EVALUATION OF AN ANTIBACTERIAL PEPTIDE (CECROPIN A) AS A RESISTANCE AGENT IN PLANT XYLEM AGAINST *XYLELLA FASTIDIOSA*

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ABSTRACT

Cecropin A (Gudmundsson, et al., 1991) is a bactericidal peptide that is a potential source of resistance against *X. fastidiosa*, the causal bacterium of Pierce's disease. *In vitro* assays in our laboratory demonstrate that cecropin A is bactericidal against *X. fastidiosa*. We are utilizing the model plant, *Arabidopsis thaliana*, to evaluate the feasibility of using cecropin A expressed in transgenic plants as a resistance agent in plant xylem against *Xylella fastidiosa*. Because *X. fastidiosa* is a xylem-limited bacterium (Purcell and Hopkins, 1996; Hopkins, 1989), xylem-specific expression of cecropin A is required for effective resistance to Pierce's disease. To achieve our ultimate goal of producing grape rootstocks that confer Pierce's disease resistance to both the rootstock and the grafted scion, it will be necessary for cecropin A to circulate in the xylem vessels. To obtain expression of cecropin A in plant xylem, we are using a signal peptide sequence taken from a protein that naturally occurs in plant xylem. Glycine-rich proteins that are localized in plant xylem (Keller, et al., 1989; Morvan, et al., 2003) have been isolated and characterized (Sakuta and Satoh, 2000; Le Provost, et al., 2003). We are testing the rice glycine-rich protein signal peptide sequence, which confers vascular-specific expression in transgenic plants (Liu, et al., 2003), to determine if it will be effective to target cecropin A to plant xylem.

INTRODUCTION

In early October 1999, University of California President Richard C. Atkinson established a task force of experts to help find solutions to Pierce's disease (Report of the Pierce's Disease Research and Emergency Response Task Force). Members of the Task Force believe that disease resistance, over the long term, offers the only sure protection for grapes from the ravages of the *X. fastidiosa* bacterium. The Task Force reported, however, that there is insufficient time for conventional plant breeding practices, which could take 20 years or more to breed resistance into grapes. Thus, the application of genetic engineering and other biotechnological techniques to insert disease-resistance genes into plants will be required.

By conventional breeding, it would be nearly impossible to produce varietals that are identical in every way to popular varietals such as Chardonnay, Pinot Noir, and Cabernet Sauvignon, except for one difference, resistance to Pierce's disease. Even by genetic engineering, it will be extremely difficult to produce Pierce's disease-resistance varietals that are identical to the currently most popular varietals in every other way but resistance. Thus, a strategy that is more likely to produce a consumer friendly result will be to generate a new rootstock that is Pierce's disease-resistant, and that also confers resistance to the grafted scion. This will require the identification and examination of Pierce's disease-resistance genes that can be introduced into rootstocks either by genetic engineering or by conventional breeding. These genes will have to encode gene products that will confer resistance not only to the rootstock, but also to the scion that is grafted onto it.

Cecropin A (Gudmundsson, et al., 1991) is a bactericidal peptide that is a potential source of resistance against *X. fastidiosa*, the causal bacterium of Pierce's disease. *In vitro* assays in our laboratory demonstrate that cecropin A is bactericidal against *X. fastidiosa*. To achieve our ultimate goal of utilizing cecropin A as one component of a multigenic approach to developing useful grape cultivars with resistance to *X. fastidiosa*, we will need to clear several preliminary hurdles. The first obstacle, demonstrating that cecropin A indeed has bactericidal activity versus *X. fastidiosa*, has already been accomplished *in vitro* in our laboratory.

The second step toward our goal will be to successfully express cecropin A in plants. Several bactericidal peptides, including cecropin B, have already been expressed in plants. Transgenic plants expressing cecropin B exhibit enhanced resistance to bacterial pathogens (Jaynes, et al., 1993). However, our *in vitro* studies indicate that cecropin A, rather than cecropin B, has greater bactericidal activity versus *X. fastidiosa*. Therefore, we are currently producing transgenic plants to express cecropin A. We are producing transgenic *A. thaliana* plants rather than immediately transforming grapevines because it is much faster to produce transgenic *A. thaliana* compared to the time that it would take to produce transgenic grapevines.

