CHARACTERIZATION OF R GENES INVOLVED IN RESISTANCE TO *Cherry leaf roll virus* IN PARADOX HYBRIDS

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ABSTRACT

A single dominant 'R' gene ($clrv^R$), in black walnuts (*Juglans hindsii*) or 'paradox' hybrids (*J. hindsii* x *J. regia*) confers resistance to *Cherry leaf roll virus* (CLRV), the causal agent of blackline disease. The identification and cloning of the 'R' gene is expected to aid the walnut breeding program by improving the accuracy and efficiency of current PCR-based screening methods of the progenies of backcrosses and is also expected to help generate novel rootstocks tolerant to infection by CLRV. In our attempt to identify the $clrv^R$, 37 degenerate primers were modified from previously published studies or were newly designed to amplify homologs of known 'R' genes from genomic DNA extracted from paradox cultivar Burbank. About 150 primer pairs were tested using gradient PCR, and amplicons specific to cultivar Burbank, but not to cultivar Chandler, were cloned, sequenced and analyzed for similarities to published 'R' gene sequences. Many sequences were screened and four of these showed significant similarities to other sequences in the NCBI BLAST database. Of these, the most promising sequence showed an almost 80% similarity to a published Hazelnut 'R' gene sequence in the database. However, subsequent screening of virus resistant back cross selections and other English walnut varieties did not find the amplified product unique to cultivar Burbank. Future studies will explore alternate strategies to identify the $clrv^R$ gene.

INTRODUCTION

Blackline disease in walnut is caused by the development of a hypersensitive reaction (HR) resulting in the necrosis of the graft union of English walnuts (*Juglans regia*) grafted on black walnuts (*J. hindsii*) or 'Paradox' hybrid (*J. hindsii* x *J. regia*) rootstocks. The HR is due to the resistance of black walnuts to infection by a walnut strain of *Cherry leaf roll virus* (CLRV-W; Mircetich and Rowhani, 1984). It is generally believed that the blackline problem in walnuts can be managed by: (1) planting self-rooted English walnuts, (2) incorporating CLRV resistance to English walnuts, and/or (3) breeding walnut rootstocks to tolerate the virus. Field experiments conducted so far have given mixed results on the use of self-rooted English walnuts versus those grafted on 'paradox' hybrids. Grant and McGranahan (2005) and Hasey et al. (2007) found nut production was lower in self-rooted English walnuts than those on 'Paradox' hybrid. However, Connell et al. (2007) did not find any difference in per tree nut production between self-rooted Chandler trees and those grafted on 'Paradox'. Even if the English walnut trees can perform as well as those grafted on 'Paradox' hybrids, their performance is questionable in areas infested with root lesion nematodes and not much is known about their performance in areas where CLRV is prevalent. Resistance to CLRV-W in *J. hindsii* is governed by a single dominant 'R' gene and it was previously thought that the best approach to managing the blackline problem was to make English walnuts resistant to CLRV-W through repeated backcrosses of *J. regia* with progenies of 'Paradox' x *J. regia* hypersensitive to CLRV-W (McGranahan et al., 1997). However, a breeding initiative started in the 80's has yet to identify varieties suitable for walnut

production. This can be attributed to difficulties in screening the progenies of back crosses and a screening method that relies on grafting bark chips from infected trees (Woeste et al., 1996). To aid the screening activities, a SCAR marker that exhibited 7% recombination with resistance has been identified (Woeste et al., 1996). However, certain traits of *J. hindsii* retained in the progenies have posed additional problems with obtaining lines that produce nuts of acceptable quality.

Based on contemporary research on other crop plants and tree fruits identification of the 'R' gene, $clrv^R$, responsible for CLRV resistance appears to be the best approach. This allows a two prong approach toward management of blackline. A marker for the actual R gene can provide a tremendous boost to the breeding program and can help develop novel rootstocks silenced for resistance by RNAi-mediated approaches. Using degenerate primers, several NBS-LRR class resistance gene candidate sequences have been cloned from a hybrid of *Citrus grandis* and *Poncirus trifoliate* (Deng et al., 2000). Of the cloned sequences, one fragment was found to be associated with resistance to *Citrus tristeza virus* and another was associated with resistance to Citrus nematode *Tylenchus semipenetrans*. Functional characterization of 'R' genes has been demonstrated by gene silencing approaches. Using this approach, an 'R' gene in *Glycine tomentella* that confers resistance to *Phakopsora pachyrhizi*, causal agent of soybean rust, has been characterized (Padmanabhan et al., 2008). Cloning and identification of the 'R' gene is expected to assist the UCD breeding program by aiding PCR-based screening of the progenies of backcrosses. It is also very likely to help generate novel rootstocks that may tolerate CLRV-W infection and thus extend the productive years of walnut orchards in areas where the disease is prevalent. Once this approach achieves the long term objective of identifying the CLRV 'R' gene, it can be extended to identify 'R' genes for other major pathogens of walnut using appropriate sources of resistance.

