ATTEMPTED CLONING OF THE *PHYTOPHTHORA INFESTANS* PYR-GENE FOR USE IN A HOMOLOGOUS TRANSFORMATION SYSTEM.

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INTRODUCTION

The development of a successful transformation system is necessary for the genetic manipulation of *Phytophthora infestans* at the molecular level. Such a system provides a helpful tool in research on the mechanisms underlying pathogenicity in *P. infestans*. Up to our knowledge, successful transformation of *Phytophthora* has not yet been accomplished although several attempts using heterologous gene constructs as selection markers have been carried out. The question whether failure is caused by the nature of the selectable marker or the conditions of transformation cannot be answered in this way. This question can better be adressed in a homologous transformation system.

A succesful homologous transformation system for *Aspergillus niger* is based on the *pyr*A gene coding for orotidine-5'-phosphate (OMP) decarboxylase, a key enzyme in the biosynthesis of pyrimidine nucleotides. This transformation system requires OMP-decarboxylase deficient mutants. These mutants can readily be obtained after mutagenesis by a positive selection method which is based on the resistance of these mutants to 5-fluoro-orotic acid (FOA). After transformation of the obtained uridine auxotrophic mutants (*pyr*$^-$) with OMP-decarboxylase (*pyr*) gene constructs, transformants can be selected on uridine-less medium.

We have concentrated our efforts on the development of a similar transformation system for *P. infestans* starting with the isolation and characterization of the *P. infestans pyr* gene.

CONSTRUCTION OF *PHYTOPHTHORA INFESTANS* GENE LIBRARY

Chromosomal DNA of *P. infestans* (isolate 88069) was isolated as described by Wernars *et al.* (Curr. Genet.; 9 (1985) 361-368). High molecular weight DNA was partially digested with Sau3A and fragments of 15-23 kb were isolated after centrifugation on a 10-40% sucrose gradient. Threehundred nanograms of these fragments was ligated to 250 ng of λ arms of the replacement vector λ EMBL3 (Frischauf *et al.*, J. Mol. Biol.; 170 (1983) 827-842), which yielded a genomic library of ca. 700,000 recombinant plaques.

The calculated genome length of *P. infestans* is approximately 2.5×10^8 bp based on microfluorometric estimates of nuclear DNA content. In order to achieve a 99% probability of having a given DNA sequence represented in a library of 15 kb sequences of the *P. infestans* genome, 80,000 recombinant plaques should be screened.

CLONING AND PARTIAL CHARACTERIZATION OF THE PUTATIVE *P. INFESTANS PYR*-GENE

The *P. infestans* genomic library (100,000 recombinant plaques) was screened under heterologous hybridization conditions (58°C, 2xSSC) using a 645 bp SalI-fragment (pGW645) of the coding sequence of the *A. niger pyr*A gene (Goosen *et al.*, Curr. Genet.; 11 (1987) 499-503). Several strongly hybridizing clones were isolated and characterized further by restriction digest analysis. The approximate location of the putative *pyr*-gene on the strongest hybridizing clone λ*pyr*5, was established by hybridization with the coding sequence of the *A. niger pyr*A gene. The results are summarized in Fig. 1.

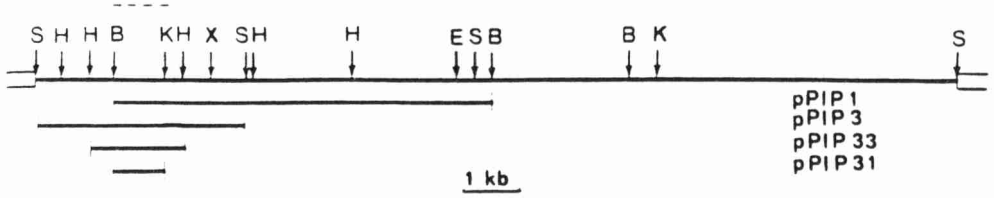

Fig. 1: Restriction map of the genomic DNA region of *P. infestans* comprising the putative *pyr*-gene which is cloned in the phage λ*pyr*5. The thick line represents *P. infestans* DNA, the open bar λ EMBL3 sequences. The dotted line indicates the approximate location of the putative pyr gene. The underlined *Bam*HI (B), *Sal*I (S), *Hind*III (H) and *Bam*HI/*Kpn*I (B/K) fragments showed strong hybridization to the *A. niger pyr*A DNA under heterologous hybridization conditions (58°C, 2xSSC) and were subcloned in pTZ19u. E = *Eco*RI and X = *Xba*I.

A Southern blot containing genomic *P. infestans* DNA digested with *Hind*III, *Sal*I, *Pst*I, *Sst*I, *Bam*HI, and *Xba*I was probed with the ^{32}P-dATP labeled 0.9 kb *Bam*HI/*Kpn*I insert from pPIP31. This probe hybridized strongly to a single band in all the lanes on the blot (65°C, 0.2xSSC). The hybridizing 1.7 kb band in the *Hind*III lane and the 7.5 kb band in the *Bam*HI lane were both present in the λ*pyr*5 clone. After deprobing, the Southern blot was probed with the *A. niger pyr*A gene using heterologous hybridization conditions (58°C, 2xSSC)). This probe hybridized to exactly the same *Hind*III, *Sal*I, *Pst*I, *Sst*I, *Bam*HI, and *Xba*I bands on the Southern blot.

DISCUSSION

The OMP-decarboxylase gene from *A. niger* hybridizes strongly to the subcloned 0.9 kb B/K fragment pPIP31 subclone of the *P. infestans* genomic library. The flanking 1.5 kb B/S and 1.7 kb K/S fragments in pPIP3 do not hybridize with the coding sequence of the *A. niger pyr*A gene which indicates that the corresponding coding sequence of the putative *P. infestans pyr*-gene is located on this 0.9 kb B/K fragment. Considering the length of the flanking fragments (1.5 cq 1.7 kb) it is most likely that the complete gene, including promotor and termination signals, is comprised in the 4.1 kb *Sal*I fragment as subcloned in pPIP3.

In order to prove that the putative *P. infestans pyr*-gene indeed encodes for OMP-decarboxylase, 90% of the sequence of the 0.9 kb B/K-fragment from pPIP31 was determined by dideoxy sequencing. After running a sequence homology test, no significant homology could be determined between the *P. infestans* and *A. niger pyr*-sequence. So far we can not explain why the *A. niger pyr*-probe hybridizes at 58°C to the 0.9 kb B/K-fragment whereas there is no significant homology between the sequences. The homology may be located on the unknown 10% of the 0.9 kb B/K-fragment. The hybridization of the *A. niger pyr*-probe may also be due to several short (up to 25 bp) highly homologous parts of the sequence. Further analysis of the subcloned B/K fragment should give more data but based on the information we have now, it is unlikely that the 0.9 kb B/K-fragment in pPIP31 contains a OMP-decarboxylase coding sequence.