BIOLOGICAL CONTROL OF BLACKLINE DISEASE OF ENGLISH WALNUT

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ABSTRACT

Blackline is an important disease of grafted orchard walnut trees in California. The causal agent is cherry leafroll virus (CLRV), and necrosis of cambium and formation of cankers at the graft union are the critical deleterious effects of this pollen-borne virus. General introduction into orchards of CLRV-resistant English walnut scions, if available, would provide an excellent long term solution to the problem of blackline disease because CLRV no longer would be present in walnut pollen. Alternatively, rootstock resistant against, or tolerant of, CLRV will allow even infected trees to continue in production. We are investigating a source of apparently CLRV-tolerant Paradox rootstock and three possible sources of genes for engineering resistant against CLRV in English walnut. A selection of Paradox, designated P 5/15, demonstrated tolerance to CLRV introduced from its English walnut (cultivar Tehema) scion in a 10 year test of one tree. New results show the generality of this tolerance to CLRV introduced from other English cultivars, and experiments in progress are designed to test a new strategy for the application of P 5/15 to controlling blackline disease. In other plant virus systems, interference with virus replication has been achieved by introducing certain nucleotide sequences into host plant cells. In this context, we are testing sequences from a satellite RNA, from the coat protein gene of CLRV, and from the long, untranslated 3' region (3'-UTR) of CLRV. We report the 3600 nucleotide sequence of the coat gene and 3'-UTR of CLRV RNA 2.

OBJECTIVE

Cherry leafroll virus (CLRV), the causal agent of blackline disease of grafted English walnut, is pollen borne and infects pistillate flowers of the scion. Spread of CLRV in the infected scion is at a rate of a few tens of cm per year, and the infection has little or no apparent effect on the appearance or productivity of the scion. Black and Paradox rootstocks are resistant to CLRV. However, this resistance is expressed in part by death of inoculated cells and necrosis at the graft union, as CLRV from the scion reaches rootstock cambium cells, results in girdling of the tree in the advanced stages of the fatal blackline disease.

Our goal is to develop control measures for blackline disease of grafted English walnut orchard trees through identification, improvement and/or engineering of

(i) English scions resistant against CLRV and/or
(ii) rootstock tolerant of, or resistant against, CLRV.

Because of the known propensity of viruses to mutate and overcome the resistance of potential hosts, it is prudent to attempt to obtain more than one genetic source of resistance against any economically important virus.
PROCEDURES

We describe here our procedures in three areas:

A. analyses and applications of an apparently CLRV-tolerant selection of the walnut rootstock Paradox,

B. tests of the possible use of the satellite RNA of tobacco ringspot virus for interference with CLRV replication, including tests of these sequences in walnut shoots in culture, and

C. analyses of the nucleotide sequences of CLRV RNA 2 and constructions of plasmids designed to express these sequences in walnut trees. The sequences encode the coat protein and form the 3'-UTR of the virus genomic RNAs

Parallel sections appear in the RESULTS AND CONCLUSIONS section, below.

A. CLRV-Tolerant Rootstock

Background. In earlier research, Tehema English walnut cultivar had been grafted onto several selections of Paradox and subsequently inoculated with walnut-8 strain of the virus. Although most of the graft unions of this set of trees began to fail within two years of inoculation, due to blackline, the graft union of the tree with Paradox selection designated P 5/15, as rootstock, remains functional as of this writing, 10 years later. Only a narrow, sharp line has developed at the graft union, with no indication of canker development in the Paradox 5/15 rootstock. The existence of the narrow, hair-like black line is itself encouraging because it suggests a hypersensitive type of resistant reaction against CLRV. This notion is supported by ELISA of cambium scrapings. Although the Tehema scion samples gave strong ELISA signals using anti-CLRV serum, the P 5/15 samples did not give a detected signal.

Research in progress is designed to evaluate both the generality and the utility of the application of P 5/15 to the blackline problem. Although Paradox selection P 5/15 presented a startling and very encouraging result, the generality of its tolerant reaction, for example in graft unions with varieties of English walnut other than Tehema, was unknown. Current technology does not allow routine rooting of Paradox selections, and vegetative increase of P 5/15 for use directly as a rootstock with unaltered genetic characteristics is problematical. However, our experience suggests that various Paradox selections readily are propagated by budding on other Paradox rootstock germinated from seed. We are testing the application of P 5/15 as an interstock to protect rootstocks from the most devastating effects of CLRV.