OBJECTIVE

Amplify 'R' genes in *J. hindsii* using degenerate primers, clone, sequence, and characterize the 'R' gene pool. This is part of our long term objective, which is to identify the $clrv^R$ gene and use this as a marker in our breeding program and to develop strategies for preventing blackline disease.

PROCEDURES

Primers and PCR. Thirty seven degenerate primers were designed by either modifying previously published primer sequences or created based on sequences of known resistance genes (Table 1). Primers were custom ordered from Invitrogen Inc. (Carlsbad, CA). Genomic DNA was extracted from leaves of English walnut cultivars Chandler and Burbank' using a Plant DNAeasy kit from Qiagen Inc. Gradient PCRs, using about 150 different degenerate primer pair combinations, were performed using genomic DNA for the presence of candidate resistance gene sequence fragments. PCR conditions were 1 cycle of 94° C for 3'; 40 cycles of 94° C for 1', 40° C to 60° C for 1', and 72° C for 1'; 1 cycle of 72° C for 5'.

Amplified products were analyzed by agarose gel electrophoresis in TAE buffer. PCR products unique to the genome of 'paradox' hybrid (absent from English walnut) were identified, excised

from the gel, and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA), or PCR products were directly purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, Ca). Purified PCR products were cloned using the TOPO TA pCR2.1 Cloning Kit and OneShot *E. coli* competent cells (Invitrogen Inc., Carlsbad, CA). Recombinant clones were identified on LB agar plates with ampicillin and X-gal and six randomly chosen clones were grown in liquid LB/Ampicillin suspension cultures overnight. Plasmids were purified from *E. coli* cell cultures using the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and cloned products were sequenced.

Bioinformatic analyses. The sequence of the amplified product was subjected to BLAST and TBLAST searches at the National Center for Biotechnology Information, for hits in nucleotide database and translated protein databases, respectively.

RESULTS AND DISCUSSION

In our first attempt to identify $clrv^R$ we screened nearly 150 degenerate primer combinations from a pool of 37 primers specific to 'R' gene homologs of various crop species (Table 1). It is possible that quite a few of the primers may be polymorphic when challenged with walnut genomic DNA and hence a gradient PCR, with annealing temperatures varying from 40 to 60 C, was attempted to increase the efficiency of primer binding. In most of the PCR assays, the amplified products from cultivars Chandler and Burbank genomic DNA were similar when compared. However, when Primer NBSF11, a forward primer modified from a citrus degenerate primer sequence (Deng, et al., 2000) and designed to be complimentary to the nucleotide binding site (NBS) region of an NBS-LRR gene, was paired with primer NBSR5, also designed to compliment the same region (Deng et al., 2000), a unique band of about 750 base pairs (bp) was amplified from 'paradox' DNA (Fig. 1). When primer NBSF11 was paired with GLPLR4, designed to amplify the NBS region of a resistance gene in sugar beet (Tian et al., 2003), a ~1.1 kilo base (kb) band was amplified from Burbank (Fig. 2). We obtained several other products that were unique to cultivar Burbank, including a 500 bp product from primer pair NBSF11 and NBSR1 (both designed to amplify the NBS region of a resistance gene in sugarcane (Glynn et al., 2008). Primers HRT-AT-F5 and HRT-AT-R5 were newly designed in our lab to amplify homologues of members of the HRT/RPP8 resistance gene family from *Arabidopsis thaliana* (Cooley et al., 2000) and produced a unique (~450bp) band from paradox DNA (Fig. 3).

Out of about 20 candidate sequences created from those cloned products, four revealed matches to other sequences when analyzed using the BLAST search function in the NCBI database (Table 2). Of interest was one sequence that had a 78% similarity to an NBS-LRR gene of hazelnut. To confirm the significance of this finding, these primers were used again to check for amplification using DNA extracted from six backcrosses confirmed in our lab to be hypersensitive and six backcrosses confirmed to not be hypersensitive to CLRV. We found that the primers were able to amplify the same 500 bp fragment in both the hypersensitive and non-hypersensitive backcrosses. Therefore, this putative R gene sequence is not a likely candidate for $clrv^R$. It is a little disappointing that only one sequence of significant homology to a putative R gene was found. However, it is possible that the $clrv^R$, might be polymorphic in cultivars Chandler and Burbank and thus the primers might be yielding the same size products. As mentioned earlier, identifying $clrv^R$ would allow the development of gene silenced rootstocks that would be tolerant

to CLRV. Clearly, we may not end up with more potential 'R' gene homologs by this approach and we wish to explore other approaches to identify *clrv*^{R}.

