

Identification of candidate target proteins of type III effectors

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Abstract

Many plant-associated Gram-negative bacteria, such as *Pseudomonas syringae* pv *tomato* DC3000 (*Pto*_{DC3000}), use a type III secretion system (T3SS) to establish a host-microbe relationship with compatible plants. The T3SS resembles a molecular injection mechanism that allows the bacteria to deliver type III effectors (T3Es) directly into the cell of the host plant. These effectors interact with native proteins inside the plant cell in order to dampen the plant's immunity mechanisms. We are determining the interaction of two T3Es from *Pto*_{DC3000} and their candidate target proteins using a yeast two-hybrid assay.

Introduction

Plants have two levels of immunity, the first of which is known as PAMP-triggered immunity (PTI). PTI is activated when plant cells detect conserved pathogen-associated molecular patterns (PAMPs) associated with the bacteria. Once PTI is activated, a battery of responses, including changes in gene expression, is initiated by the plant cell to protect itself from bacterial invasion. One example of an output of PTI defense is callose deposition in the cell wall (Thomas *et al.*, 2009). A successful pathogen delivers T3Es into host cells in order to dampen PTI outputs. T3Es dampen plant immunity by interacting with defense-associated proteins inside the plant cell. AvrPto is an example of a T3E that inhibits the plant's ability to deposit callose in the cell wall by interaction with the resistance protein (R protein) Pto in plant cells (Abramovitch and Martin, 2005).

We have identified the open reading frames (ORFs) of two *Pto*_{DC3000} T3Es (HopAY and HopW). We have obtained from a collaborator a cDNA library that was constructed from a pathogen-challenged plant believed to express the target defense-associated proteins of HopAY and HopW. We will characterize the interaction between these two T3Es and their target defense proteins using a yeast two-hybrid assay.

Materials and Procedures

The yeast two-hybrid assay is used to analyze protein-protein interactions. This system utilizes a Gal4 transcription factor that is composed of two separable domains, referred to as the activation domain (AD) and the binding domain (BD). Physical interaction of the two domains is necessary in order to activate transcription. The Gal4 transcription factor is responsible for the activation of transcription of reporter genes controlled by the BD binding sites. Candidate proteins are fused both domains by cloning. Once proteins are fused to their respective domains, a mating or cross over of the two domains will be done. Positive protein-protein interactions form a bridge between the two domains, which brings the domains into close proximity to activate transcription of reporter genes. There are three reporter genes used to determine positive interaction. The *lacZ* reporter gene is a colorimetric test for interaction. The two other reporter genes are heterotrophic tests for interaction (Walhout and Vidal, 2001).

Results

In order to proceed with the yeast two-hybrid procedure, the selected T3Es ORFs had to be cloned into Gateway-ready yeast two-hybrid vectors. The HopAY ORF was first cloned to an empty gateway vector (pDONR207) in *E. coli* cells. The transformants were plated on gentamicin-containing media for selection. Colonies that grew on

selection were tested via PCR for final confirmation. Two colonies were selected for PCR screening and both colonies showed positive results (Figure 1).

HopAY and HopW in pDONR207 were isolated and sequenced to ensure that the ORFs were being properly cloned into the empty vector. Both constructs were cloned again to a Gateway-ready yeast two-hybrid BD vector (pPC97). The transformants were plated on media containing ampicillin for selection. Random colonies were selected and confirmed via PCR (Figure 2).

Continuing the yeast two-hybrid screening, T3E constructs were transformed into a competent yeast strain, Mav103. Similarly, the cDNA library will be transformed into another yeast strain, Mav203. Both yeast strains are haploid and are opposite mating types, which allows future mating experiments (Walhout and Vidal, 2001).

Effector ORF constructs in pPC97 were amplified and isolated through gel extraction techniques. These constructs were then transformed into Mav103 cells. The transformants were plated on yeast media lacking leucine for selection. We used a heterotrophic test in order to determine transformation success. Three random yeast colonies were selected and replated on media lacking tryptophan (-Trp). All colonies that did not grow on -Trp plates were screened via PCR. We confirmed that most screened colonies had a successful transformation of the effector genes (Figure 3).

To assess the expression of effector genes in yeast cells, yeast colonies that tested positive for the effector genes were analyzed through a Western blot assay. The outcome of the assay had high background levels that could potentially be hiding the expected results. The results derived from the Western analysis were inconclusive.

Discussion

We cloned and transformed HopAY and HopW ORFs into yeast cells in order to proceed with a yeast two-hybrid analysis. Western analyses on yeast cells were inconclusive, which suggest that the effector genes are not expressed in yeast cells or that they are expressed but background levels is hiding the desired results. High background levels could be due to high antibody concentration or lack of specificity.

Future work on this project involves cloning the plant cDNA library to a Gateway-ready yeast two-hybrid AD vector in *E. coli* cells. This construct will then be transformed into Mav203 cells. It is necessary to have a highly efficient transformation in order to have an overall representation of the cDNA contents.

We can proceed with mating experiments once expression of effector genes in yeast cells is confirmed. Mating involves pairing Mav103 cells expressing BD-effector gene fusions with Mav203 cells expressing cDNA library proteins. Matings will be plated on selective yeast media lacking both leucine and tryptophan. Single colonies that grow on selective media will replated on three other plates with different selectivity. Colonies that grow on two out of three plates will be considered to have a positive protein-protein interaction. Once single colonies are confirmed for interaction, screening via PCR and sequencing will be done to identify protein sequences in plant cells that interact with HopAY or HopW.