Experimental. In June of 1988, sets of three bark patches were applied to two Chandler and three Ashly English walnut trees as indicated in Fig. 1A. The central patch of the set is from an English walnut tree infected with walnut-8 CLRV; the other two patches are from Paradox selections: P 5/15, the
Figure 1
Tests of a CLRV-tolerant selection of Paradox

Budded future scion Aug., 1990 (Chandler, Tehema or Vinia)

Interstock P 5/15, budding, Aug., 1989

Rootstock Paradox, planted Dec., 1988

Rootstock Paradox, planted Dec., 1988

30 to 60 cm

10 cm

P 3/27 ("cankered")

CLRV-infected bark patch

P 5/15 ("hairline" blackline)

Ashly or Chandler

Spring, 1991
Cut and prune

August, 1991
Inoculate, bark patches

Summer, 1992
Read results
selection under test, and P 3/27, a control known to develop blackline cankers when its English scion is infected by CLRV. Each set of the three bark patches was applied at three sites on each tree for a total of 15 replications of the CLRV inoculation. It is known from previous experiments that CLRV will migrate both upward and downward from the infected bark patch with equal facility, so that the P 3/27 and P 5/15 patches can be expected to be exposed to approximately equal challenges of CLRV.

Fig. 1B illustrates our interstock strategy for protecting rootstock grafts from the effects of CLRV infection of the scion, in experiments begun in 1988. This strategy, if successful, will make the rooting of P 5/15 unnecessary. In 1989, more than 20 Paradox seedling rootstocks were budded with P 5/15 or, as a control, with P 3/27. In August of 1990 the P 3/27 or P 5/15 stem of each tree was budded with Tehema, Vinia, or Chandler English walnut at a suitable site 30 cm to 60 cm above the union with the Paradox seedling rootstock. The result is the introduction of P 5/15 (or P 3/27) as an interstock of 30 to 60 cm between Paradox seedling rootstock and the English walnut scion.

B. Interaction of Satellite RNA of Tobacco Ringspot Virus with CLRV

Background. A satellite RNA of a plant virus is able to replicate only in association with specific viruses, designated as supporting viruses. A characteristic of some satellite RNAs, a characteristic potentially of agronomical importance, is their ability to reduce the titer of the supporting virus and the severity of symptoms that the supporting virus alone induces. One of the most well studied satellite RNAs, and one that is very effective in ameliorating the symptoms of infections by its supporting virus, is the satellite RNA of tobacco ringspot virus (sTobRV RNA).

Ponz et al. (1987) observed a new interference phenomenon in which sTobRV RNA reduced the titer of CLRV in inoculated leaves when CLRV and sTobRV RNA were co-inoculated to cowpea (Vigna unguiculata). This was an unexpected result because CLRV does not support a detected increase in sTobRV RNA. The interference was limited to the inoculated leaf, and CLRV spread to and increased in the uninoculated trifoliate leaves. Presumably the co-inoculated, but not replicating, sTobRV RNA was confined to the inoculated cells and was not able to influence the CLRV that had migrated from those cells. To test for a possible systemic suppression of CLRV in walnut by sTobRV RNA, rather than only the localized interference that might be expected from co-inoculation of sTobRV RNA and CLRV, transgenic, sTobRV RNA-expressing English walnut tissue was generated.

Experimental. Plasmid constructions were prepared in Escherichia coli and introduced into Agrobacterium tumefaciens for transfer into walnut embryos in culture. The design of plasmid inserts has DNA copies of satellite RNA sequences arranged so that the positive polarity of the RNA will be transcribed under control of the cauliflower mosaic virus 35S promoter. The DNA segment engineered to be transferred to walnut cells also includes two other genes: kanamycin phosphotransferase (for antibiotic selection of walnut transformants) and β-glucuronidase (for screening of transformants). Details of the constructions and procedures appear in last year's report. Transformation was as described by McGranahan et al. (1988) using engineered A. tumefaciens.
C. Characterization of the CLRV Coat Protein Gene and 3' Untranslated Region

