MOLECULAR CHARACTERIZATION OF APPLE STEM GROOVING VIRUS INFECTING APPLE

PROJECT REPORT

By

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CERTIFICATE

This is to certify that this project report entitled, **"Molecular characterization of a *Apple stem grooving virus* infecting Apple"**, submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science** in the subject of **Biotechnology** of Guru Nanak Dev University, Amritsar, is a bonafide research work carried out by **Mr. Ashun Chaudhary** son of **Shri Parshotam Lal** from 24th May 2007 to 21st July 2007 under my supervision and that no part of this report has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place:Palampur
Dated:

(A.A. Zaidi)
Scientist and Head
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INTRODUCTION

CLASSIFICATION OF APPLE
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Rosales
Family: Rosaceae
Subfamily: Maloideae
Genus : *Malus*

The apple is a small deciduous tree reaching 5-12m tall with a broad, often densely twiggy crown. The leaves are alternately arranged, simple oval with an acute tip and serrated margin slightly downy below, 5-12cm long and 3-6cm broad on a 2-3cm petiole. The flowers are produced in spring with the leaves, white, usually tinged pink at first 2.5-3.5cm diameter, with five petals. The fruit mature in autumn and is typically 5-9cm diameter (rarely up to 15cm). The centre of fruit contain five carpel's arranged star-like, each carpel containing one or two (rarely three) seeds. *M.pumila, M.communis, M.sylvestrus and M.domestica* are commonly cultivated in India. *M.pumila* fruit eaten as dessert , also consumed cooked , dried and made into apple sauce , apple butter , jam and jelly, marmalade and a source of pectin , also employed for apple juice , apple syrup and cider .Apple murrabba used as a heart tonic . Rost-bark, anthelmintic hypnotic and refrigerant. Vitamins, salts and organic acids are concentrated in and just below the skin. Wood used for tool-handles, knobs, mallet heads, rulers and turnery. .*M.sylvestris* leaves eaten as a vegetable young carpel's and seeds also eaten. Plant a good source of cartone and calcium, mucilaginous, demulcent and emollient, used in pulmonary and urinary affections and in external application for inflammations and abscesses. Flower, malvae, used for gargles and mouth washes. (The useful plants of India Smt.Kamala Ramachandra

Project coordinator K.Kashyapa). At least 55 million tones of apple were grown in worldwide in 2005 with a value of 10 billons. China is leader. Apple *(M. domestica)* is one of the oldest fruits known to man and is the major cultivated fruit grown in the temperate regions of the world. Apple was probably first domesticated in the Caucasus, but spread fast over Europe, USA and and other countries (saucer, 1993). Apple is called as premier table fruit .Everyone has heard this popular quotation." An apple a day keeps the doctor away". This Focuses mans attention on the importance of apple in the daily diet. Apple is a good source of carbohydrates, protein, minerals, pectin and vitaminB6. Pectin of apple helps in preventing arteriosclerosis, a major heart disease in which deposition of cholesterol and hardening of arteries occurs. Eating apple is believed to reduce incidence of dental carriers, help control obesity and supply energy for heavy exercise. Apple skin has been reported to check proliferation of cancer cells in human liver by 57% and in colon region by 43% (yahia, 1994).Apple is a temperate crop crop which require cool temperatures to achieve optimum growth and development. Buds require a temperature in winter falls below 7C with adequate snowfall & winter rains are suitable for apple cultivation. However, extremely cold temperature of -10 C and -20C kill the blossom. Soils which are loamy, rich in organic matter, nutrients with a pH range of 5.8-6.2 are ideal. At present, about 84 countries in the world are engaged in apple cultivation with a total production of 6,30,85,000 T (FAO,2005). Apple is the fourth widely produced fruit in the world after banana, orange and grapes. China is the largest apple producing country in the world followed by USA. While India occupies 10th place with a total production of 14,70,100T (FAO, 2005) . In India apple are mainly grown in the states of Jammu and Kashmir (J$K), Himachal Pradesh (H.P), Uttarakhand and to

