BIOLOGICAL CONTROL OF BLACKLINE DISEASE OF ENGLISH WALNUT

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ABSTRACT

Under project number 87 WMB 2 we have been studying a small ribonucleic acid (RNA) molecule that interferes with the replication of cherry leafroll virus (CLRV). CLRV is the causative agent of walnut blackline. The small RNA is termed a "satellite RNA" because of its inability to replicate when inoculated alone. Earlier we had co-inoculated CLRV and satellite RNA to cowpeas. We were unable to detect a significant increase in either CLRV or the satellite RNA in the inoculated leaves. This was an unexpected result since it indicates that the satellite RNA interfered with CLRV replication without itself replicating. We now have shown that the satellite RNA also interferes with CLRV replication in walnut trees. In other, federally-supported, research the satellite RNA nucleotide sequence was incorporated into the nuclear DNA of an herbaceous host of CLRV, lettuce, such that the satellite RNA was synthesized in lettuce plant cells. These lettuce plants resisted CLRV. Thus if satellite RNA sequences can be introduced into walnut cells, which we propose to do, it may be possible to generate CLRV-resistant walnut trees. We also have obtained improved plasmid clones of the CLRV RNA sequences which may be applied in a sensitive procedure for detecting CLRV.

INTRODUCTION

CLRV is a member of the nepovirus group of plant viruses. Another member, tobacco ringspot virus (TobRV), is sometimes found associated with a small satellite RNA, the satellite RNA of tobacco ringspot virus, referred to hereafter as STobRV RNA. STobRV RNA is one of the most well-studied of the plant virus satellite RNAs. As is characteristic of satellite RNAs, STobRV RNA replicates only in plant tissue that is infected with TobRV. Thus TobRV is considered to be a supporting virus for STobRV RNA. STobRV RNA acts as a parasite of TobRV, greatly reducing the titer of TobRV in several hosts. In the cowpea (Vigna unguiculata), which usually suffers a fatal stem necrosis in TobRV infections, co-inoculation of STobRV RNA with TobRV saves the plants, which continue to grow and eventually produce seed.

Initially, we attempted to adapt STobRV RNA to CLRV as a new supporting virus. Although these attempts were unsuccessful, we discovered a new and potentially useful satellite RNA phenomenon in the course of these experiments. STobRV RNA, though not supported in its replication by CLRV, protected cowpeas against CLRV. Cowpea leaves co-inoculated with CLRV and STobRV RNA showed no detected increase of CLRV. CLRV was able to spread to and multiply in the uninoculated, trifoliate leaves that had not been inoculated with STobRV RNA. The translation of CLRV RNAs in vitro also was greatly reduced by STobRV RNA, providing a possible mechanism for the anticalrv action of the satellite RNA: interference with the production of CLRV-specified proteins. These results have been published (F. Ponz et al., Virology 160, 183-190 (1987)).
The ability of STobRV RNA to protect plants against CLRV raises the possibility that English walnut trees can be protected against CLRV infections if they can be caused to generate STobRV RNA continuously and in all CLRV-susceptible tissues. Two approaches to producing STobRV RNA in walnut trees are

(a) to genetically transform walnut cells with a properly engineered deoxyribonucleic acid (DNA) form of the STobRV RNA nucleotide sequence and from these cells regenerate STobRV RNA-producing walnut trees

and

(b) to infect standing walnut trees with a mixture of STobRV RNA and TobRV, the virus that supports the replication and spread of STobRV RNA.

The former approach is designed to produce CLRV-resistant walnut lines, the latter to protect susceptible trees that are either uninfected or infected but not yet seriously damaged.

OBJECTIVES

Our principal immediate objective was to determine whether STobRV RNA can interfere with the replication of CLRV in English walnut. We have answered this question in the affirmative. Longer term goals are to exploit such an interference using one or both of the two approaches, the first aimed at the future production of CLRV-resistant English walnut stock and cultivars and the second at slowing or preventing the appearance of blackline in existing, producing trees that have become infected with CLRV.

Transformed English walnut cultivars should be able to generate STobRV RNA from DNA copies of STobRV RNA installed in the nuclear DNA. Transformation obviously is the method of choice in the long term, because of its potential for producing CLRV-resistant cultivars of English walnut.

If STobRV RNA could be made to increase in walnut trees, by introducing TobRV to support the increase of the satellite RNA, it is conceivable that walnut trees already infected with CLRV, but not yet showing blackline symptoms, could be protected.

In support of the latter strategy, our subsidiary objective was to improve the sensitivity of detection of CLRV in walnut trees and pollen using nucleic acid hybridization. Towards this end, we have obtained improved plasmid clones of CLRV RNAs.

