Lukoševičius. Ripens early July. Fruit round, 6g, attractive, very tasty. Skin yellowish with a red blush. Flesh medium firm, juicy, sweet. Stone 0.3g, easily removed. Tree moderately vigorous, resistant to cherry leaf spot (*Coccomyces hiemalis*). Produces heavy crops yearly.

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Cross Protection Against Virus Diseases in Fruit Trees

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Abstract

Fruit trees are commonly infected with plant viruses. Several methods have been used to eradicate viruses from plant tissues including chemotherapy, thermotherapy, *in vitro* propagation, and a combination of some of these protocols. Recent advances in molecular techniques have provided a new approach for developing virus resistant genotypes. Genetic engineering of virus resistance into plants has been accomplished using several strategies including satellite-RNA-mediated resistance, antisense RNA-mediated resistance, and coat protein-mediated resistance, among others. Current advances in using coat protein-mediated resistance have proven promising in protecting several agronomic crops against virus infection. More recently, a number of fruit crop species have been transformed with coat protein genes of important plant viruses and promising results have been obtained. This is a general review of cross protection strategies used in combatting virus diseases and the current advances made in genetic engineering of virus resistance in fruit trees.

Most fruit crops are susceptible to virus diseases; in most cases, viruses cause reductions in yield and/or fruit quality resulting in small, deformed fruits. Viruses multiply in plant cells or tissues and spread throughout the whole tree, producing disease symptoms. Some genotypes are tolerant to virus infection and the virus may spread after it multiplies without causing disease symptoms. Viruses are transported from cell-to-cell and within vascular tissues, and therefore nuclei, chloroplasts, and mitochondria are easily infected (52). Most viruses, such as cowpea mosaic virus and turnip yellow mosaic virus, attach themselves to certain membranes in the cytoplasm (52).

Viruses contain the genetic information specifying the symptoms produced, therefore different viruses induce different symptoms on a species or in different varieties or cultivars of a single species. This diversity can be used for selection and breeding for resistance (52). Backcrossing to cultivated and wild varieties of a plant can lead to an improved variety selected for a desired combination of characters. By identifying resistant genotypes and crossing them with commercially important cultivars, breeders working with agronomic crops were able to develop disease resistant plants. Some plant breeders transferred genes from a non-cultivated plant species to a crop variety in a related species via interspecific hybridization. This approach was later extended for transferring genes from wild species to cultivated relatives in the same genus via intergeneric hybridization (15).

McKinney (31) reported that when a tobacco plant was infected with a mild strain of tobacco mosaic virus (TMV), it did not develop severe disease symptoms upon superinfection with a highly virulent strain of TMV. The strategy of purposely infecting plants with a mild virus strain to protect against a severe strain is called

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"cross-protection." This approach was used to minimize damage of commercial plants by virus infection. Tomato plants were protected against severe strains of TMV and tomato mosaic virus (ToMV) by infecting them with an avirulent strain of the virus (41). Upon superinfection of a plant by a severe strain of a virus, the rate of virus spread is reduced and some plants do not develop symptoms at all. However, there may be some disadvantages for such cross-protection (3). First, infection by the mild strain may cause significant yield loss; second, a severe strain might evolve from the mild strain leading to more serious disease incidence; third, the challenge virus may act synergistically with the protecting strain to cause a more severe disease.

Importance of Control of Virus Diseases in Fruit Trees

Virus infection of vegetatively propagated fruit trees is serious and widespread. Sometimes, symptoms are relatively mild, or the virus may be latent (i.e. infection without symptoms) in infected cultivars. Once a tree is infected, it will remain infected for life. Symptoms and crop losses caused by the virus in an individual tree may vary from season to season, but in cases of severe infections or in order to prevent virus spread to adjacent healthy trees, the grower may have to remove infected trees. Loss of income due to tree loss, costs of replacement, and delayed years to fruiting, can seriously hurt a fruit grower.

Many viruses infect fruit trees and cause yield loss or damage to fruits (Table 1). Another economic consideration reflecting the importance of plant viruses on fruit trees is the high cost of preventive or control measures required to avoid infection. These include chemical sprays to control insect vectors, breeding for disease resistance, and virus indexing and certification to provide healthy planting stock.

Current Methods for Controlling Virus Diseases in Fruit Trees

Different approaches have been used to control virus diseases and their spread in fruit trees. These approaches include production of virus-free stocks, chemotherapy, *in vitro* propagation of plants, and thermotherapy. In the following section, each of these methods will be discussed.