some extent in Northern Eastern states. Among all the fruits grown in India apple alone accounts for about 6% of the area and 3.5% of production (Yadav, 1997). H.P occupies 1st place in apple area and 2nd place in production after J and K. First commercial cultivation of apple in the state was started in the late 1800's but the revolution came only after introduction of delicious varieties in 1918 which changed the livelihoods of apple farmers. The net returns for a typical apple farmer having land of 1ha amounted to Rs 50,000 -60,000 per year (Tejpratap, 2003). The apple farming also improved employment opportunities, for landless households and women (Verma and Pratap, 1992). The area under the crop is increasing exponentially year by year but the increase in productivity is very low. There are many factor responsible for low productivity (1.9MT/ha) and one of the major constraints seems to be the use of non-certified & poor quality planting material with special reference to viral infection. A number of viral pathogen is known to cause slow decline in trees (Nemeth, 1986). Recent observations indicated that apple plantations in the state are hugely affected by infection of Apple Stem Grooving Capillovirus (ASGV). To improve the quality of germplasm, productivity and minimize viral infection in different cultivars proper diagnosis and control is essential. In addition diagnosis countries where strict quarantine laws have been imposed. During the last two decades much advancement has been made in the development of diagnostic for the detection of viral diseases. Biological detection using indicator plants is the first and foremost method being employed for viral detection. Later serological methods like enzyme linked immunosorbent Assay (ELISA) become popular. Apart from these immunodiagnostics, molecular biology has also provided techniques like tissue blotting by use of DNA/RNA probes and PCR for viral detection etc. These techniques have

shown great potential as far as specificity and sensitivity is concerned. The viral coat protein (particularly the N-terminal regions) is implicated in some biological properties, host range & cross protection. The coat protein sequence is also correlated to pathogenecity of virus (German-Retana *et al.,* 1997). The information generated from coat protein gene can be useful to study particle assembly, structural parameter & development of diagnostic kit against the virus. Keeping in mind the importance of coat protein gene of ASGV and non availability of any information on molecular characteristics of ASGV from India it was thought worthwhile to conduct investigations on ASGV with following objectives.

1. Amplification of coat protein gene of a virus infecting apple
2. Cloning and Sequencing the amplified coat protein gene
3. Analysis of sequence using bioinformatics tools such as BLAST search

REVIEW OF LITERATURE

Apple stem grooving virus first described by Lister et al, (1965). Later by Bancroft and Nadakavukaren (1965), Desequeria (1965, 1967) and Lister (1969). Apple is infected by a number disease of which apple stem grooving virus is the one of the most important virus which causes huge loss to the crop. Apart from apple, ASGV also infects other stone fruits like plum, apricot, plum et. (Dunez and Delbos, 1988, Nemeth, 1986, Mink, 1989). On an average the incidence of ASGV on apple is an high as 40% (verma and Sharma, 2002) Cytopathology Virions found in cambium, probably. .

TRANSMISSION:

It is not transmitted by mean of any vector. The true transmission way is not known. But it is transmitted by grafting on infected rootstock. Virus can be transmitted by mechanical inoculation (from apple, especially in spring, by inoculating extracts from buds, young leaves or petals ground in inoculation buffer (Appendix).

SYMPTOMS AND HOST RANGE OF ASGV:

The virus does not cause obvious symptoms in commercial cultivars; it induces symptoms in indicator plants and certain size controlling rootstocks. Infected Virginia Crab frequently develops long grooves on the woody stems, which become apparent after the bark is removed. If the grooves are clustered, sunken or flattened areas are visible without removing the bark. Swelling of the scion stem at the union with a sensitive rootstock often occurs. These unions break easily revealing plates of necrotic xylem above the graft. The scion component may eventually die, while the rootstock remains unaffected. If the union is severely affected, leaves become small, pale green, and drop prematurely (Nemeth 1986).