References

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3. Walhout, A. J., and Vidal, M., 2001, High-throughput yeast two-hybrid assays for large-scale protein interaction mapping. *Methods*, v.24, p.297– 306

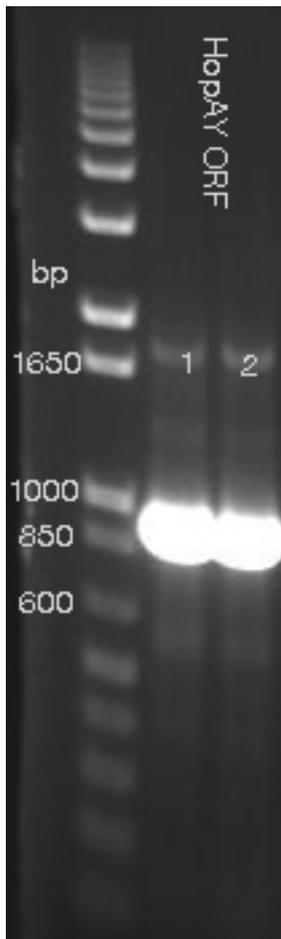


Figure 1: HopAY ORF cloned into pDONR207 in electrocompetent DH5 α *E. coli* cells. Two colonies were selected for PCR screening and they had an expected size of 750bp. The PCR-amplified product of the transformants showed products between 850-1000bp. The outcome product is larger than expected because the primers used add an extra ~200bp to the amplified product.

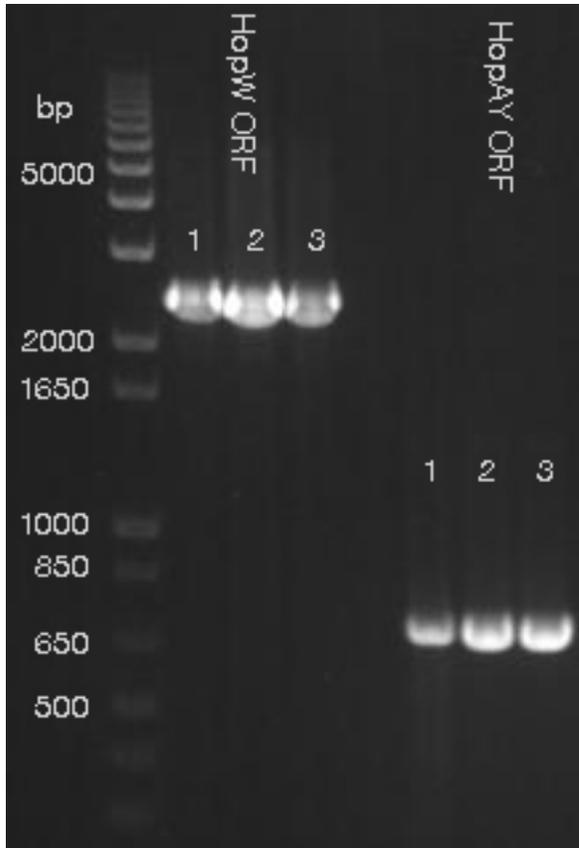


Figure 2: HopAY and HopW ORFs cloned into Gateway-ready yeast two-hybrid binding domain vector. Three colonies were selected from each plate. The expected size for HopW ORF is 2325bp and the HopAY ORF is expected to have a size of 750bp. PCR products for HopW ORF had sizes around 2300bp and HopAY ORF products had sizes between 650-700bp.

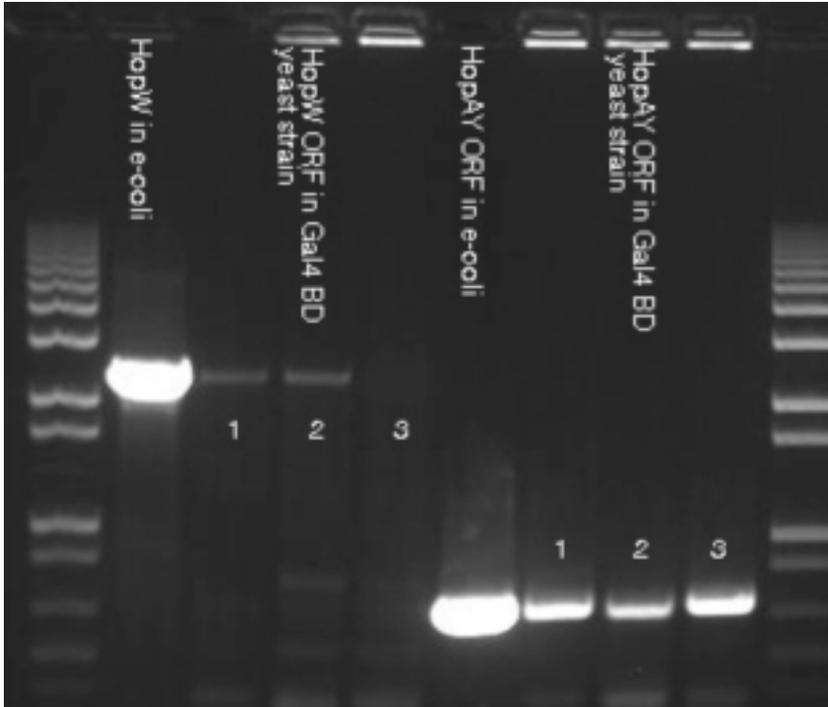


Figure 3: HopAY and HopW transformed into yeast competent cells (Mav103). Controls are T3Es ORFs cloned into *E. coli* cells, which can be referenced to Figure 2. Numbered bands represent the genotype of transformants that grew on yeast selective media lacking leucine. HopW line #3 does not show any bands, meaning that the transformation of HopW ORF was unsuccessful in that specific colony. All other numbered bands match to their corresponding control band.