1) Virus-free stocks

One of the most successful methods of control is the exclusion of virus diseases from new orchards. The accuracy of the diagnostic procedure determines the reliability of this technique. Fridlund (11) developed a uniform and rapid method for detecting *Prunus*, *Malus* and *Pyrus* viruses in North America using greenhouse indexing. This was done by planting healthy rootstocks in plastic containers; inoculations were made by simultaneously double-budding two inoculum buds and one woody indicator bud to each healthy seedling. Seedlings were cut back after one week to force indicator buds to grow and maintained at constant temperatures in the greenhouse. Indicators usually showed symptoms four weeks following inoculation.

An Interregional Research Project (IR2) was initiated to obtain virus-free cultivars and clones of deciduous fruit trees, verify their freedom from viruses, maintain healthy stock in isolated repositories, and distribute small amounts of budwood to research centers and/or industry (12). In addition research was conducted on techniques for identifying and detecting viruses and host plants. Greenhouse indexing is accurate, efficient, and more economical than field indexing. The average time for symptom development in woody indicators can be reduced from one year to three or four weeks, therefore avoiding problems associated with herbaceous indicators and reducing chance errors due to climate and other environmental factors (11).

Table 1. Some examples of viruses that infect deciduous fruit trees.

Group	Virus	Fruit Crop
Capillovirus	Apple stem grooving	apple
Caulimovirus	strawberry vein banding	strawberry
	blueberry red ringspot	blueberry
Closterovirus	apple chlorotic leaf spot	pear, apple, peach, apricot, quince
	grapevine leaf roll	grape
Ilarvirus	apple mosaic	apple, plum
	tulare apple moaic	apple
	black raspbery latent	black raspberry
	prune dwarf	peach, plum, sour cherry
	purnus necrotic ring spot	peach, sweet cherry, plum
Nepovirus	blueberry leaf mottle	blueberry, grape
	grapevine bulgarian latent	grape
	grapevine chrome mosaic	grape
	grapevine fan leaf	grape
	raspberry ringspot	raspberry, strawberry, red currant, cherry, grape
	strawberry latent ringspot	strawberry, raspberry, blackberry, black currant, red currant, cherry, elderberry, grape, plum, peach
	peach rosette mosaic	grape, peach
	cherry leaf roll	cherry, blackberry
	cherry rasp leaf	cherry, peach, apple
Rhabdovirus	raspberry vein chlorosis	red raspberry, loganberry
	strawberry crinkle	strawberry
Ungrouped	black raspberry necrosis	red and black raspberry
	blueberry shoestring	blueberry
	raspberry bushy dwarf	red and black raspbery, loganberry, boysenberry

Serological methods such as immunosorbent electron micoscopy (IEM) (22) and enzyme-linked immunosorbent assay (ELISA) (32 & 37) have been used for virus indexing. IEM has been used to detect apple chlorotic leafspot virus (ACLSV) and plum pox virus (PPV) (22). Using polyclonal antibodies, ELISA has been used to verify the presence of 41 isolates of ilarviruses in *Prunus* and *Malus*, representing the entire symptomatic and serological range of prunus necrotic ringspot virus (PNRSV), apple mosaic virus (ApMV), and prune dwarf virus (PDV) (32). Reactions with components of healthy plants often develop with polyclonal antibodies in ELISA tests. Poul and Dunez (37) described the production of monoclonal antibodies against ACLSV as well as their characterization and use for virus detection using the double antibody sandwich ELISA. Their results showed that monoclonal antibodies can improve the sensitivity and specificity of the detection assay over polyclonal antibodies.

2) **Chemotherapy**

Virus diseases in orchards are primarily controlled through use of virus-free stocks. However, established healthy trees might become infected with viruses via vector such as pollen and aphids, among other agents. Consequently, there is a need to develop

practical and effective alternatives to controlling virus diseases in deciduous fruit trees. Several studies have been conducted to determine whether certain chemicals can be used successfully against viruses and virus-like agent (VLA) in woody hosts. Ribavirin, a guanosine analogue, was applied as a foliar spray to two-year-old *Prunus serrulata* L. trees infected with the non-sap-transmissible VLA of green ring mottle (GRM) (18). Weekly applications of 500ppm ribavirin prevented symptom development on newly developing foliage, and gradually eliminated the infective agent from previously infected older wood. After one year, ribavirin treatments were discontinued and VLA and its symptoms were not detected in shoots or limbs. However, this treatment was not successful in eliminating PNRSV from *Prunus persica* L. (18).