**Background.** Research with many plant viruses has shown that the coat protein gene, when engineered to be part of the plant nuclear DNA, confers resistance on the plant. That is, the production of virus coat protein in each cell of the plant, before those cells encounter the virus, prevents or greatly delays virus increase after inoculation of the usual virus dose. In other, less extensive research, including a study in this laboratory on tobacco ringspot virus, the untranslated 3' portion (3'-UTR) of the virus RNA, when applied as an independent RNA molecule, was able to interfere with virus replication. We investigated both the coat protein gene and the 3'-UTR of CLRV as possible sources of resistance genes against CLRV.

CLRV has a genome composed of two large RNA molecules, RNA 1 of about 7000 nucleotide residues (7 kilobases, i.e., 7 kb) and RNA 2 of about 6 kb. Analogy with other viruses strongly suggests that the coat protein gene will be encoded in RNA 2 and that the 3'-UTRs of the two genomic RNA molecules will be very similar in sequence. Furthermore, the coat protein gene was not expected to be expressed independently. Genomic RNAs of members of the nepovirus group, to which CLRV is very similar, are translated into polyproteins, and it is the specific proteolytic cleavage of the polyprotein that releases functional virus proteins during the infection process. Thus, the amino acid sequence of the CLRV coat protein is expected to be synthesized as part of a precursor polyprotein. We determined the sequence of bases in more than half of CLRV RNA 2 in order to deduce the amino acid sequence of a portion of the polyprotein and to reveal how far the 3'-UTR extends from the 3' end of the molecule. However, determining the exact location of the coat protein amino acid sequence within the polyprotein and the precise cleavage sites will require results from other experiments.

The expression of CLRV coat protein in transgenic walnut tissue will be approached in a manner similar to that taken for the expression of biologically active sToBRV RNA. However, the constructions are more complex because not merely RNA synthesis but also efficient synthesis of protein is required in the transformed walnut cells.

**Experimental.** We obtained a 6000 kb, nearly full length copy DNA clone of RNA 2 of CLRV, the genomic RNA of the virus that already was known to encode the coat protein. Segments of this DNA were separately transcribed into RNA molecules that thus correspond to different segments of RNA 2. Each of these RNAs was supplied to an *in vitro* translation system. Some of these CLRV RNA 2-derived RNAs directed the synthesis of a polypeptide just slightly smaller than authentic CLRV coat protein. This polypeptide reacted with antibody prepared against CLRV particles, confirming its identity as a coat protein-related molecule.

The ability of fragments of CLRV RNA 2 to direct the synthesis of a coat protein-like molecule *in vitro* was unexpected because fragments of a messenger RNA usually will not have the proper signals to direct protein synthesis. This "illicit" translation is being examined as separate research project and is of interest here only because it allowed us roughly to localize the coat protein.
protein gene within the 6000 bases of cloned sequence, guiding nucleotide sequencing efforts and, subsequently, locating the coat protein amino acid sequence as the carboxyl-terminal portion of the CLRV RNA 2 polyprotein.

An *Agrobacterium* plasmid now under construction will carry a cDNA copy of a segment of the polyprotein that includes the amino acid sequences of CLRV coat protein. The CLRV RNA 2 nucleotide sequences are arranged to be downstream from the 5' leader sequence of tobacco etch virus (TEV) to enhance translation of the resulting messenger RNA. The combined TEV RNA and CLRV RNA 2 sequence is being inserted so as to be under control of the 35S promoter as indicated in Fig. 2.

![Diagram](image)

**Fig. 2.** Transformation vector for the expression of CLRV coat protein in walnut. Between the T-DNA left border (LB) and right border (RB) are:

- a selectable drug resistance gene (KAN)
- a screenable gene β-glucuronidase (GUS)
- a segment of the CLRV RNA 2 polyprotein that includes all of the amino acid sequence of the CLRV coat protein. This sequence is fused in translation reading frame register with the translational leader of tobacco etch virus (TEV)
RESULTS AND CONCLUSIONS

