

Identification of strawberry pallidosis virus and development of a sensitive detection method.

I.E. Tzanetakis and R.R. Martin
USDA-ARS, Corvallis, Oregon,

Introduction

Virus diseases that affected cultivated strawberry *Fragaria x ananassa* Duch. were first noted in the 1920's. Currently, more than thirty viruses and phytoplasmas have been reported in *Fragaria* (Spiegel, 1998). Although single virus infections can result in little to no observable symptoms in strawberry plantings, multiple virus infections in strawberry plants often result in a "running down" or reduction in plant performance, i.e. reduced runner production, reduced daughter plant production, reduced mother plant root mass, reduction in fruit yield, and diminished fruit quality. All of these symptoms can result in reduced yields for the commercial grower.

The majority of the work concerning strawberry viruses in North America has been done in the Pacific Northwest and California. The first report of a virus disease in an eastern strawberry field was made in 1931 (Demaree and Marcus, 1951). A search for virus-free eastern strawberry cultivars that was initiated in 1946 represents the first virus survey in eastern North America (Demaree and Marcus, 1951). The authors reported that the majority of plants selected from healthy looking fields in 16 eastern states were determined to be infected when graft indexed on 'Marshall' and the East Malling *Fragaria vesca* index plants. However, the strawberry virus situation in eastern North America is currently relatively uncharacterized.

Only a subset of the more than 30 known virus and virus-like diseases of strawberry are found in North America (Martin and Spiegel, 1998). Of particular interest to the eastern North American strawberry industry is the strawberry pallidosis disease that is suspected to be incited by a vector-borne virus currently referred to as pallidosis agent (Fulton, 1987). The disease was detected originally in the United States and Australia in 1957 (Frazier and Stubbs, 1969); however, it appears to be indigenous to North America. Although the disease has been reported in the western U.S. (Frazier and Stubbs, 1969) it is rarely seen in commercial fields in the Pacific Northwest and is not considered a primary virus problem there (R. Martin, personal observation). In contrast, graft indexing assays of 'run-down' cultivars and selections from USDA Beltsville fields, routinely produce symptoms on *F. virginiana* UC-10, but not on *F. vesca* UC-4 and UC-5 indicators suggesting strawberry pallidosis disease is present in field plantings and a potentially significant strawberry disease (Hokanson et al., 2000; Tzanetakis et al., In press).

Although strawberry pallidosis is known to be graft transmissible, little is known about how the disease is transmitted. Frazier (1975), suggested it may be spread by the leafhopper *Coelidia olitoria*, and also provided unpublished evidence that the disease is pollen-borne. Pallidosis is generally considered latent in most commercial cultivars, and only thought to produce 'economic effects' when involved in multiple virus infections. However, Converse and Volk (1990) demonstrated that a severe isolate significantly reduced root and runner production in otherwise symptomless 'Northwest' plants. Since the symptoms of pallidosis in *F. virginiana* indicators are not diagnostic, little progress has been made in determining the economic effects of the disease, the mode of transmission, and the casual agent. Yoshikawa and Converse (1990) reported the recovery of distinctive double-stranded RNA from pallidosis- infected plants that were deemed sufficiently distinct to allow identification of pallidosis in plants with multiple

virus infections.

The fact that strawberry pallidosis exists at such high levels in commercial plantings in Maryland raises serious concerns. Because the disease appears to occur at such high levels in Maryland, it is a relatively safe assumption that strawberry pallidosis is a greater problem than previously suspected in other regions of eastern North America. Furthermore, the mode of transmission and vectors for the disease need to be determined in order to enact cultural control methods in commercial plantings and nurseries.

Materials and Methods

Double-stranded RNA (dsRNA) was extracted from 21 plants that indexed positive for pallidosis agent using the graft indexing procedures. The dsRNA was cloned using protocols developed for efficient cloning of dsRNA (Jelkmann et al., 1989). Clones were sequenced and matched against the NCBI (National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD) database to help identify the virus associated with pallidosis. Primer pairs (short pieces of DNA) were developed from the sequence information and these were used in PCR to determine if the pallidosis virus could be detected directly from strawberry.

Using sequence handling software we were able to get the sequence of over 90% of the genome of SPaV and BPYV isolated from strawberry by combining overlapping sequences of various clones. The ends of both RNAs of both viruses was obtained by tailing the dsRNA and using PCR to fill in the gaps between the known sequences and the tails.

