Phytophthora endoglucanase and a Corresponding Inhibitor Protein

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ABSTRACT

Phytophthora infestans, causal agent of late blight, remains a poorly understood pathogen of potato and tomato. New genotypes or strains arise with increasing virulence in areas where these crops are cultivated. Cultivated varieties of potato and tomato are all susceptible to late blight, but the degree of susceptibility can differ. We have focused on early events in the late blight disease that may contribute to pathogen virulence and levels of host susceptibility. Rapid disintegration of host tissue suggests that cell wall modifying enzymes are an essential factor in disease. Clones for a unique family 12 xyloglucan-specific endoglucanase (XEG) were identified and characterized. The gene encodes a 22 kD extracellular protein that can specifically target the xyloglucan component of the host cell wall. Activity of this enzyme is high during disease progression, as measured by remazol blue-xyloglucan hydrolysis. The gene is present in three copies in the *P. infestans* genome, and provides the first example of a pathogen-encoded family 12 XEG. Family 12 XEGs are known to be inhibited by a specific inhibitor protein. A gene encoding an inhibitor protein that binds specifically to the Phytophthora XEG was identified in potato. The gene is wound responsive and encodes a 43 kD extracellular protein. Overexpression of the XEG inhibitor protein gene from potato, using a Potato Virus X vector, resulted in a marked reduction in the rate of Phytophthora lesion expansion on susceptible potato. Levels of expression of this inhibitor protein may contribute to the reduced susceptibility found in some cultivars and germplasm of potato.

Key words: *Phytophthora infestans*, Late blight, Potato, Tomato, Endoglucanase.

INTRODUCTION

Late blight, caused by *Phytophthora infestans*, continues to be the primary disease in limiting of potato and tomato production. Among the options for control, deployment of resistant germplasm provides the strongest sustainable management practice. Two types of resistance are available, race specific resistance (R) genes, and race-nonspecific genetic mechanisms. While the mechanisms of race non-specific resistance have not been elucidated, resistance has been evaluated as a reduction in rate of lesion expansion. We have initiated studies on the role of host-encoded inhibitor proteins in limiting late blight lesion expansion rates, and the possibility that they contribute to the non-race specific form of resistance. We are specifically investigating proteins that may inhibit extracellular enzymes produced by *Phytophthora infestans*.

Recently a new type of inhibitor was identified from tomato that interacted with a Family 12 xyloglucan-specific endoglucanase. The inhibitor protein, with structural similarity to an inactive form of protease, binds tightly to the Family 12 endoglucanase structure (York et al., 2004) resulting in blockage of the catalytic sites of the endoglucanase. The cellular target of the Family 12 endoglucanases is xyloglucan. The xyloglucans play a critical role in plant cell wall structure by linking the cellulose microfibrils together (Hayashi 1989). It could be expected that xyloglucan hydrolysis would lead to a loosening of the host cell wall structure. This was demonstrated for a Family 12 endoglucanase, where cell wall extension was activated by enzyme treatment (Yuan et al., 2001). Cell wall loosening may facilitate entry by cell wall modifying enzymes and could also lead to membrane instability and water soaking symptoms.

MATERIALS AND METHODS

Identification of family 12 endoglucanase-encoding genes

Family 12 endoglucanases share short regions of conserved amino acid sequence, such as NNLW. Surveying the available EST information for Phytophthora, potential sequences were identified through translational BLAST searches. Genomic DNA and total RNA samples were isolated using DNAeasy and RNAeasy kits from Qiagen. Primers covering the 5′ and 3′ coding regions were used in PCR reactions with PlatinumTaq polymerase, using genomic DNA as a template. Total RNA was also used as a template, following cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen). Products were cloned into TOPO TA 2.1 and subsequently sequenced.

Total DNA was digested with *Eco* R1 or *Bam* H1, electrophoretically separated and transferred to Hybond nylon membrane. Fluorescein-labelled probe derived from the clone was used to determine copy number in the *P. infestans* genome.

Identification and cloning of the inhibitor protein gene

Using the tomato xyloglucan-specific endoglucanase inhibitor (XIP) gene as a reference (Qin et al., 2003), ESTs were identified from potato using information from GenBank and TIGR databases. Analysis of current database information resulted in an EST that was lacking two nucleotides at the 5′end, which after incorporation, provided the full coding sequence. Forward and reverse primers were added to a PCR reaction mix and amplified with PlatinumTaq polymerase (Invitrogen). The single product obtained was cloned into the TOPO TA cloning vector 2.1 and the cloned product was sequenced.