Ribavirin was added to a tissue culture medium to eliminate systemic infection of apple shoot cultures with ACLSV (17). Sequential indexing showed that all treated shoots were virus-free following subsequent transfers to a ribavirin-free medium, then to a greenhouse, and finally to the field. A sugar-free triazole base of ribavirin was similarly tested and found to be ineffective (17). Ribavirin completely suppressed symptom expression when injected into orchard trees during the fall and to greenhouse-grown grafted trees infected with ApMV (4). Injections of ribavirin in the spring reduced symptom expression but did not completely control the ApMV infection. Injection of the antiviral compound into trees showing symptoms of scar skin or dapple apple disease, or direct application of this compound to individual fruits did not produce significant changes in fruit symptoms.

From a practical stand point, foliar applications of ribavirin offer an advantage over thermotherapy, meristem culture, micrografting, or virus-indexing, as it is a cheaper, faster, and simpler method. The main limiting factor for the general use of ribavirin is the narrow range of plant viruses controlled by this compound.

3) ***In vitro* propagation of plants**

In vitro propagation can be used for inactivation of viruses in woody plants and for studying plant-virus interactions. Sweet cheery clones infected with PNRSV, PDV, and CLSV viruses were cultivated *in vitro* on a Murashige and Skoog (34) medium rich with hormones, such as adenine sulphate, kinetin, indoleacetic acid and 2-isopentene (6). Intensive shoot proliferation by repeated subculturing resulted in significant decrease of the virus content in shoots. The hormonal composition of the culture medium seemed to play an important role in the competition between cellular and viral multiplication and hence, resulted in reduction in virus multiplication.

4) **Thermotherapy and chemotherapy of *in vitro* cultures**

In vitro propagated clones of sweet cherry infected with ACLSV, PNRSV or a complex of PNRSV and PDV were subjected to a gradual increase of temperature up to 32-34°C (7). Surviving shoots were recovered and transferred to a rooting medium and moved to the greenhouse. One month-old plants were indexed by ELISA. Heat treatment was successful with only one of the two cultivars tested. Therefore it is possible to combine tissue culture and heat treatment techniques to obtain better results than with either treatment alone. The disadvantage of this approach is that some plants are more sensitive to heat treatment than others (7). Moreover, some viruses multiply faster at high temperatures; while others require lower temperatures for survival (13).

The additive effect of combining tissue culture and chemotherapy to eliminate ACLSV, PNRSV and PDV from infected sweet cherry was also tested (7). A virazole concentration of 50-100 mg/l was effective in eliminat-

ing ACLSV from all shoots, but had no influence on PDV replication and only a slight effect on PNRSV multiplication. Cyanoguanidine appeared to stimulate shoot development but had no antiviral activity against these viruses. Therefore, eliminating viruses with heat therapy and chemotherapy depends on the virus, method of elimination, and probably the genetic background of the plant host.

5) **Genetically engineered plant virus resistance**

Recently, recombinant DNA techniques have provided new alternatives for genetic improvement of agricultural crops. The development of regeneration and gene transfer systems for fruit crops is an important ongoing effort in various research programs. These technologies will provide promising opportunities for introducing novel and useful genes of economic importance into fruit crops. Currently, significant progess has been made to introduce virus resistance into a number of plant species; so far this has been accomplished mainly in annual and forage crops. These promising accomplishments should be extended to important perennial fruit crops. The following strategies have been used to introduce virus resistance into plants.

a. **Satellite-RNA-mediated resistance**

Harrison (19) transformed tobacco plants with a cDNA copy of cucumber mosaic virus (CMV) satellite RNA. Upon inoculation of transformed plants with the helper virus, development of disease symptoms and CMV accumulation were suppressed. Inoculation with a closely related virus, tomato aspermy virus (TAV), showed a similar effect but with no decrease in TAV accumulation. These responses suggest that symptom suppression does not necessarily depend on a decrease in virus replication. Tobacco plants that expressed a full-length satellite RNA of tobacco ring spot virus (TRSV) or its complementary sequence as RNA transcripts showed phenotypic resistance when infected with TRSV (14).

b. **Antisense RNA-mediated protection**

Tobacco plants were transformed with a copy of CMV coat protein (CP) gene cloned in an opposite orientation (antisense) (5 & 45). Transgenic plants expressing the CMV-CP antisense transcript were protected upon inoculation with CMV. This protection was overcome by the presence of high concentration of the helper virus in the inoculum. Similar results were obtained upon inoculation of transgenic plants that express the potato virus X-antisense CP with PVX (20). It was reported that plants expressing transcripts complementary to the TMV-CP sequence and containing the tRNA-like structure at the 3'-end of the transcript were better protected than those without the tRNA-like structure, the replicase binding sequence (39). In all cases, protection by the CP-antisense copy was overcome by high concentrations of the inoculum.

c. **Coat protein-mediated resistance**

"Coat protein-mediated resistance" refers to the resistance caused by the expression of a virus CP gene in transgenic plants (2). To induce this type of resistance, the genomic organization of the virus has to be known. Resistance has been developed against viruses that belong to eight different groups described below.