A. CLRV-Tolerant Rootstock

Assessment of the results for the experiment diagrammed in Fig. 1A is necessarily destructive. Two trees (total of 6 experimental replicates), one Ashly and one Chandler, were sampled in July of 1990, with decisive results. In each case, the P 3/27-derived barkpatch developed typical blackline cankers. In fact, it is apparent from the P 3/27 patches on the remaining three trees that all P 3/27 patches are likely to be lost to blackline canker. In contrast, the six P 5/15 patches that were examined showed only narrow, black hairlines, and the patch graft unions show no sign of failing. These results support, for graft unions with English cultivars Ashly and Chandler, the tolerance of Paradox selection P 5/15 previously observed for a graft union to cultivar Tehema. Potentially, the application of P 5/15 is likely to be very general. Observations on the remaining three threes will continue through the 1991 season with the objective of observing the degree further development of blackline, if any, at the P 5/15 patch graft unions.

As is indicated by Fig. 1B, we plan to continue to prepare the interstock trees, initiated in 1988, through 1991, with inoculation by CLRV strain walnut-8 in August, 1991. Results from least some of the trees are likely to be known by late summer, 1992.

Thus, results to date are very encouraging with regard to the possible application of P 5/15 as a practical solution to the problem of blackline disease of grafted walnut cultivars.

B. Interaction of Satellite RNA of Tobacco Ringspot Virus with CLRV

Several transformed walnut lines were produced and shoot material was regenerated. We obtained several lines of walnut shoots that expressed sTobRV RNA uniformly. Line "TG-60" was found to produce intact, biologically active sTobRV RNA as evidenced by the ability of total RNA extracts from TG-60 to initiate an increase in sTobRV RNA when co-inoculated with TobRV to bean (Phaseolus vulgaris) seedlings. Walnut tissue was challenged with CLRV at a series of inoculum concentrations. Unfortunately, CLRV infection of TG-60-derived walnut shoots was not found to be significantly reduced, compared with infection of control, untransformed English walnut shoot material.

C. Characterization of the CLRV Coat Protein Gene and 3’ Untranslated Region

We have established that the coat protein gene ends 1579 bases from the 3’ end of CLRV RNA 2 (Fig. 3), and we have identified region of the polyprotein that includes the coat protein. The most likely site of the amino terminus of the coat protein in the CLRV RNA 2 polyprotein is shown in Fig. 3, based on comparisons with the sequences of polyproteins of other viruses. The molecular weight of the corresponding protein is about 56,000, as expected for CLRV coat protein. Experiments in progress are design to locate the amino terminus of the coat protein precisely. Unfortunately, the amino terminal
The unusually long, 1579 base 3'leader sequence of CLRV RNA 2 makes this sequence a good candidate as a possible CLRV-interfering sequence. Plasmid constructions are in progress that are designed to integrate into the genomic DNA of walnut trees sequences that will be expressed either as CLRV coat protein or as RNA corresponding to the 3'-UTR of CLRV RNA 2 or the complementary, "antisense" version of this RNA. Small regenerated walnut trees will be tested directly for their ability to resist challenge by CLRV.

As indicated above (Fig. 2), the first A. tumefaciens strains for expression of CLRV polyprotein segment containing the coat protein now are being prepared. The second generation construction will be engineered to express coat protein amino acid sequences without the other sequences of the CLRV RNA 2 polyprotein.

REFERENCES


Fig. 3. Nucleotide sequence and derived polyprotein amino acid sequence of a portion of the
CLRV RNA 2 polyprotein.

