium* and *Dendrobium Phalaenopsis.* Nath *et al*. (1988) reported meristem tip culture of certain orchid species (*Aerides*, *Odoratum*, *Cymbidium alloifolium*, *Dendrobium amoenium*, *Dendrobium moscatum* and *Phaloenopsis manii*) and obtained virus-free propagated plants after 6 weeks. Decruse *et al*. (2003) micropropagated *Vanda* through meristem tip culture. Lim *et al*. (2008) reported elimination of CymMV and *Odontoglossum ring spot* using two methods in order to generate virus-free plants: meristem culture and thin section culture with chemotherapy. Meristem excised from (0.10 to 1.00 mm) axillary shoots of an infected monopodial orchid hybrid (Mokara char Kuan 'Pink') and cultured in modified Vacin and Went medium generated plantlets free from virus infection from larger meristem explants only. In contrast, a high percentage of virus-free plantlets and protocorm like bodies (PLBs) with ribavirin treatment yielded resistant plants. Resistant plantlets were further tested with ELISA and PCR techniques.

TRANSGENIC APPROACHES FOR VIRUS RESISTANCE

Coat protein gene

The use of viral coat protein (CP) gene as a transgene for producing virus-resistant plants is one of the greatest successes of plant biotechnology. The effectiveness of the CP gene in conferring virus resistance can be affected by both the amount of CP in transgenic plants and by the concentrations of virus inoculum (Singh 2003). Many crops have been reported to show high levels of resistance in comparison to untransformed plants. Powell-Abel *et al*. (1986) first reported virus-resistant transgenic tobacco expressing the TMV CP gene. In orchid, Kuehnle and Sugii (1992) reported transformation of *Dendrobium* in which tungsten particles coated with plasmid Pga482GG/cpPRV4 containing the Nos-NPTII and *Papaya ringspot virus* (PRV) CP genes and transformed by particle gun. Li *et al.* (2005) reported gene stacking in *Phalaenopsis* in which resistance to both viral and bacterial pathogens was achieved when PLBs were originally transformed with CymMV CP cDNA and then retransformed with sweet pepper ferredoxin-like protein cDNA (pFLP) by *Agrobacterium tumefaciens*. Transgene integration in transgenic *Phalaenopsis* lines was confirmed by southern blot analysis for both CP and pFLP genes. Chen *et al*. (2005) reported genetic transformation of viral capsid protein gene in *Dendrobium*. To generate virus-resistant varieties the CymMV-CS CP gene was transformed into *Dendrobium* protocorms through particle bombardment and transformants were selected on medium supplemented with 20 mg/l hygromycin. The presence of the transgene was confirmed by PCR, Southern, Northern and Western blot analysis. Borth *et al*. (2006) obtained virus-resistance genetic transformation in *Dendrobium*. Transformed plants were evaluated for CymMV resistance after mechanical inoculations in greenhouse experiments under controlled conditions. Five transgenic orchid lines transformed with the CP or the MP genes tested became all infected, whereas 5/25 lines transformed with the mutated MP gene showed partial resistance to CymMV infection.

Post transcriptional gene silencing

PTGS is a specific RNA degradation mechanism of any organism that takes care of aberrant, unwanted excess or foreign RNA intracellularly in a homology-dependent manner. The elicitor double-stranded RNA (dsRNA), commonly produced during viral infection, is degraded to 21-25 nucleotides and termed small interfering RNA or siRNA with the help of a variety of factors (Ketting and Plastere 2000). A complex of cellular factors namely RNA-dependent RNA polymerase, RNA helicase, translation elongation factor, RNase, etc. along with the small 21-25 nt RNA acting as the guide RNA (Hammond *et al*. 2001) that degrades RNA molecular bearing homology with the elicitor RNA. This degradation process initiating from a concerned cell having the elicitor RNA, spreads later within the entire organism in a systemic fashion. This process generally evolved as a plant defense mechanism towards invading viruses containing RNA or DNA genomes (Smyth 1999). When the viral RNA is either the elicitor or target of post transcription silencing mechanism (PTGS), the degradation mechanism is known as virus-induced gene silencing (VIGS). Jen *et al.* (2004) studied transgene silencing in *Phalaenopsis* expressing the CymMV CP gene imparting RNA-mediated resistance. A DNA cassette containing a CymMV CP cDNA and a NOS terminator placed downstream of a maize ubiquitin promoter was transformed into *Phalaenopsis* by particle bombardment. Southern blot analysis of transgenic plants confirmed the successful integration of the CP gene and a low level of CP mRNA transcript signal in northern blot analysis. Transformed plants exhibited enhanced resistance to CymMV infection confirmed by RT-PCR and ELISA. Five among the 13 tested lines showed CymMV protection in more than 50% of their progeny. With siRNA analysis CymMV resistance was RNA-mediated using PTGS in silenced transgenic *Phalaenopsis* plants.

Movement protein

Movement protein (MP) is essential for cell-to-cell movement of plant viruses (Dasgupta *et al*. 2003). These proteins modify the gating function of plasmodesmata thereby allowing the virus particle or their nucleoprotein derivatives to spread to adjacent cells (Lapidot *et al*. 1993). This phenomenon was first used to engineer resistance against TMV in tobacco by producing modified MP, acting partially as a transgene. The conferred resistance is believed to be based on the competition between wild type virus-encoded MP and the preformed dysfunctional MP to bind to the plasmodesmatal sites (Malyeshenko *et al*. 1993). The above resistance was moreover seen to be effective against distantly related or unrelated viruses, for example resistance against TMV could be achieved in tobacco using the MP derived from *Brome mosaic virus* suggesting functional conservation of this protein among several viruses (Cooper *et al*. 1995). Five transgenic *Dendrobium* lines transformed with the CP or MP gene against CymMV were obtained by Broth *et al.* (2006). Obsuwan *et al*. (2009) obtained transgenic *Dendrobium*, CymMV using an MP gene with a site specific-mutation (mut-11) under the control of a ubiquitin promoter and inserted using biolistics into two *Dendrobium* varieties, *Dendrobium* x Jaquelyn Thomas (Uniwai Mist) and *Dendrobium* x Jaquelyn Hawaii (Uniwai Pearl).

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