Over-expression of XIP.

To test the role of the inhibitor protein on infection and spread of *Phytophthora infestans*, the coding region for XIP was introduced into a Potato virus X vector (Faivre et al., 2004). The XIP gene was amplified with primers containing *Nru 1* and *Sal 1* sites and recloned into TOPO TA 2.1 vector. Insert was removed by digestion and

placed into the PVX vector. Linearized PVX vector was used to generate infectious transcripts for inoculation onto *Nicotiana benthamiana*. After propagation for one week, virus was transferred from tobacco to six week old potato (*Solanum tuberosum* cvs. Kennebec and Green Mountain) by mechanical inoculation. Plants were maintained in greenhouse conditions for two weeks. Expression of the XIP gene from PVX was monitored by reverse transcriptase-PCR using primers that spanned the region at the junction of the PVX vector and the XIP gene.

Disease evaluation on detached leaflets

Fourteen days after inoculation, systemically infected leaves were excised and placed abaxial side up on moist paper towels. Leaves were inoculated with a sporangial suspension of *Phytophthora infestans* US race 8, and incubated in enclosed containers. Disease symptoms were evaluated visually at 5 and 8 days post-inoculation. Immature tubers were harvested and inoculated in the same manner as the detached leaves.

Assay for xyloglucan-specific endoglucanase activity

Suspensions of Remazol Brilliant Blue linked xyloglucan (Megazyme) were prepared as a 0.1% w/v mixture in water. Samples (0.5 ml) were aliquoted into microfuge tubes, sealed and stored at room temperature. Tissue samples were extracted with three volumes of water, microfuged and 0.1 ml samples of supernatant transferred to tubes containing labeled xyloglucan.. Incubations were carried out overnight at room temperature. Samples were then precipitated with equal volumes of ethanol, and microfuged for five minutes. Xyloglucanase activity was observed as a reduction in pellet size and increase in soluble blue dye.

RESULTS

Identification of family 12 homologs in *Phytophthora infestans*.

Performing BLAST searches using the conserved domains from Family 12 protein allowed for identification of a Family 12 endoglucanase in *P. infestans*. The gene encodes an acidic protein of about 24 kD (Figure 1a). As expected, the gene lacks introns in the genomic coding sequence. Comparison of the *Phytophthora* sequence showed 69% amino acid conservation with an *Aspergillus nidulans* homolog. Southern analysis revealed at least three copies in four isolates analyzed. Homologs of the Family 12 xyloglucan-specific endoglucanase are also present in other *Phytophthora* species.

Identification of a potato encoded XIP.

Clones were obtained from genomic DNA by PCR amplification and shown to be identical to the EST sequences, indicating a lack of introns. The gene encodes a basic protein of about 48 kD (Fig. 1b.). The clone showed 98% amino acid conservation with the tomato homolog, 80 % conservation with an extracellular dermal protein from carrot (Satoh et al., 1992), and 78% conservation to the same protein in Arabidopsis. In carrot, inhibitor production was up-regulated by wounding and stress, suggesting an active role in plant defense.

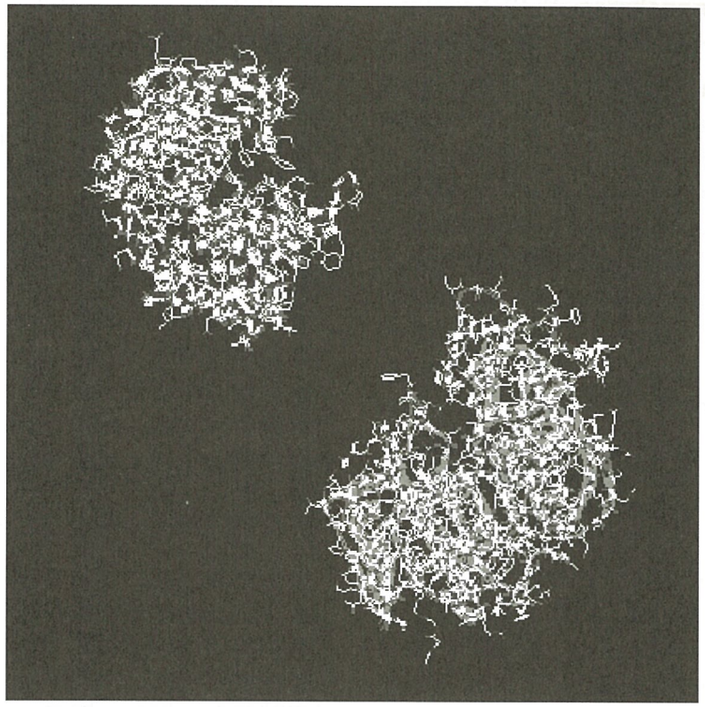


Fig. 1. Models of egl12 (left) and XIP (right) developed with Swissmodel.