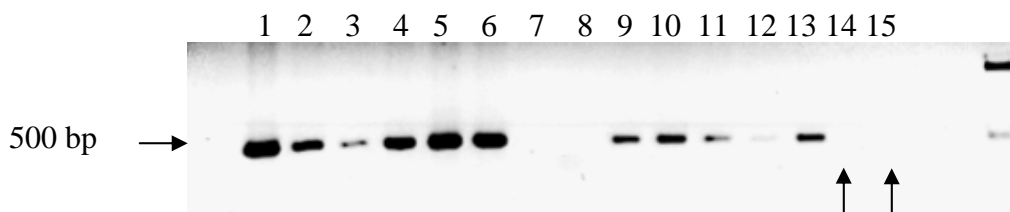
Results

The dsRNA extracted from pallidosis infected strawberry plants contained 6 – 10 bands in agarose gels. Some bands were present in all dsRNA extracts while others seemed to have varying amounts of dsRNA depending on source plant and time of year. The dsRNA pattern suggested the virus might be a member of the *Closteroviridae* family, genus *Crinivirus*, of plant viruses. Sequence information confirmed that SPaV was indeed a member of the closterovirus family since it contains a heat shock protein that is present in all members of this virus family but not found in any other plant viruses. There is also high homology of the other pallidosis genes with genes of viruses belonging to the *Crinivirus* genus. Over 130 clones have been sequenced and we now have information on each of the genes of pallidosis associated virus. Based on the sequence information primer pairs have been developed for several purposes; first to develop a diagnostic test for pallidosis and secondly to fill in the gaps in the sequence. Primer pairs for detection were made in the heat shock protein, the major and minor coat proteins and the methyltransferase genes. The complete sequence was obtained by tailing the dsRNA then using RT-PCR to fill in gaps between sequences obtained with the initial cloning and the gaps between the added tails and known sequences.

Total RNA was extracted from infected and healthy strawberries using a single step procedure. The RNA was then precipitated and washed with 70% ethanol before being dried and used in the laboratory assay. Since PCR is used to amplify DNA the first step in the procedure is to use reverse transcriptase (RT) to make a DNA copy of the extracted RNA. Then this DNA is amplified by PCR so that it can be visualized in an agarose gel. One set of primers that was developed gave a positive test in the RT-PCR reaction in 37 of 38 samples that tested positive for pallidosis by grafting. These samples included field material from the survey done in

Maryland in 1999 and standard isolates.

Figure 1. Detection of strawberry pallidosis associated virus by PCR.



PCR reactions were run on an agarose gel. Lanes 1-6 and 9-13 contain PCR reactions from pallidosis positive plants, lanes 7 and 8 are blank, lanes 14 and 15 (arrows) contain PCR reactions from healthy strawberry plants and the last lane contains markers

Working with the 38th isolate based on graft results a second virus capable of causing pallidosis disease in strawberry has been identified. This second virus is *Beet pseudo yellows virus* (BPYV) (Tzanetakis et al., 2003) which has also been completely sequenced (Tzanetakis and Martin, 2003). Working with Dr. Wintermantel at the USDA-ARS lab in Salinas, CA we have shown that SPaV and BPYV are transmitted by the greenhouse whitefly. Whiteflies have become common in strawberry fields in CA over the past 4-10 years and may explain why these two viruses have become so important there.

Strawberry pallidosis associated virus (SPaV) and BPYV have been found as major components in a strawberry decline in California production fields in 2003 but they do not appear to be involved in a strawberry decline observed in the WA and B.C. (Tzanetakis and Martin, unpublished data). Also, both viruses were present in samples from Maryland (1-38 above) with about 25% of those plants being doubly infected with the two viruses.

Summary

There is good evidence that strawberry pallidosis is caused by a closterovirus and that we can now detect the virus using PCR. This test, though much better than grafting, is relatively expensive. Therefore, the next goal is to develop an ELISA test for this virus that could be used in certification programs. Also, even though grafting can be used to detect pallidosis it is not very sensitive compared to PCR or ELISA. In our sequencing project, strawberry mild yellow edge was detected in plants that indexed negative for strawberry mild yellow edge on *F. vesca* indicators, suggesting that graft indexing can miss some virus infections. Now that we have a good laboratory assay for SPaV and BPYV in strawberry we have initiated studies to identify the vector of the virus and preliminary results show that the greenhouse whitefly can transmit SPaV, it has been known as a vector for BPYV for some time. A rapid sensitive detection method for pallidosis and knowing the vector of the virus will allow for the development of methods to control this virus in strawberry nurseries, commercial fields and breeding programs. Testing of strawberries for SPaV and BPYV from other states is being carried out.

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