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Successful back-inoculation confirms the role of black currant reversion associated virus as the causal agent of reversion disease

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Abstract

Reversion is the most wide-spread and serious virus-like disease infecting black currant but the causal agent of the disease has not been described. Recently, we have isolated a new nepovirus from reversion-infected black currant and by using immunocapture-RT-PCR detection, we have shown that the virus is consistently associated with reversion disease (Lemmetty et al., *Phytopathology* 87: 404–413, 1997). These data suggested that the virus, tentatively called black currant reversion associated virus (BRAV), could be the causal agent of reversion disease. Here we report that the isolated virus was successfully inoculated back to healthy black currant plants by slash inoculation of *in vitro* propagated young recipient plants. Vein pattern symptoms identical or very similar to the reported early symptoms of reversion disease were produced in the virus-infected plants. Using immunocapture-RT-PCR, the virus was again detected from symptomatic but not from inoculated symptomless plants or from the mock-inoculated or uninoculated controls. Production of the acute reversion symptoms demonstrates that BRAV is the causal agent of reversion disease, and we therefore propose that the virus be named black currant reversion virus, abbreviated BRV.

Introduction

Reversion is the most significant of the virus and virus-like diseases of black currant (*Ribes nigrum* L.). It is transmitted by the black currant gall mite (*Cecidophyopsis ribis* Westwood), and experimentally by grafting. In sensitive black currant varieties the first (acute) symptom of the disease is a yellow line pattern, often called a vein pattern. The ragged yellow line can be either broad or narrow (Adams and Thresh, 1987). The vein pattern symptom usually occurs one year after inoculation, early in the growing season (Thresh, 1963; Bremer and Heikinheimo, 1980; Bremer, 1983; Adams and Thresh, 1987; Atroshchenko,1992), but its appearance depends on the host cultivar, virus isolate, inoculation method (graft vs mite inoculation) and incubation conditions (Krczal, 1976; Adams and Thresh, 1987; Jones, 1994; Lemmetty et al., 1997). The line patterns usually occur only in some leaves of inoculated shoots and are concealed by later growth (Thresh, 1963). More persistent reversion symptoms include decreased hairiness and increased color intensity of the flower buds and flowers, and gradual change of leaf shapes. A distinct symptom, associated with the severe form of the disease, is malformation of flowers, affected flowers having ten elongated, sepal-like petals instead of the normal five petals. These symptoms occur erratically (Adams and Thresh, 1987; Jones, 1994). All disease isolates gradually reduce the yield of the infected bushes, but the rate and level of yield loss vary according to the aggressiveness of the disease isolate (Cropley et al., 1964; Thresh, 1966; Krczal, 1976).

The main obstacle to studies of the reversion disease has been the failure of many different approaches to

isolate or detect its causal agent (reviewed by Jones, 1994 and Lemmetty et al., 1997). However, we have recently reported isolation and partial characterization of a new nepovirus from black currant infected with the severe form of reversion (Lemmetty et al., 1997; Latvala et al., 1998). By immunocapture-RT-PCR (IC-RT-PCR) method, this virus was shown to be associated with numerous positive reversion infections originating from different parts of the world, and to occur in gall mite vectors and in the other host species of reversion, but not in samples of healthy black currant or other *Ribes* species (Lemmetty et al., 1997; Latvala et al., 1997). This virus, tentatively named black currant reversion associated virus (BRAV) was strongly suspected to be the causal agent of the reversion disease, but final confirmation of this required fulfilment of Koch's postulates. Here we report the results of successful back-inoculation of purified BRAV in black currant and production of the acute vein pattern symptoms of reversion disease.

Materials and methods

Virus

The BRAV isolate used in back-inoculation tests was the same as in previous studies (Lemmetty et al., 1997). The virus was propagated in *Chenopodium quinoa* Willd and purified as described (Lemmetty et al., 1997). After sucrose gradient centrifugation, the virus was pelleted and resuspended in 0.05 M sodium citrate buffer, pH 7.0. Virus suspensions diluted 1 to 10 in sterile water were used for inoculation tests.