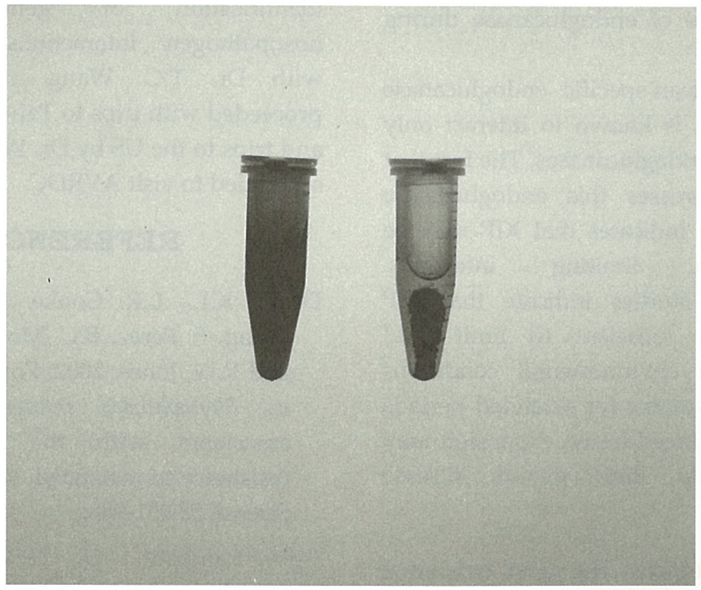


Fig. 2. Xyloglucanase activity measured by remazol brilliant blue xyloglucan.

Over-expression analysis with PVX vector

Expression of the inhibitor protein resulted in a 25-35% reduction in water-soaked lesion area five days after inoculation. Continued reduction was seen at eight days post-inoculation. Tuber inoculations also resulted in lower tissue damage in the presence of PVX-expressed inhibitor protein. Tuber infection symptoms were different. Tubers with enhanced inhibitor protein were firm and darkened, while the normal infections were not discolored, but water-soaked. Assays for xyloglucan-specific endoglucanase activity, using Remazol dyed xyloglucan revealed strong activity in infected tissues (Fig. 2).

To date, no Family 12 endoglucanases have been identified in *Phytophthora*, although other endoglucanases have been identified (McLeod et al 2003). This study also provides the first evidence that any phytopathogenic micro-organisms actively express this type of endoglucanase during infections.

The xyloglucan-specific endoglucanase inhibitor protein is known to interact only with Family 12 endoglucanases. The fact that *P. infestans* expresses this endoglucanase during infection indicates that XIP may be important in limiting infections. Overexpression studies indicate that XIP levels may be sufficient to limit field infection where environmental conditions are not often favorable for extended periods of time, thus reduced lesion expansion may be sufficient to limit overall disease significantly.

This study provides the first evidence that xyloglucan-specific endoglucanase inhibitor protein can play a role in late blight disease development

Differences in steady state or induced levels of this protein in various cultivars and germplasm may contribute to durable, non-R gene mediated resistance. Further work will study various cultivars and germplasm to determine if a correlation exists between the levels of XIP present and the level of resistance to late blight and other diseases. Further cloning and screening of sequences from *P. infestans* will be needed to determine the expression level of each copy of family 12 endoglucanase, and determine if a correlation exists between expression levels and pathogen virulence (Deahl *et al.*, 2002).

Exchange of Scientific Information and Technology

Our laboratory and Dr. Deahl's laboratory work together on issues related to molecular characterization of *P. infestans*. Emphasis has been on molecular identification of pathogen populations and identification of genes regulating host:pathogen interactions. Collaboration with Dr. T.C. Wang at AVRDC has proceeded with trips to Taiwan by Dr. Deahl and trips to the US by Dr. Wang. Dr. Jones is scheduled to visit AVRDC.

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