UUUUUUACGCUUAAAGCAGUACGACCAAA CAUUUAGUUUGCUCCUGAUUGGCUGGGUUGUUUG AAAGGUCAGUACUGCCAGCAAAACUCUAC 90
F F I R L K L M H Q H C S A P D G R V L K G H D L E H K L Y
UCGUAUUGGAGGGAUUGCCCCAGGACCAAGU GUCUAUUGGCAAGCCCUGUAACACAUAGC 180
SDWRIAPGPS V Y W H P S T H S P G H D R T I S R A
CUGGCGACUGCAAGACAAAGGACCCGGAGACAU GUCUUGCCUGCCCCACAGGACAGGAA 270
LAT AQ I Q G Y N I R A V Q Q G NAT V F A P Q G G H V E
GGAAACCCAGUCACGUAAACCAAGGGGA GCGUGGAAACACUCUGGAAACACGCGA 360
G T P S A D L O M G A T E L T V Q T G T H W R L Q S A S
AGCCGAAUUUGGGGGCGACCCUGCCACACGG CCAGAAUUGGGAUUGAUUGCAGAUCGAGA 450
SRF V V E G T S R Q R E P R R M S S V R V D R G D F P D Q
GGAGCAUGGCGCCAGAACCACCAACCCCGAUAA AUAUGGCCUUGGCAGCAUGCAUGCAUGUA 540
G A R G Q Q Q P N M P L Q S G I L P S M H W S A A T P F 56,330
AAAGUGGCGCAUGGACAGGGGCGCCGGAAGAUCUC AACUCUGGCCCGUCGUCCUGCCACAUU 630
K C A A E A E N S I L A R S L R E A W I N E R A T S D A W I
AAAGGCGACUGCAACCAAGCCUCACCUUUU CUUGUAAGGGAACACACGUGCGACUGGUG AUAUAIUAGCUGCUAGACUGUGA 720
K W Q R E Q R S T F L V E G T I A M S V I N I A G T T L G L
GUGUGCCACCGCUACACCGGGCGGAGCAUU UUUAAAUAAUUCCCUAGUGCUGCGGCAAG AUAUAGCCAGAAGGGGUUGAUUGAUUGA 810
V S D A F N R A E H L N N F P S A L G Q N M P Q K V F P L S
AAUCCUGACUGGAGAAUUUGGGGGCUU UUGAGAUGAUGUUAGUGCAUGGGAUUGCAUGA CCAUGACGGUGAGCAUGGAGUGAAGA 900
N P L E R N F S F S M S E L L G Y T M H P A S A Y E D V Q
UUAUACUCUUAAUGGCUAACAAAGCUAGAU GGUCUUGGCGAGCAGUUGGGGGGCAU AUCUUGUGCGAGAUGGAAGAGUGUGUCGCU 990
F I L Y V L N T N D V C A A D W G H I L W Q V K D D A A
GAACCUAUGAGCGUACAGUAAUCCUGGCAU CCAGGGAUGGCGCCUCGUCUGGAUACUGG CGUGGCGCCAGCAAGUGGCGAGGCA 1080
E P Y E L Q L P V P R D G A R L D I W R G P A T M V Q G T
UCCCUGUACACGACAAUUGGCAUUUGGGCG UUCCUGGCAACGAAAUGUGCAAGGGCAA UACUUGCCUCAAAUGAUCACCCAGGA 1170
F P Y T T N V N L G F A E P R S V Q T G Y A P I T S F H Q A
GCCUGUCAUACCUAAUGUGGAGAACACAGG ACCAAUCCAGGGCUGAUGUAUAAAGUGGCC UCGUGUUAGGUGAGAAUGAGACUAGUCUGUG 1260
A L S Y Y I S Y G G I H G R L I K I G S G L V Q D I A L
GCCUGUGCCGAUGUAUUAGCUGACAGGUGCC GCUUAAUGGAAAUAUUCAAGGGCUGCAU GCCUGGUCAGCAUGGCGGAGGGGAAGUUU 1350
A M W H D N A D M V A Y R E I I K V P H V L L T G G E G E F
GCUCUCUCACCAUGGCGGGCUGCCUUGGCAU ACUUCUACAGCGCCAGCGCGCCACCAUA GCUGUGUGUGUGUGGUGUGGGCGLAGCUG 1440
A L P I N A P F F G T R T D R G L T A V C L V S G V V A
CCUAGGACUGCCACAGCCCCAUCAGAUGG AGUAGUUAUUUGGUAAGGGGGAUGGGGUUGAUAU GCAGAGAAUACCCUGUGUGACAGAUGGAG 1530
PKDCSAPYRF M I Y F D R V E F N A Q L P P V I A N R
CUGCAGUUCUCUGGGCAGUCUUUGGUGAAC UCUGCCACAGGGAAUCUGCCAGGCAAAAGC ACCUGUAGUACAGUCUCGUCCGUUCCGGAU 1620
L Q F L W A S F S N F A P V V P A N R T W M I P C R L S D