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Table 1. List of primers used for amplifying 'R' gene homologs from English walnut cultivar Chandler and Burbank clone of Paradox.

Primer	Nucleotide sequence	Source (reference)
NBSF1	GGIGGIGTIGGIAAIACIAC	Sugarcane
NBSF4	GGITYIGGIAARACWACIC	(Glynn et al. 2008)
NBSR1	ARIGCTARIGGIARICC	
NBSR2	ARIGCRTARTGCATRAA	
KINF2	TTYGGIWSIGTITAYAARGG	
KINR1	ATICCRAAISWRTAIACRT	
NBSF5	GGTGGGGTTGGGAAGACAACG	*Faba bean, Chickpea*
NBSR3	CAACGCTAGTGGCAATCC	(Palomino et al. 2006)
NBSF6	GGIAAAACRACIYTIGC	Sugarbeet
NBSF9	AGGTAAAACTACTTTGGC	(Tian et al. 2003)
NBSF10	TIHTIGAYGAYGTITGG	
KINF3	GGAGGCATCGGGAAAACAAC	
KINF4	ATHHTHYTICAICAIMG	
KINR2	ATICKRCTICCYTTYTT	
GLPLR1	CCAIACRAGRTCIADIA	
GLPLR2	IACIGCIAGIGGIAGICC	
GLPLR3	AGCCACCAAAGGAAGCCC	
GLPLR4	TGCTAGAGGTAATCCTCC	
TIRR2	RAARCAIGCDATRTGIARIARRAA	
TIRF1	GTIYTRGAYGAYGTIGA	
TIR4	RAAIARISWRCARTAIGCRAA	
CCR1	RTAIAGRAARCAISKYAG	
CCR2	YTICTIGAYGAYGTIGA	
NBSF11	GGDGTDGGIAARACWAC	Citrus
NBSR5	AGIGCHAGICGIAGICC	(Deng et al. 2000)
HRT-AT-F1	GGIATHGGIAARACIAC	*Arabidopsis thaliana*
HRT-AT-F2	GGIYTIGGIAARACIAC	(Cooley et al., 2000)
HRT-AT-F3	GGIWSIGGIAARACIAC	
HRT-AT-F4	GGIGTIGGIAARACIAC	
HRT-AT-R1	RAICGIRAIGGIRAICC	
HRT-AT-R2	RAICGIRAIGGIAARCC	
HRT-AT-R3	CAICGIRAIGGIRAICC	
HRT-AT-R4	CAICGIRAIGGIAARCC	

Primer	Nucleotide sequence	Source (reference)
HRT-AT-F5	GGIATGGGIGGIATHGGIAA	
HRT-AT-R5	TGIRAIRAITACTTYACCCC	
HRT-AT-F6	YTIGARAARYTITGGGA	
HRT-AT-R6	TTYCGICTRCGICTRTT	
HRT-AT-R7	CTYRAIRAIGTYAARRAITT	
HRT-AT-F7	AARTTYGARGGIGGIAA	
HRT-AT-R8	TTYCTYTTYACCCTYKCITT	

Table 2. BLAST Analysis results of sequences from five amplified products unique to 'Paradox' genomic DNA.

Primer pairs	**BLAST search results**
NBSF11 + NBSR5	Some similarity to mitochondrial genome sequence
NBSF11 + GLPLR4	Some similarity to *Vitis vinifera* genome sequence; predicted protease gene
HRT-AT-F5 + HRT-AT-R5	87% similarity to NADH dehydrogenase subunit 5 (mitochondrial genome) of watermelon (*Citrullus lanatus*)
NBSF11 + NBSR1	78% similarity to hazelnut (*Corylus avellana*) NBS-LRR gene