Test plant material and inoculation method

Young, tissue culture-propagated black currant, cv. Mortti (Öjebyn × Wellington XXX) plants were used. The material was originally propagated from certified healthy Mortti shoots, obtained from the Laukaa Research and Elite Plant Unit of the Agricultural Research Centre of Finland. Micropropagation was initiated from bud apexes, and cultured under the conditions described by Graham and McNicol (1991). Propagated rooted shoots were transferred to soil and grown in a greenhouse. Plants used for inoculation were 20–30 cm tall. Before inoculation, the plants were tested by IC-RT-PCR for the presence of BRAV.

Inoculation and incubation of the inoculated plants

Six potted healthy plants were inoculated in late October and eight plants in early November 1996 by the modified 'slash inoculation' method described by Adams et al. (1995): a sterile razor blade was dipped into the diluted virus suspension and then vertical cuts approximately 3–5 mm long were made into the bark of the recipient black currant. The blade was recharged, and 20–30 cuts were made into each plant. At each inoculation one plant was mock-inoculated with buffer, and one plant was kept as uninoculated healthy controls. After inoculation, plants were maintained in the greenhouse at 18–20 °C, with an 18 h photoperiod. The lighting was continued until November. From December to February the temperature was lowered to 12 °C. At the beginning of the new growing season the plants were pruned. Development of disease symptoms in the plants was monitored throughout the growing season.

IC-RT-PCR

The presence of BRAV in black currant leaves was detected by IC-RT-PCR as described by Lemmetty et al. (1998), using oligonucleotide primer pair annealing to viral RNA and cDNA, respectively, in genome positions 1–15 and 198–210 (numbering from the 3′ end of RNA2). Aliquots (35 µl) of the PCR reactions were electrophoresed in 2.0% Seakem agarose gels in Tris-acetate-EDTA buffer (40 mmol Tris-acetate and 1 mmol EDTA, pH 8.0). The gels were stained with ethidium bromide and visualized using UV light.

Results

Symptoms

The first leaf symptoms were observed five months after slash inoculation in four Mortti plants after leaf emergence, in March 1997. The very first symptom in two plants was a dark-brown banding near the leaf margin (Figure 1A), which grew out of the leaf as it expanded. Later, one of these leaves showed a distinct yellow vein pattern. The other two plants showed faint yellow line and ring-like patterns. By the end of