Potexvirus. Tobacco plants transformed with potato virus X coat protein gene (PVX-CP) were found to be protected against PVX infection and accumulated lower levels of the virus than untransformed plants (CP^-) (20). Inoculation of transgenic plants with PVX-RNA did not overcome resistance. Likewise, analysis of the PVX-CP^+ potato plants for resistance to inoculation with PVX showed a delay in symptom development and a reduction in the accumulation of the virus (21). A correlation was observed between the level of CP expression and the reduction in virus accumulation.

Table 2. Summary of virus resistance conferred by viral coat protein genes in fruit crops.

Virus	Group	Host	Reference
Plum pox virus (PPV)	Potyvirus	Apricot	23 & 24
		Plum	46
Grapevine fanleaf virus (GFLV)	Nepovirus	Grape	--*
Papaya ringspot virus (PRSV)	Potyvirus	Papaya	9
		Plum	46
Citrus tristeza virus (CTV)	Closterovirus	Carrizo citrange	33
		Sour orange	33

*This unpublished work was conducted by Moet & Chandon Company, Paris, France.

Cucumovirus. Expression of cucumber mosaic virus coat protein gene (CMV-CP) in transgenic tobacco plants caused a reduction in virus accumulation and symptom development upon inoculation with the challenge virus (5). This reduction was found to be independent of virus concentration in the inoculum.

Carlavirus. Mackenzie (28) reported absence of symptom development and lack of virus accumulation and systemic spread of potato virus S (PVS) upon inoculation of PVS-CP$^+$ tobacco plants with PVS-ME strain. Transgenic plants were also protected against inoculation with PVS-RNA.

Tobraviruses. Nicotiana plants transformed with tobacco rattle virus (TRV)-TCM strain coat protein gene were found to be resistant to infection with TRV-TCM strain but not with TRV-PLB strain (49). The primary structure of the coat proteins of these two strains is identical but differs in the RNA2 noncoding 3'-terminal sequence. Transformed plants showed a high degree of resistance to infection with pea early browning virus (PEBV) due to the homology between RNA2 sequence of TRV-TCM and PEBV (49).

Potyviruses. Plants expressing soybean mosaic virus coat protein (SMV-CP) were protected upon subsequent infection with two serologically unrelated potyviruses, potato virus Y (PVY) and tobacco etch virus (TEV), that are pathogenic to tobacco (48). Potato plants transformed with PVY-CP and PVX-CP overcame infection with PVX and/or PVY by mechanical inoculation (25). Plants were also resistant to infection with PVY by viruliferous green peach aphids.

Transgenic *Nicotiana benthamiana* plants expressing the coat protein gene of watermelon mosaic virus II (WMVII) or zucchini yellow mosaic virus (ZYMV) showed protection against six other potyviruses (35). Apparently, transgenic plants expressing a potyvirus coat protein gene show at least a noticeable level of protection against symptom development when challenged by other potyviruses. Similar results were obtained when tobacco plants expressing papaya ringspot virus (PRSV) coat protein were challenged with three other potyviruses (26).

Alfalfa mosaic virus (ALMV). Protoplasts of ALMV-CP$^+$ plants showed protection when inoculated with ALMV virions (27). However, infection of these protoplasts with ALMV RNAs1-3 overcame the resistance conferred by ALMV coat protein expressed in plants (51).

Tobamovirus. Tobacco mosaic virus (TMV) CP-mediated protection against infection with TMV was observed in TMV-CP$^+$ plants (3 & 40). Infection of U_1-TMV CP$^+$ tobacco plants with TMV U_1 strain or PV230 strain, which is serologically related to U_1 strains showed less development of local lesions and lower virus accumulation than that of control plants (36). Powell

(38) confirmed the need for the presence of the coat protein rather than the coat protein mRNA sequences for protection using specific mutagenesis to delete the initiation codon from the gene.