Its natural host is *M.sylvestris* CV Virginia crab. Strain E36 causes grooves in stem , abnormal graft union to be brown, fragile, swollen and sunken area on stem .(Lister 1965).It is reported to infected about 20species, usually symptomlessly in 9 dicotyledonous families *viz*. Aizoaceae, Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Lamiaceae, Fabaceae, Rosaceae, Scrophulariaceae, Solanaceae. The diagnostically susceptible host species are *Malus .sylvestris, Chenopodium.quinoa, Nicotiana.glutinosa and Phaseolus vulgaris. C. quinoa* is the most useful plant for propagation. Zucchini squash is also reported as a propagation host for some isolates (Uyemoto & Gilmer, 1971). Transmitted through seed to progeny seedlings of lily (1.8%) and C. quinoa (2.5 - 60.0%) (Inouye *et al.,* 1979.) To transmit the virus from fruit trees*, N. occidentalis*, a symptomless host is useful. *N. glutinosa* can be used to eliminate contaminating viruses from apple such as Apple chlorotic leaf spot virus and Tobacco mosaic virus. *C.quinoa* is main propagation host and *N.glutinosa, P.vulgaris are maintaince* host for culturing. Ecology and control is first Studies by Mink and Shay (1962). The disease has been eliminated from some apple scions by heat therapy (30 days at 36ºC; de Sequeira and Posnette, 1969)

Best tests for diagnosis

This virus is not transmitted to *Solanum tuberosum,* like potato virus T (Salazar and Harrison, 1978), nor to *Nicotiana glutinosa* like apple chlorotic leaf spot virus. Symptoms in *Chenopodium quinoa, Phaseolus vulgaris,* Russian apple R-12740-7A and Virginia Crab distinguish apple chlorotic leaf spot and apple stem grooving viruses (de Sequeira and Cropley, 1968; de Sequeira and Posnette, 1967). Chenopodium quinoa is best for isolating this virus because other apple viruses such as tobacco mosaic and apple

chlorotic leaf spot virus replicates poorly in that species and their local lesions develop 2-3 days later. When both viruses are present, symptoms are much more severe.

Geographical distribution:

Spreads in Australia, China, India, Italy, Japan, the Netherlands, New Zealand, Portugal, and the UK (probably occurs wherever apple trees are cultivated).Natural spread in citrus plants is reported in China and Japan (Ke & Wu, 1991; Miyakawa, 1980; Miyakawa & Matsui, 1976; Su & Cheon, 1984; Zhang *et al.,* 1988).

ASGV Properties in sap are Its Thermal Inactivation Point (TIP) is 60-63 °C, Longevity of the Infectivity of sap in Vitro (LIV) is 2 days, Dilution end point (DEP), log10 minus 4. Leaf sap contains few virions. Electron microscopy: best stained with UA or UF; breakage occurs in PTA (Lister *et al.,* 1965; de Sequeira and Lister 1969b).

Purification method

Purified from infected C. quinoa (de Sequeira & Lister, 1969b) by homogenizing 100g leaves in 0.01M or 0.05M phosphate buffer (pH7-8) containing 0.15% sodium thioglycollate or 1% 2-mercaptoethanol. Clarify the extract by low speed centrifugation and squeeze through cheesecloth. Add a bentonite suspension (30-40 mg/ml in 0.01M phosphate buffer, pH7-8) slowly and clarify by centrifuging at low speed. Repeat this clarification step until the supernatant fluid is clear. Precipitate the virus from the supernatant fluid by adding PEG (mol. wt 6,000) to 4-8 %(w/v) and NaCl to 0.02M, incubate for 1 hr and centrifuge at low speed. Resuspend the pellet in 0.01M phosphate buffer (pH 7.0) and clarify by centrifuging at low speed. Concentrate the virus by

ultracentrifugation and purify further by sucrose density gradient centrifugation. Yields are 50 - 250 µg/100g leaf tissue.