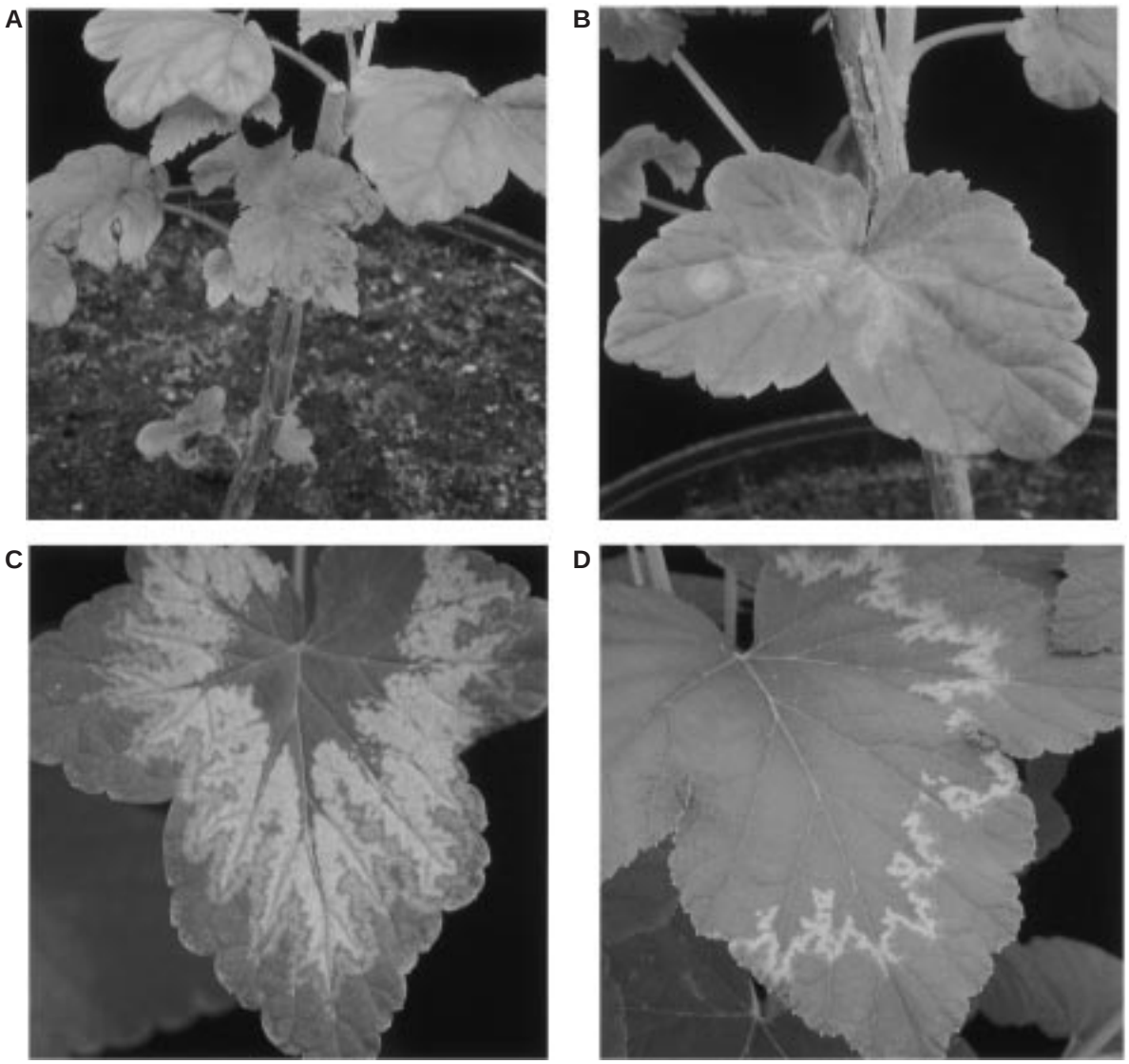

Figure 1. Various leaf symptoms observed in the slash-inoculated black currants. A, Necrotic line pattern observed in two plants about 5 months after inoculation. B, Various line patterns, and a ring-pattern observed in one plant. C and D, Vein patterns representing the typical early leaf symptom of reversion about seven months after inoculation.

May, leaf symptoms occurred in six of the 14 slash-inoculated plants. Symptoms included yellow line patterns and a ring-like pattern (Figure 1B). In addition, there were small faint chlorotic rings in the young upper leaves. Two plants showed very distinct bright yellow vein patterns, identical to the published vein pattern symptoms of reversion disease by the end of May (Figure 1C,D). Mock-inoculated plants and uninoculated controls remained symptomless. No vein pattern symptoms were produced in the infected plants in the following spring (1998). So far the plants have produced no flowers, and therefore there is no observations of flower symptoms.

IC-RT-PCR detection of BRAV

Samples of all 18 black currant test plants were tested by IC-RT-PCR before slash inoculation, and found to

