RICE CRC

FINAL REPORT

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Title of Project :	Bloodworm resistant rice
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Summary

The aim this project was to determine if it was feasible to control bloodworm infestations in rice using transgenic plants expressing an insecticidal toxin. Without control bloodworm can cause massive damage to the plant stand, up to 85% plant loss in years of high infestation. Currently bloodworm are controlled through the use of chemicals applied at the time the paddy is planted. The advantage of a transgenic plant approach is that it will eliminate, or at least severely reduce, the need for pesticide application and the consequent impact on beneficial insects and animals. Avoidance of pesticide application will improve water quality.

We have shown that the insecticidal toxins from *Bacillus thuringiensis israelensis* (Bti) are toxic to bloodworm. Whole *Bti* was highly toxic to bloodworm with an LC_{50} of 45ng of toxin per ml of medium, which is a far greater toxicity than that of other Bt strains for their target insects. I have cloned the individual toxin genes from Bti and expressed five in Bt (*cry4A, cry4B,cry10A, cry11A* and *cyt1A*) and the relative toxicities of the individual toxins and their mixtures were determined. The most toxic protein was Cry11A, closely followed by Cry4B, with LC_{50}s of 550 and 980 ng toxin per ml, respectively.

After the identification of *cry11A* as a candidate gene, a construct was made to transform rice and test the expression of the native coding sequence. One callus line (of twenty) was found to contain detectable quantities of Cry11A protein. However, once regenerated into a transgenic plant this line does not express the toxin well. This was expected because native Bt *cry* genes are not well expressed in plant tissue because of the differing codon usages by Bt and rice and potential negative acting elements of the gene itself when expressed in another organism. A synthetic rice optimised gene was designed to increase the amount of protein produced within rice plants and so obtain plants with enough toxin production to be insecticidal.

The synthetic gene was coupled to a strong constitutive promoter and transformed into rice to ascertain if sufficient expression could be obtained to kill bloodworm. Rice transformation was performed on callus, which is an undifferentiated lump of rice cells not capable of survival outside tissue culture. Ten transgenic callus lines were bioassayed at Yanco. All of the calli had at least some activity against bloodworm and two look to have very good levels of activity against bloodworm. This is significant for several reasons:

The expression levels achieved are able to kill bloodworm, meaning that it is possible to express enough of the toxin to be lethal to insects. The resynthesised gene retains the toxicity of the parental gene.

The large number of lines that are demonstrating toxicity indicates that even moderate expression *in planta* enables control of bloodworm at least in the laboratory. This gives a greater range of options for deciding on the level of expression acceptable in the field.

The speed at which the insects die implies that they stop feeding quickly. In some insecticidal plants it takes days to stop feeding and die, in this time they are able to do considerable damage to the plant. With a rapid acting toxin, it is more likely that the damage caused between feeding and death will be minimal.

The demonstration of bloodworm activity is a major step forward and we are now in a strong position to continue the project, Plants expressing Cry11A have been generated and demonstrated to have high level bloodworm activity. All insects exposed to the Cry11Asyn transgenics were killed. A root specific promoter has been cloned and used to drive the expression of Cry11Asyn. Root specific expression is seen as a desireable characteristic because it limits the expression of the transgene in seed and therefore peoples exposure to Cry11A protein. Genes with potentially interesting expression profiles were identified through EST expression data in public databases, the expression pattern and level were experimentally determined using real time PCR and subsequently cloned. Expression analysis will be performed once plants are generated.

1. Background

Bloodworm (*Chironomus tepperi*) are a non biting midge that colonise ephemeral water pools. The adult bloodworm are large for Midge's and able to travel long distances to lay eggs in new pools of water, *C. tepperi* does not lay eggs in water that has had prior colonisation of bloodworm or to a lesser extent mosquitoes. Without control bloodworm can cause massive damage to the plant stand, up to 85% plant loss in years of high infestation. Currently bloodworm are controlled through the use of chemicals applied at the time the paddy is planted as it can be assumed that bloodworm eggs are present when the field is planted. The advantage of a transgenic plant approach is that it will eliminate the need for pesticide application and the consequent negative impact on beneficial insects and animals. Avoidance of pesticide application will improve water quality and eliminate concerns over the movement of pesticides through the water table and prevent OHS issues associated with the handling of chemical concentrates prior to application.

2. Objectives

Reduce the Rice industries reliance on chemical based insect control with the development of bloodworm resistant rice

3. Introductory technical information concerning the problem or research need

Bacillus thuringiensis is a bacterium that has had a long history of use as a biocontrol agent. It has been proven safe specific and effective for more than fifty years.

4. The Methodology

Bloodworm (Chironomus tepperi) are nematoceran diptera that belong to the same group as mosquitoes and blackflies. After paddy flooding bloodworm lay their eggs in the water and the larvae eat the roots of the rice seedlings during the early part of establishment. The root feeding behaviour of the bloodworm results in root damage and subsequent plant drowning due to lodging of the seedlings. Rice plants that are able to resist the attack of bloodworm in the field would be of large economic and social benefit to the community as no chemicals would need to be applied to the crop and no secondary effects of groundwater leaching of chemicals or exposure of workers to chemical concentrates would occur.

Bacillus thuringiensis produces "Crystal" or "Cry" toxins at a particular stage of growth and formulations of that stage are used to kill insects. *Bacillus thuringiensis israelensis* (Bti) is an effective biocontrol agent for the control of both blackflies and mosquitoes which are closely related to bloodworm. This was the starting point for the project to determine if Bti was toxic to bloodworm and what, if any, toxins it produced were toxic to bloodworm. Bti was found to be highly toxic to bloodworm (Hughes, Stevens, et al. 2005, Stevens, Akhurst, et al. 2004) and that Cry11A was the most active component of the toxin mixture present in Bti.