Expressing antibacterial peptides in grapevines is likely to provide resistance versus *X. fastidiosa* only if the expression products are correctly targeted to xylem tissues. Therefore, we are conducting several experiments to determine if the promoter, signal peptide, intron, antibacterial peptide, and transcription terminator combination that we have chosen will be successful to express active antibacterial peptide in the xylem of a model plant that is easily and rapidly transformable. Once we have succeeded in expressing an active antimicrobial peptide in xylem, then we will have the confidence to proceed to the next step in producing transgenic grapevines that are resistant against *X. fastidiosa*.

Another major hurdle will be to develop systems for expression of cecropin A in plant xylem. Because *X. fastidiosa* is a xylem-limited bacterium (Purcell and Hopkins, 1996; Hopkins, 1989), xylem-specific expression of cecropin A will be required for effective resistance to Pierce's disease. To achieve our goal of producing grape rootstocks that confer Pierce's disease resistance to both the rootstock and the grafted scion, it will be necessary for cecropin A to circulate in the xylem vessels. To obtain expression of cecropin A in plant xylem, we are using a signal peptide sequence taken from a protein that naturally occurs in plant xylem. Glycine-rich proteins that are localized in plant xylem (Keller, et al., 1989; Morvan, et al., 2003) have been isolated and characterized (Sakuta and Satoh, 2000; Le Provost, et al., 2003). We are testing the rice glycine-rich protein signal peptide sequence, which confers vascular-specific expression in transgenic plants (Liu, et al., 2003), to determine if it will be effective to target cecropin A to plant xylem.

OBJECTIVES

1. Express cecropin A in plants.
   A. Utilize *Agrobacterium tumefaciens* to transform *Arabidopsis thaliana* with a cecropin A gene.
   B. Assay putative transgenic plants via PCR, Southern blots, and western blots to verify the foreign gene insertion and expression.
2. Assay plants expressing cecropin A to determine their resistance versus plant pathogenic bacteria.
   A. Assay transgenic plants expressing cecropin A versus the bacterial plant pathogen *Pseudomonas syringae* pv tomato (Pst).
   B. Assay extracts of transgenic plants expressing cecropin A to determine if they have enhanced bactericidal activity versus *X. fastidiosa*.
3. Develop systems for xylem-specific expression of cecropin A in plants.
   A. Transform *A. thaliana* with a gene construct linking a cecropin A gene with the rice glycine-rich protein signal peptide sequence (GRP).
   B. Assay putative transgenic plants via PCR, Southern blots, and western blots to verify the foreign gene insertion and expression.
   C. Assay xylem sap and xylem tissues from transgenic plants to determine if the GRP has directed the foreign protein to be expressed in xylem.

RESULTS

For transformation of the model plant, *A. thaliana*, the sequence of the cecropin A gene from the Giant Silk Moth (*Hyalophora cecropia*) was codon modified (Perlak, et al., 1991) to conform with the codon usage of *A. thaliana*. Two plant transformation plasmid vectors, pCAMBIA1305.1 and pCAMBIA1305.2, (Roberts, et al., 1998) were modified by replacement of the GUSPlus gene with either a codon-modified mature cecropin A gene, or a codon-modified pro cecropin A gene (Figs. 1, 2). The plasmids pCAMBIA1305.1 and pCAMBIA 1305.2 are similar, except that the pCAMBIA1305.2 contains a rice glycine rich protein signal peptide. The plasmid construct with pCAMBIA1305.2 is made such that the cecropin A gene product will be fused with the rice glycine rich protein signal peptide sequence for targeting to the xylem. The transformation of *A. thaliana* with these plasmid constructs is currently in progress (Clough and Bent, 1998).

