DEVELOPMENT OF NOVEL SOURCES OF RESISTANCE TO 
BEET CURLY TOP VIRUS 
THROUGH VIRUS-INDUCED GENE SILENCING

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In recent years curly top disease, caused by Beet curly top virus (BCTV), re-emerged in California, resulting in significant economic losses for sugarbeet production in the San Joaquin Valley. Curly top has affected California agriculture for over a century, and no cost effective control methods have been developed that effectively and reliably prevent losses. During the summer of 2001, Beet curly top virus (BCTV) reemerged as an important, economically damaging pathogen of sugarbeet, tomato, and pepper throughout widespread areas of the western United States. These areas included California, the Snake River Valley of Idaho and the southwestern desert of west Texas and New Mexico. More recently curly top has been problematic in the Rocky Mountain region. The wide host range of BCTV, abundance of the beet leafhopper vector (Circulifer tenellus), and increasing acreage of uncultivated land in some areas is making curly top management increasingly difficult. The present California management strategy focuses on the large-scale use of insecticides to control the leafhopper vector in rangeland, and the use of insecticidal treatments on crops.

In an effort to control the beet leafhopper, and indirectly BCTV, California growers (all affected crops) pay $1.5 million annually (2004 figures) for the spraying of 80,000-200,000 acres of uncultivated land with insecticide. The insecticide applications are directed at the overwintering breeding hosts (annual and perennial weeds) of the leafhopper to decrease the spring populations of the vector. Many California growers have become heavily dependent on the spray program. Although it is somewhat difficult to measure the efficacy of the insecticide treatments, this control measure is thought to work well in certain years and locations, and be inadequate in others. This was demonstrated in 2001 and 2003, when in spite of the heavy application of insecticide to leafhopper overwintering grounds, leafhopper populations and curly top disease incidence reached levels not seen for over a decade. Such outbreaks occur periodically and usually continue for a few years as was observed this decade, depending largely on environmental conditions, but also influenced by available weed and crop hosts, cropping patterns and management practices to name a few. Since the leafhopper vector needs only a brief feeding interval to introduce the virus into a healthy plant, treating sugarbeet with insecticides will not effectively block virus transmission, but may reduce overall numbers of leafhoppers.

The inability to manage curly top through traditional means necessitates the use of novel approaches, including molecular genetics. These methods have shown promise with related viruses in other hosts, and should be effective for curly top in sugarbeet as well. New advances in technology are leading to approaches that may ultimately be useful for biotechnology-based control. It is in the best interest of the sugarbeet industry to explore new avenues for virus control and prevention, as this may ultimately reduce reliance on chemical control of the beet leafhopper, and lead to effective management of a virus that has been a chronic problem for over a century.
Many plants induce a natural process known as virus-induced gene silencing (VIGS) upon infection by viruses. VIGS causes selective, specific degradation of viral genome sequences, as well as any additional sequences inserted into it. This can occur either during or after production of RNA. A number of different structural features on nucleic acids have been implicated as possible triggers. These include abnormal double stranded RNA molecules (double stranded RNA is something that is produced during RNA virus replication), tandem insertions of the same DNA sequence, and specific structural features on the nucleic acids to name a few. VIGS can initiate even in the first cell the virus infects, preventing whole plant infection, and in many transgenic systems it has been demonstrated that the silencing signal can be transmitted systemically throughout the plant. Recent studies have shown that when small pieces of DNA corresponding to a target gene present in transgenic plants are blasted into a leaf (using a device called a gene gun that essentially shoots DNA into a leaf), systemic silencing (suppression) of the target gene can be detected 2 to 3 days after bombardment. Even though the small pieces of DNA that were delivered by the gene gun only occurred in a few cells, the target gene was suppressed throughout the plant. Although most studies on gene silencing have been done with RNA viruses, silencing also occurs with DNA viruses. BCTV is a DNA virus, however it does produce RNA as a template for synthesis of virus proteins. Recent studies indicated that silencing based approaches have been effective for other geminiviruses (BCTV is also a geminivirus), such as African cassava mosaic virus and Tomato yellow leaf curl virus. As a result, it may be possible to develop methods to suppress infection by BCTV using similar approaches and a common virus-based vector for delivery of constructs to plants for testing.

Our initial goal is to develop strategies for control of BCTV in sugarbeet using VIGS. Ultimately we wish to develop methodologies that will allow this system to be delivered to plants using both traditional plant transformation and using alternate methodologies that might be effective even without the development of GMO sugarbeet; however, the first step is to demonstrate the effectiveness of the method for controlling this important viral disease. Progress has been made demonstrating that two genetic constructs can reduce severity of curly top in the model host, Nicotiana benthamiana when treated 3 days prior to curtovirus inoculation. One construct appears to exhibit nearly complete control of Beet mild curly top virus (BMCTV) if sufficient time for activation of gene silencing occurs prior to exposure to BMCTV. Success for control of Beet severe curly top virus (BSCTV) was less impressive, but results suggest only minor modifications may be needed to achieve complete control of both viruses. Additional constructs are in development with further testing of new/modified constructs anticipated by summer 2007.