Once Identified as a target Cry11A was used to generate GM plants and their effectiveness tested.

Literature

Hughes, P. A., Stevens, M. M., Park, H. W., Federici, B. A., Dennis, E. S.Akhurst, R. 2005. Response of larval Chironomus tepperi (Diptera: Chironomidae) to individual Bacillus thuringiensis var. israelensis toxins and toxin mixtures. J Invertebr Pathol. 88(1), 34-9.

Stevens, M. M., Akhurst, R. J., Clifton, M. A.Hughes, P. A. 2004. Factors affecting the toxicity of Bacillus thuringiensis var. israelensis and Bacillus sphaericus to fourth instar larvae of Chironomus tepperi (Diptera: Chironomidae). J Invertebr Pathol. 86(3), 104-10.

5. Detailed results

We have shown that the insecticidal toxins from Bacillus thuringiensis israelensis (Bti) are toxic to bloodworm. Whole *Bti* was highly toxic to bloodworm with an LC_{50} of 45ng of toxin per ml of medium, which is a far greater toxicity than that of other Bt strains for their target insects. There are four crystal toxin genes in Bti (*cry 4A, 4B, 10A* and *11A*) and two non-specific cytolytic toxin genes (*cyt1A* and *cyt2B*); of these genes, all except *cry10A* and *cyt2B* are expressed in the native strain.

I have cloned the individual toxin genes from Bti and expressed five in Bt (*cry4A, cry4B,cry10A, cry11A* and *cyt1A*) and the relative toxicities of the individual toxins and their mixtures were determined. The most toxic protein was Cry11A, closely followed by Cry4B, with LC_{50}s of 550 and 980 ng toxin per ml, respectively. Mixtures of the toxins showed little interaction; this was unexpected, as in mosquitoes there is significant synergism. The interaction between Cry11A and Cry4B was slightly antagonistic, and there is some synergism between Cry4A and Cyt1 however this is at a level of toxicity 100fold less than that of whole Bti.

A construct was made to express the native Cry11A gene in rice and transgenic (GM) callus made. No expression of Cry11A could be detected by any means, Bt genes can be difficult to express in plants because bacteria do not process RNA in the same way plants to nor do they use the same group of codons to translate the RNA into proteins. The differences in how RNA is used in Bt and plants can cause the misreading of the bacterial type RNA in plants and lead to very poor expression. To correct the misinterpretation of the Cry11A gene it was resynthesised to make it an optimal gene for expression in rice, almost every base in the entire gene was changed while keeping the same amino acid sequence. The new gene Cry11Asyn was transformed into rice in the same manner as the native gene, however the RNA from the gene was detectable using Real time PCR and the callus was highly toxic to bloodworm. Plants were generated from the Cry11A callus and these plants were tested in Canberra for their ability to resist bloodworm feeding see figure 1.

Figure 1.

From the figure it is easy to see that the Cry11A expressing plant suffers no damage when bloodworm feed on the seedlings. Behavioural changes are apparent within an hour of the insects beginning feeding and bloodworm death usually follows within 48Hrs.

The promoter used to drive the expression of Cry11A in Figure1 is a strong promoter that drives expression throughout the plant. This is not ideal for industry use as considerable Cry11A production is found in the mature seed. Various genes were analysed for expression pattern in rice to determine if a strong promoter with significant root activity and little seed activity could be found. Two genes were found with greater than 50 fold higher expression in the root than the seed. The promoter and first intron of a metallothionein variant 3 has been cloned and used to drive the expression of Cry11A. Plants from this construct have not yet been analysed.

6. Discussion

The objective of this research was to produce rice capable of withstanding feeding damage from bloodworm. Although the native Cry11A gene is poorly expressed in rice plants, after extensive modification a synthetic rice optimised gene Cry11Asyn was transformed into rice. Rice plants expressing Cry11Asyn are totally protected against attack from bloodworm. In most GM plants expressing Cry genes from Bt the insect continues feeding for a considerable period of time before feeding stops, in our GM rice no damage is visible to seedlings at all and bloodworm are visibly distressed with 1-2hrs after exposure. The quick cessation of feeding is beneficial as little damage occurs in this time and suggests that the effectiveness of the plant is very high. It is highly likely that the appropriate expression of Cry11A in a rice plant would lead to the effective control of bloodworm in the field.

7. Implications and recommendations

Rice plants expressing Cry11A are capable of controlling bloodworm effectively. The implication is that bloodworm resistant rice is feasible. Although searches for allergenicity are negative for Cry11A and significant safe use history exists for Bti, allergen and toxicity testing would need to be carried out before commercial release would be possible. Optimisation of the GM rice plants to lessen the expression of the gene in the endosperm would also be a desirable characteristic and would require further exploratory research with a high probability of success. The gene that was introduced into rice was a synthetic gene modified for increased expression in plants, for Bt genes this process has been patented by several companies who currently are fighting in various courts to determine the ownership of the modification process. It is highly likely that royalties would need to be paid to one of these companies for “use” of the modified gene.

On current estimates of cost of control for insects in rice fields, about $2-2.5million are spent each year on bloodworm control (in a full non drought affected season). At this rate 6-8 years whole industry control money would be needed to commercialise Cry11A rice. Significant environmental and OHS safety benefits exist for crops with no insecticidal applications and it is not currently feasible to put a dollar value on the benefits.

8. A description of the Project Intellectual Property and of any commercially significant developments arising from the Project

No protected intellectual property has been generated in the process of this project and all non protected material has been published or is in the process of being published.

9. A dissemination strategy and communications or extension plan for the Project Intellectual Property

Several articles have already been published as part of this project and others are in the final stages of writing. No further action is planned for the dissemination of information as it is freely available.