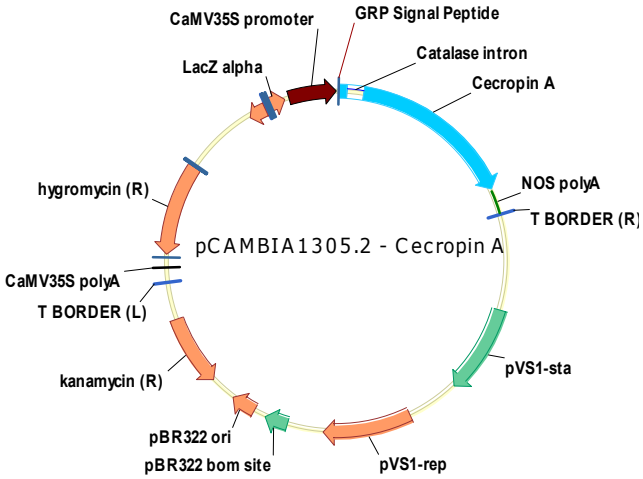

Figure 1. pCAMBIA 1305.1 plasmid vector containing Cecropin A gene.

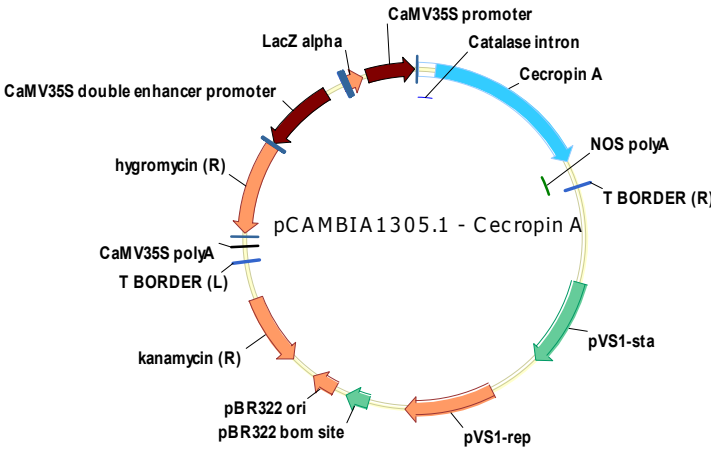

Figure 2. pCAMBIA 1305.2 plasmid vector containing Cecropin A gene.

In developing a model system for testing foreign gene constructs as resistance agents versus *X. fastidiosa*, it will be very useful if a *X. fastidiosa* isolate can be identified that will develop systemic infections in *A. thaliana*. Therefore, we inoculated 50 *X. thaliana* seedlings with a Temecula grape isolate of *X. fastidiosa*. For negative controls, 25 seedlings were

inoculated with *Escherichia coli*, which is not expected to infect *A. thaliana*, and 25 seedlings were mock inoculated with PD3 bacteriological medium (Campanharo, et al., 2003). They will be assayed by ELISA and PCR for the development of systemic infections.

CONCLUSIONS

Our recent laboratory results reveal that cecropin A has bactericidal activity *in vitro* against *X. fastidiosa*. Our project will test the antibacterial activity of cecropin A in transgenic plants. This is a preliminary step to determine if cecropin A would be a good candidate for expression in transgenic grapevines as a resistance agent versus *X. fastidiosa*. Expression strategies that result in xylem-specific expression are desirable. An antibacterial peptide produced in a grape rootstock that circulates in xylem would be ideal, because it would have great potential to allow the grafted scion to also be resistant to *X. fastidiosa*, without having to alter popular grape varietals.

We expect that cecropin A expressed in plants will have antibacterial activity. GRP signal peptide has already been demonstrated to direct foreign gene expression into vascular tissues (Liu, et al., 2003). We expect that the GRP signal peptide will also be effective for directing cecropin A into xylem, and that the cecropin A will circulate in xylem vessels. The confirmation of this hypothesis is one of the major goals of this project. If the answers are positive, then we would be confident to recommend cecropin A as an excellent candidate for production of grape rootstocks with resistance versus *X. fastidiosa*. For purposes of resistance management, we would recommend that cecropin A be one component of a multigenic resistance strategy.

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