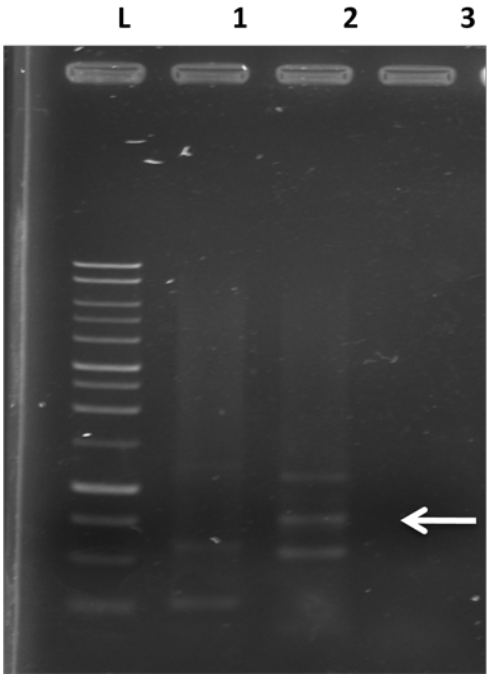

Figure 1. Agarose gel showing PCR products amplified from walnut genomic DNA with forward primer NBSF11 and reverse primer NBSR5. Lanes marked L, 1 kb ladder (Promega Inc); 1, English walnut Chandler; 2, Paradox cultivar Burbank; 3, water control. White arrow points to a unique band (~750bp).

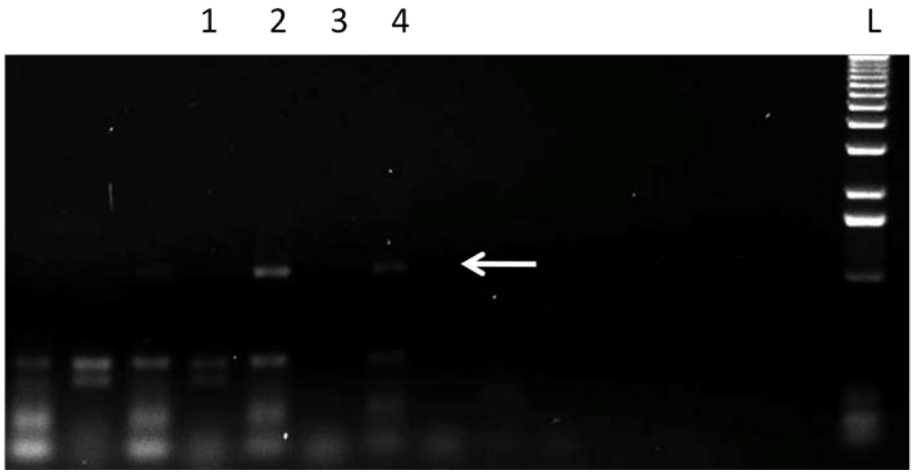

Figure 2. Agarose gel showing gradient PCR products amplified from walnut genomic DNA with forward primer HRT-AT-F5 and reverse primer HRT-AT-R5. Lanes marked 1 & 3; English 'Chandler' walnut; 2 & 4, Paradox clone Burbank; L, 1 kb ladder (Invitrogen Inc). Arrow points to unique band (~1.1 kb).

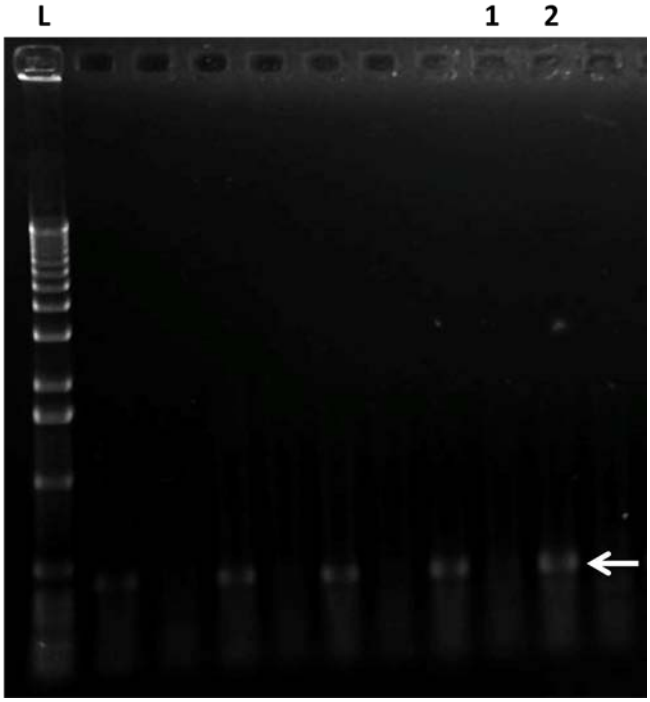

Figure 3. 1Agarose gel showing gradient PCR products amplified from walnut genomic DNA using forward primer NBSF11 and reverse primer GLPLR4. Lanes marked L, 1 Kb ladder (Invitrogen Inc); 1, English walnut Chandler; 2, Paradox clone Burbank; walnut DNA. Arrow points to a unique band (~450bp).