RESULTS

Five three year old Hartely English walnut trees on Northern black walnut rootstock were inoculated at bark patches 10 cm above the graft union with CLRV. On the opposite side of the trunk, the inoculation was with CLRV plus
STobRV RNA. As is indicated in Table 1, all five trees showed accumulation of CLRV below the patch inoculated with CLRV alone, as assessed by enzyme-linked immunosorbent assay (ELISA). In four of the five trees no significant increase of CLRV was observed when CLRV and STobRV RNA had been co-inoculated. These results strongly support the notion that walnut trees may be made CLRV-resistant, either by genetically-transformed them to express STobRV RNA or by co-inoculation with TobRV and STobRV RNA to foster the replication and spread of STobRV RNA. The trees from this experiment are being maintained and will be inspected at the appropriate times for the possible appearance of blackline, expected below the sites of inoculation of CLRV alone but not below the sites at which CLRV plus STobRV RNA had been inoculated.

In a similar experiment, we inoculated one-year-old walnut trees with CLRV, TobRV, and STobRV RNA in various combinations. We expected that the combination of TobRV and STobRV RNA would show strong protection against co-inoculated CLRV because of the increase in STobRV RNA that should be fostered by TobRV. However, extensive analyses by ELISA, of samples taken at several times after inoculation, showed that in this particular experiment we had very poor transmission of CLRV and no replication of TobRV in the inoculated cambium tissue. Because the cambium of trees used in this experiment was in less than the optimal physiological condition for inoculation with viruses, we were unable to draw any conclusion about the ability of TobRV to replicate in walnut cambium.

If the strategy, of using TobRV-supported STobRV RNA to protect walnut trees, is successful, we anticipate that a more sensitive test for CLRV infection of orchard trees will be needed. Nucleic acid molecular hybridization promises to provide the desired increased sensitivity. A new vector was obtained for cloning RNAs with the structure of nepovirus RNAs. CLRV has two RNAs, RNA 1 and RNA 2. Full-length, or nearly full-length clones of RNA 2 have been obtained. Other clones have more than half of the RNA 1 sequence. These long clones should allow CLRV to be specifically and more effectively detected in extracts of walnut pollen and other walnut tissues than has been possible in the past with ELISA and other detection procedures.
Table 1. Satellite Tobacco Ringspot VirusRNA
Protects Walnut Trees Against Cherry Leafroll Virusa

<table>
<thead>
<tr>
<th>Inoculum</th>
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<tbody>
<tr>
<td>Tree No.</td>
<td>CLRV</td>
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<tr>
<td>1</td>
<td>1.78</td>
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<tr>
<td>2</td>
<td>1.41</td>
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<tr>
<td>3</td>
<td>1.23</td>
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<tr>
<td>4</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>5</td>
<td>1.82</td>
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aELISA absorbance values were determined from homogenized
samples of bark. As a check, samples were taken from trees that
had bark patches rubbed with buffer alone. The average and
standard deviation of ELISA values were 0.09 +/- 0.01 for five
trees.

DISCUSSION

Our results on the protection of English walnut against CLRV by co-
inoculated STobRV RNA are very encouraging. This is especially true when they
are considered in conjunction with what we have learned using transgenic
lettuce plants. Lettuce plants were exposed under conditions for
transformation to a "dimeric" construction of the STobRV RNA sequence. Other
research in our laboratory has shown that such a dimeric construction should
generate, after RNA processing reactions, unit length STobRV RNA.

Several regenerated lettuce plants have been shown to have been
transformed successfully, as evidenced by production of STobRV RNA detected by
molecular hybridization tests. Recently the selfed progeny of one such plant
were tested for resistance to CLRV. If the parent plant had a single copy of
the STobRV RNA sequences, this would be expected to behave as a single
dominant gene. The satellite RNA sequence should segregate 3:1 in the seed
set on the transformed plant. We observed that seven of nine seedlings
germinated from the seed of one transformed lettuce plant, when artificially
inoculated with CLRV and assayed one week later, showed no detectable CLRV in
the uninoculated leaves to which CLRV would be expected to spread. The
corresponding uninoculated leaves of eleven of eleven control lettuce plants
were systemically infected and were supporting a strong increase in CLRV, as
detected by ELISA. Thus the satellite RNA sequences behaved as expected for
an engineered dominant gene for resistance to CLRV.
The research on transgenic lettuce plants was in collaboration with the laboratory of Dr. Richard Michelmore. That portion of the research conducted in the laboratory of George Bruening is supported by federal grants. We are more completely characterizing this system. The results suggest that English walnut transformed with STobRV RNA sequences should resist CLRV.

Although we have not been able to infect walnut trees with TobRV thus far, further attempts along these lines will be made. If such infections can be induced, and if, as expected, they are not accompanied by unduely harmful symptoms, we will again attempt to support STobRV RNA replication with TobRV in walnut.