AES/CE MAR 84Workgroup/Department:

University of California
Division of Agricultural Sciences

PROJECT PLAN/RESEARCH GRANT PROPOSAL

Project Year 2009 Anticipated Duration of Project three years

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Project Title: Characterization of R genes involved in resistance to *Cherry leafroll virus* in 'Paradox' hybrids.

Keywords
Commodity(s) walnut, blackline, paradox, Relevant AES/CE Project No.

Problem and its Significance:

Blackline disease in walnut is caused by 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 resistance of black walnuts to infection by the walnut strain of *Cherry leaf roll virus* (CLRV-W; Mircetich and Rowhani, 1984). Decline of mature trees due to blackline is a major problem in California's walnut orchards. The virus is spread from pollen produced by infected trees that land on female flowers of healthy trees in the orchard. Wind helps to disseminate the pollen several miles away leading to infected nuts and spurs (Mircetich and Rowhani, 1986). In a statewide survey conducted in California in 1985, blackline incidence was found in at least 16 counties and the incidence was as high as 54% in Contra Costa County (Reil et al., 1985). Walnut culture in Contra Costa County has been on the decline. In English walnuts, symptoms of CLRV infection are mild and systemic infection may take several years to reach the graft union. Once blackline shows up in an orchard, there is not much a grower can do to prevent the spread of the virus except removing dead trees. Replanting is not an option when CLRV-W infected English walnuts are present in the vicinity. The virus belongs to Genus *Nepovirus* in the Family *Comoviridae*. The members of the Genus *Nepovirus* are generally transmitted by nematode species belonging to *Longidorous* or *Xiphinema*. However, CLRV is not known to have a nematode

vector in California and its spread is primarily through infected nuts and pollen.

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 less in self-rooted English walnuts when compared with 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'. However, 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 by 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 and this can be attributed to difficulties in screening the progenies of back crosses and the 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 to obtaining lines that produce nuts of acceptable quality.

Identification of the 'R' gene, $clrv^R$, responsible for CLRV resistance appears to be the best approach based on contemporary research on other crop plants and tree fruits. 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 it can help develop novel rootstocks silenced for resistance by RNAi-mediated approaches. This proposal is to obtain extramural support for identification of the 'R' gene as an alternative to the use of the SCAR marker. Our ongoing research is intended to clone and identify the avirulence factor in CLRV, the viral protein that actually causes the hypersensitive reaction in *J. hindsii*. This proposal is intended to isolate and characterize the 'R' genes present in *J. hindsii* and to identify the cognate 'R' gene for CLRV by the coexpression of the viral protein that causes HR in black walnuts and the 'R' gene in herbaceous hosts. Once the gene is identified we intend to develop a molecular screening method to identify the backcross progenies with R genes. This approach might help us to trim down the screening time in the backcrossing program and to develop walnut lines with superior nut qualities and resistance to CLRV. In addition, gene silencing approaches can be initiated to modify 'Paradox' hybrids to suit the needs of walnut producers in California.

The genome of CLRV-W is made of RNA-1 and RNA-2, ca. 9000 b and 7500 b, respectively. However, the nucleotide sequence of these RNAs is yet to be determined. It is essential to determine the genome and evaluate individual viral proteins for their ability to initiate HR in black walnuts. Our ongoing work is expected to unravel the viral elicitor of HR.

Higher plants have several 'R' genes and each one of these may help detect diverse biological challenges that the plant has to encounter during its growth and survival. *Arabidopsis thaliana*, a

model plant whose genome has been completely determined, contains about 200 genes with similarities to plant resistance genes and about 75% of these appear to be complete (Meyers et al., 2003). 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 associated with resistance to *Citrus tristeza virus* and another was associated with resistance to Citrus nematode *Tylenchus semipenetrans*). Functional identification 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.

This proposal is expected to meet the requirements for consideration under both 'genetic improvement of walnut scions for CLRV resistance' and 'genetic improvement of rootstocks for tolerance to blackline'.

Objectives:

1. Amplify 'R' genes in *J. hindsii* using degenerate primers, clone, sequence, and characterize the 'R' gene pool.
2. Clone full-length sequences of the putative genes and determine the complete sequence of the 'R' genes.
3. Insert 'R' genes into binary vectors for transient expression.

Plans and Procedures:

Amplification of 'R' genes from *J. hindsii*: Degenerate Primers used for isolating NBS-LRR class resistance genes in citrus (Deng et al., 2000), *Medicago truncatula* (Zhu et al., 2002), and sugar beets (Tian et al., 2004). Walnut genomic DNA will be isolated from suckers of *J. hindsii* trees showing blackline disease. The primers will be ordered from commercial vendors and PCR products will be generated using Elongase enzyme mix (Invitrogen Inc., Carlsbad, CA) and cloned into plasmid vectors. At least ten recombinant colonies will be sequenced for each PCR product and subjected to BLAST analyses.

Identification of 'R' genes associated with CLRV resistance: Once putative 'R' genes are identified, using specific primers, PCR analysis will be done to evaluate the presence of each of these genes in nucleic acid extracts prepared from *J. hindsii*, 'paradox' hybrids, *J. regia*, and selected backcrosses to filter out 'R' genes not associated with CLRV resistance. Subsequently, inverse PCR will be performed to clone the complete gene sequences of the remaining 'R' genes, and analyzed for completeness of the gene.

Identification of CLRV resistance gene: All the 'R' genes that are complete will be inserted into

binary vector for evaluation by agroinfiltration. We hope that at this stage, we will have the genome sequence of CLRV available. The cDNA sequence of the viral elicitor is inserted into another binary vector for transient expression by agroinfiltration. It has been demonstrated that *Nicotiana benthamiana*, a herbaceous plant that can be easily grown and tested under greenhouse or controlled environment conditions, can respond to presence of the matching pair of 'R' gene and the avirulence factor (Takken et al., 2001; Van der Hoorn et al., 2000). The CLRV avirulence factor in combination with the panel of complete 'R' genes will be coinfiltrated into *N. benthamiana* leaves to study development of HR.

Confirmation of CLRV resistance gene: Specific primers for the candidate 'R' gene will be designed and PCR-based analysis will be performed to establish association of the gene in walnut varieties and backcrosses that show HR phenotype with respect to CLRV. Further confirmation will be obtained by RNAi-mediated silencing of the 'R' gene in *J. hindsii* and 'Paradox' hybrids.

The present proposal is to obtain support for Objective 1. Depending on the progress, additional support will be requested for objectives 2 and 3 in subsequent years. The principal investigator is a well-trained molecular virologist with extensive research experience on plant-virus interactions at molecular level.

Deliverables: Information on diverse 'R' genes present in *J. hindsii* and/or 'Pradox' hybrids, and molecular markers for $clrv^R$.

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BUDGET REQUEST Budget Year 2009

Funding Source: California Walnut Board

Salaries and Benefits

Postdocs/RA's (50%)	_____		$___18,000_____
SRA's	_____		$_____
Lab/Field Assistance	_____		$_____
Subtotal		Sub 2	$_____
Employee benefits (33%)		Sub 6	$___5,600_____
		SUBTOTAL	$__23,600_____
Supplies and Expenses		Sub 3	$__ 1,000_____
Equipment		Sub 4	$_____
Travel		Sub 4	$___ 400_____
		TOTAL	$_ 25,000_____

Department account number _____

_____ Date _____
Originator's Signature

COOPERATIVE EXTENSION County Director _____ Date _____

Program Director_____ Date _____

AGRICULTURAL EXPERIMENT Department Chair_____ Date _____
STATION

LIAISON OFFICER _____ Date _____

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