Particle morphology

Virions is filamentous not enveloped, usually flexuous with a clear modal length of 600-700 nm and 12 nm wide. Pitch of basic helix is 3.4-3.7 nm (Lister and Bar-Joseph, 1981).Its sedimentation coefficient is 112 S and Isoelectric point pH 4.3 (ionic strength of 0.1).

Biochemical properties

ASGV genome (monopartite) is consisting of a 6·5 kb, single-stranded, plus-sense RNA that is 5'-capped and 3'-polyadenylated. ASGV RNA contains two overlapping open reading frames: ORF1 (6·3 kb) and ORF2 (1·0 kb), which encode proteins of molecular mass 241 and 36 kDa, respectively (Yoshikawa *et al.,* 1992,Ohira *et al.,* 1995.The larger ORF1 encodes an apparently chimeric polyprotein containing at least two conserved regions: the replicase (Rep) region contains several domains (methyltransferase, papain-like protease, NTP-binding helicase and RNA-dependent RNA polymerase domains) characteristic of viral Rep, and the coat protein (CP) region is located at the C-terminal end and contains motifs typical of CP (Yoshikawa *et al.,* 1992 ORF2 encodes a protein with conserved motifs for both movement proteins (MP) and viral proteases (Yoshika,Ohira *et al.,* 1994 wa *et al.,* 1992 Ohira *et al.,* 1995.The expression strategies of these ORFs are not yet well understood. By using an antiserum against purified ASGV particles, the expected 241 kDa polyprotein for ORF1 was not detected in a plant infected with ASGV; only a small protein thought to be the CP was detected (Yoshikawa & Takahashi, 1992). These results suggest that CP is expressed from sub genomic RNA

rather than polyprotein processing of the 241 kDa protein. The mechanism of expression of ORF1 thus remains to be elucidated. Construction of a full-length cDNA clone, pITCL, of ASGV lily strain (formerly Citrus tatter leaf virus lily strain: Nishio *et al.,* 1989; Yoshikawa *et al.,* 1993, Ohira *et al.,* 1995) that produces infectious viral RNA transcripts (ASGV-wt) in vitro has been described (Ohira *et al.,* 1995), allowing mutagenesis of the ASGV genome.

Virus (es) with serologically related virions

Many isolates are reported from apple, Japanese pear, European pear, Japanese apricot, lily and citrus plants, but most are not well characterized biologically and serologically. Some isolates have been differentiated only on symptomatology (Lister, 1970). This virus and Citrus tatter leaf virus (CTLV) have been regarded as distinct viruses. However, CTLV from citrus and lily is indistinguishable from ASGV from Rosaceae fruit trees biologically, serologically, in genome organization and in nucleotide sequence (Kawai *et al.,* 1991; Magome *et al.,* 1997; Ohira *et al.,* 1995; Yoshikawa *et al.,* 1993). Isolate Li-23 from lily can infect citrus plants (N. Inouye, personal communication) and isolate V-3 from Japanese pear induced symptoms similar to those by CTLV from citrus on Rusk citrange (Iwanami et al. 1991). From these results, CTLV is regarded as an isolate of ASGV. Virus isolates from apple, Japanese pear and European pear trees comprise at least two to four variants that differ considerably from each other in nucleotide sequence (Magome *et al.,* 1997; Yoshikawa *et al.,* 1996). The composition of sequence variants within a tree differed among leaves from different branches, showing that each sequence variant is distributed unevenly within

an individual tree (Magome *et al.,* 1999). Apple chlorotic leaf spot virus (Lister *et al.,* 1965; de Sequeira, 1967). is serologically unrelated virions. In cross-protection experiments in Virginia Crab apple the virus seemed to be unrelated to apple stem grooving virus (de Sequeira and Cropley, 1968).