Ilarviruses. Van Dun (50) reported resistance and low tobacco streak (TSV) accumulation in TSV-CP$^+$ tobacco plants inoculated with TSV. However, these plants were not resistant to infection by ALMV or ALMV-RNA. This demonstrated that endogenously-produced TSV coat protein is capable of activating the ALMV genome but does not cross-protect against this virus (50).

Seed-borne viruses. Beet necrotic yellow vein coat protein gene (BNYVV-CP) was cloned into a binary vector and used to transform sugar beet hairy roots via *Agrobacterium rhizogenes* (8). Transformed hairy roots could not be infected with BNYVV to confirm their resistance to viral infection, perhaps due to physiological differences between normal and transformed roots.

Outlook for Genetically Engineered Virus Resistance in Fruit Crops

As presented in the above sections genetic engineering of virus resistance has been successfully demonstrated in various annual and forage crops. Therefore, it is important that this approach be evaluated for its ability to protect fruit crops against virus diseases. Efforts for the development of regeneration and gene transfer systems for fruit crops such as apple, strawberry, grape, peach, *Rubus,* among others, are important for the introduction of novel genes (16). Currently, there are a few successful efforts in fruit crops that have been reported whereby the coat protein gene of a plant virus have been engineered into fruit crops resulting in the development of virus-resistant genotypes. A summary of these reported efforts is presented in Table 2.

Plum pot virus (PPV), a member of the potyvirus group, causes heavy yield losses in plum, peach, and apricots grown all over Europe (10). The complete nucleotide sequence of PPV-RNA has been determined (29). The coat protein gene of PPV has been isolated, cloned, and characterized (30 & 43). Transgenic *Nicotiana benthamiana, N. clevelandii,* and *N. tabacum* plants expressing PPV coat protein were engineered by *Agrobacterium tumefaciens* (42 & 44). When challenged with PPV, plants showed a reduction in accumulation of the virus and inhibition of the systemic spread. Immature apricot embryos have been transformed with PPV coat protein gene (23 & 24). Polymerase chain reaction (PCR) was used to verify the introduction of the PPV coat protein gene into apricot embryos. Transformed plants showed a clear band corresponding to the relevant sequence within the coat protein gene (23). Much more work needs to be done to develop regeneration protocols from mature somatic tissues of apricot in order to introduce the CP gene into economically important cultivars. Transgenic plum plants carrying the potyvirus papaya ringspot virus (PRSV) coat protein gene have been developed (46). One plant has shown resistance to PRSV based on symptomology, ELISA tests, and reverse transcriptase-PCR assays. Plum plants have also been transformed with the PPV-CP gene and are currently being evaluated for protection against PPV infection (46).

Immature zygotic embryos of papaya have been transformed with the PRSV-CP gene (9). Putative transgenic R_0 papaya plants were assayed for PRSV-CP expression and for presence of the NPT-II and PRSV-CP genes using PCR and genomic blot hybridization analyses. Four R_0 transgenic lines carrying the PRSV-CP gene have shown varying degrees of resistance to PRSV. Citrus tristeza virus coat protein gene (CTV-CP), a member of closteroviruses, has been cloned and sequenced (47). Internodal stem sec-

tions of citrus seedling have been transformed with a plant expression vector containing the CTV-CP gene (33). Transgenic plants of Carrizo citrange and sour orange expressing the CTV-CP gene have been identified based on glucuronidase (GUS) gene expression, PCR, and Southern analyses as well as immunoblot analysis with antibodies to the coat protein. These plants are currently being tested for resistance to CTV infection (33).

In a recent unpublished report, transgenic Chardonnay grapevine plants carrying the coat protein gene of the grapevine fanleaf virus (GFLV) have been developed which are reportedly resistant to infection by the virus. This work has been conducted by Moet & Chandon Company in Paris, France.

Recently, the coat protein gene of apple mosaic virus (ApMV) has been isolated, cloned, and characterized in our laboratory (1). The ApMV coat protein gene will be transferred into apple and plum. Other groups are also working on isolating coat protein genes of other important plant viruses such as the blueberry scorch virus, tomato ring spot virus, raspberry mosaic virus, leaf roll virus, and stem pitting virus, among others. These advances will provide new opportunities for genetically engineering virus resistance into various fruit crops.

All the above reported advances in introducing coat protein genes of some viruses into various genotypes of fruit crops are very promising, and provide good examples of the useful strategies of genetic engineering in developing new genotypes of important commercial fruit crops with resistance to plant viruses.

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