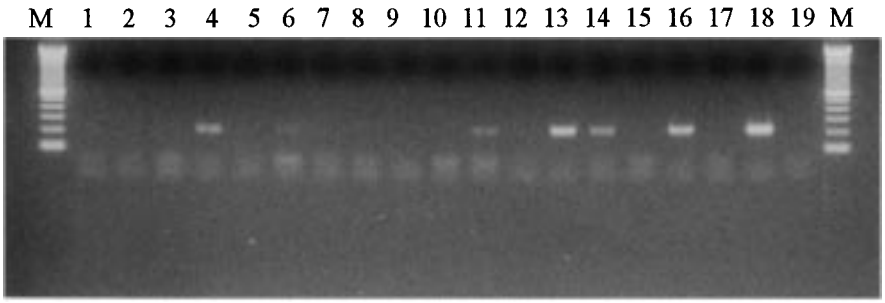

Figure 2. Detection of the BRAV specific, 210 bp fragment, amplified from the infected plants by IC-RT-PCR. Positive detection was obtained from six plants (lanes 4, 6, 11, 13, 14, 16), all of which also showed various reversion related leaf symptoms. Lane 1: uninoculated control; lanes 2 and 3: mock-inoculated controls; lane 18: positive control for BRAV; lane 19: a buffer control; lane 'M': a 100 bp DNA ladder (Gibco BRL).

be BRAV negative. After the appearance of the first symptoms in March 1997, samples from the four symptomatic plants were tested, and three were found to be BRAV positive. In May, all inoculated plants and mock-inoculated plants were again tested by IC-RT-PCR, and BRAV-specific fragment of 210 nt in length was amplified from all six symptomatic plants (Figure 2). No product was amplified from the symptomless or the mock-inoculated plants.

Discussion

Reversion disease is the most serious viral disease infecting black currant, but still, despite many research efforts, its causal agent has defied detection (reviewed by Lemmetty et al., 1997 and Jones, 1994). So far, the only method available for detecting this disease has been indexing on indicator plants, and monitoring for symptoms over a two year period. This detection method is slow and cumbersome, and has been made even more difficult by the erratic appearance of symptoms in the indicator host, and by the erratic distribution of the disease agent in the source tissues, requiring multiple samples to be used for reliable testing of only one plant.

Recently, we have reported isolation of a new nepovirus (BRAV) from reverted black currant. Associated data have strongly suggested that the virus is the causal agent of reversion disease. To prove this we made multiple attempts to mechanically inoculate the virus back in to black currant cv. Öjebyn, a cultivar commonly used for detection of reversion by grafting, but these attempts were not successful. Many woody hosts are known to be very difficult, or impossible, to mechanically inoculate with isolated viral pathogens, due to the high content of phenolic and oxidative compounds in their sap (Fulton, 1966), and possibly due to their thick and strong cell walls. These properties may also explain the high reluctance of black currant to inoculation by BRAV. The slash inoculation technique has been successfully used before for inoculation of woody plants (Kyriakou, 1992; Hadidi et al., 1997), and its effectiviness may be due to the introduction of infectious agents directly into the phloem tissues of plants. Tissue culture-propagated young plants are probably the most succeptible test material because of their soft tissues and low content of phenolic compounds.

We used black currant cv. Mortti as our test plant because it is easily propagated *in vitro* compared to cv. Öjebyn. Mortti has been produced as a cross of cultivars Öjebyn and Wellington, both of which have been reported to show vein patterns as an early symptom of reversion (Thresh, 1963; Bremer and Heikinheimo, 1980).

Some of the BRAV inoculated plants produced vein pattern symptoms identical or very similar to the reported early symptoms of reversion (Posnette, 1952; Thresh, 1963; Campbell, 1965; Bremer, 1983; Adams and Thresh, 1987). Also small faint ring-like patterns occurred in some leaves; these, too, have been reported before in connection with the reversion disease (Posnette, 1952; Campbell, 1965). However, the initial symptom observed in two leaves, a distinct necrotic line pattern has not been reported earlier. This symptom was possibly due to the high inoculum concentration, leading to a shock reaction in the infected tissue. Also a ring spot symptom observed in one leaf, has not been previously reported for reversion. The certified healthy status of the recipient plants used in

back-inoculation rules out other causal agents for these symptoms. The occurrence of variable symptoms after inoculation with the purified virus preparations indicates that the variety of symptoms earlier reported for reversion are all caused by this particular virus. Our results prove that the isolated nepovirus is the causal agent of the reversion disease. Therefore, we propose that this virus be renamed black currant reversion virus, abbreviated BRV.

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