Genetic constructs for control of the two predominant curtoviruses in North America (BSCTV and BMCTV) were designed based on viral sequences critical to virus replication and host infection, and include structures demonstrated to be effective inducers of gene silencing. The silencing constructs designed to date target sequences involved in virus replication. Studies concluded in 2006 tested 3 types of constructs, including; a small positive strand sequence, a small negative strand (or antisense) sequence, and a construct producing a structure known as a hairpin. Results demonstrated some control with both the hairpin and the antisense constructs, but no differences from untreated controls with the positive strand construct. Although both the hairpin and antisense constructs reduced severity of curly top symptoms on Nicotiana
*benthamiana* (experimental host commonly used for studies such as these), control was not sufficient. As a result, we obtained a different gene delivery system based on *Tobacco rattle virus* (TRV) from Yale University. The TRV vector was specifically designed for use in members of the *Solanaceae*, our model system for identifying constructs that induce gene silencing. Our previous systems were known to work on solanaceous hosts for other types of testing, but had to be adapted for the specific needs of this project, whereas this TRV system was expected to be more suited to our studies and would hopefully provide more complete control, rather than the partial control observed during Year 1 of this project. We inserted our two previously successful constructs, BMCTV hairpin and BSCTV antisense, into the new vector with only minor sequence modifications. These are now known as pTRV-hp25 and pTRV-CFHC1, respectively. The first set of tests with these constructs was completed in early November 2006 and results were extremely promising.

Plants were inoculated with the TRV vector carrying either a “hairpin” construct designed against BMCTV in which the inserted sequence assembles in the form of a hairpin, or an “antisense” construct, in which the inserted sequence consists of the reverse complement of a segment derived from the replication associated protein of BSCTV. Three days following treatment, untreated plants and plants treated with either of the two constructs were inoculated with either BSCTV or BMCTV. Symptom development (leaf curling, twisting, discoloration and stunting) was observed over a period of 5 weeks, and plant height was determined as an indicator of infection severity. Results indicate that in some cases, control was dramatic and highly effective, while in others it was less effective. Much of this depended on the similarity between the construct used for control and the inoculated virus. Both constructs were most effective on the virus from which the resistance construct was derived and less effective against the divergent virus. The construct pTRV-HP25 was the most effective overall. With this construct, resistance appeared to be nearly complete in some treated plants inoculated with BMCTV, with minimal symptom development and very little stunting. In other plants, control was not as dramatic, suggesting that perhaps silencing was not activated in some plants by the time virus replication began accelerating. There was very little difference in plant growth between pTRV-hp25 silenced plants and untreated controls, as well as few symptoms on silenced pTRV-HP25-treated plants, whereas untreated plants inoculated with BMCTV exhibited severe stunting and leaf curling. Importantly, we were unable to detect BMCTV in BMCTV-inoculated plants treated with pTRV-HP25, whereas plants inoculated with BMCTV, but not treated with pTRV-hp25 contained high levels of virus, based on serological virus detection tests (ELISA). Control of BSCTV with the pTRV-HP25 was not effective. In order for control to be effective against both curtovirus species it will most likely be necessary to design a similar construct to a region of the viral genome that is conserved between the two primary curtovirus species affecting agriculture in California.

Treatment of plants with pTRV-CFHC1 also showed some promise, but control was not as impressive as for the pTRV-HP25. Treatment of *N. benthamiana* 3 days prior to inoculation with BSCTV led to some decrease in stunting severity, but this was incomplete. Plants still developed substantial symptoms, but were not as severely affected as untreated plants. Like the results observed for pTRV-HP25, it appeared that silencing was not effectively activated in all plants, since some plants uniformly exhibiting reduced symptom severity. Unlike the results of pTRV-HP25 on BMCTV infection described above, we were still able to detect BSCTV in
BSCTV-inoculated plants treated with pTRV-CFHCl (data not shown). This is not surprising, since the silencing was only partially effective with this construct. As with pTRV-HP25, effectiveness of pTRV-CFHCl was most effective against the source of origin for the transgene, in this case, BSCTV. Little difference was observed between treated and untreated plants for pTRV-CFHCl inoculated with BMCTV, indicating control was most effective against the viral source of the control sequence. This experiment was repeated in Feb-March 2007, however resistance was not as strong as in the initial experiment. We are pleased with the success to date, and anticipate the next several months should allow us to follow these results with additional constructs that may be effective against both major curtovirus species.

Based on the success of the studies to date, we are developing new constructs using sequences conserved between both of the predominant forms of curly top, BSCTV and BMCTV. Since the constructs seem to work well against viruses from which the construct sequence was derived, and less well against the virus from which it was not derived, even though the two are fairly close in sequence similarity, we are focusing on areas within the same gene sharing near sequence identity between both BSCTV and BMCTV. We anticipate that the new constructs will be completed by summer 2007. Hopefully these new constructs will be effective not against one, but against both viruses responsible for curly top in the western U.S.

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