REPORT

Identification of new disease resistance genes in tobacco via functional genomics

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ABSTRACT

State of the art functional genomic tools were used to find, characterize and analyze the function of genes involved with disease resistance in tobacco plants. The identification of genes induced by the interaction of *Nicotiana megalosiphon* and tobacco blue mold was achieved by suppression subtractive hybridization (SSH), and virus-induced gene silencing (VIGS). Ethylene transcription factor (EIL2) and glutathione synthetase were identified as resistance factors to tobacco blue mold.

*I*ntroduction

The isolation of genes which are distinctively regulated in a specific tissue, life cycle stage or physiological condition is a important strategy for obtaining information on the details of the processes underlying important biological phenomena, such as cell differentiation, metabolic or morphologic changes and disease progression, as well as for studying the pathogenicity and host range of parasites.

The emergence of new affordable technologies, together with the commodity status of DNA sequencing, have granted plant biologists access to the genomics era. Research projects in progress involve large scale sequencing of cDNA libraries from a wide range of plants. These sequences provide a snapshot of the expression of genes in specific cell types or development stages, which combined with bioinformatics analyses, enable a fast and thorough assessment of the genes that are transcribed, and that are induced or expressed constitutively in transcriptomic interactions.

In order to study the molecular components of resistance to tobacco blue mold in *Nicotiana megalosiphon*, suppression subtractive hybridization (SSH) was used to prepare a cDNA library enriched with transcripts that are induced during the *N. megalosiphon - P. hyoscyami* interaction. After the identification of relevant genes, their biological role was studied by virus-induced gene silencing (VIGS) and gain-of-function experiments. This strategy yields result with potential applicability to crop improvement programs, either by providing new molecular markers for marker-assisted selection, or directly by guiding the design of transgenic plants with high disease resistance levels, or for the use of bioproducts in plant disease control.

*R*esults and discussion

Identification and isolation of genes involved in disease resistance in tobacco plants

A strategy based on the use of functional genomic tools is presented for identifying, characterizing and analyzing the function of genes involved in resistance to tobacco blue mold:

1. Establishing of the *N. megalosiphon - P. hyoscyami* f. sp. *tabacina* interaction.
2. Identifying and isolating genes that are differentially activated during this interaction, using suppression subtractive hybridization (SSH).
3. Sequencing and analyzing the sequences in international data banks.
4. Molecular characterization of the genes by Northern-blot and RT-PCR.
5. Analysis of their functional role through virus-induced gene silencing (VIGS) or constitutive expression (gain-of-function).

Establishing of the *N. megalosiphon - P. hyoscyami* f. sp. *tabacina* interaction

An infection model was established for the reproducible interaction between *P. hyoscyami* f. sp. *tabacina* and *N. megalosiphon* (this species is widely used in crop improvement programs, due to its high levels of resistance against the main tobacco diseases) (Figure 1). The leaves, stems and roots used as the starting material were inoculated either with the infectious agent or with water and evaluated at different time points (2, 4 and 6 days post-inoculation). *N. tabacum,* var. "Sumatra" was used for the control group.

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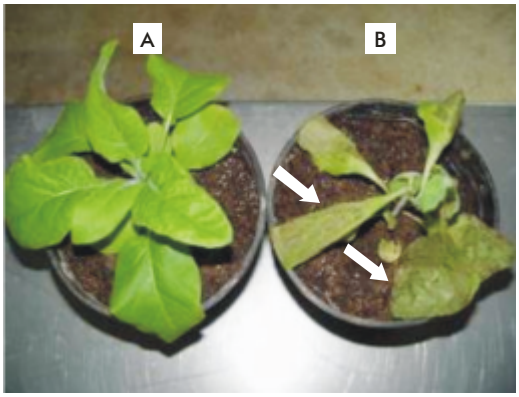

Figure 1. Infection model for the interaction of *N. megalosiphon* with *P. hyoscyami f. sp. tabacina*. *N. megalosiphon* (A) and *N. tabacum*, var. «Sumatra» (B) plants inoculated with blue mold are shown, 10 days after inoculation. The arrows indicate visible symptoms of infection on the leaves.

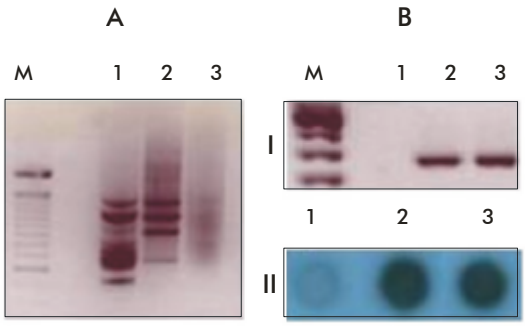

Figure 2. Method for the identification and isolation of genes expressed differentially in the interaction of *N. megalosiphon* and tobacco blue mold, using SSH. A: SSH (1), control from the kit (2) and non-subtracted sample (3). B: Check for the efficiency of the SSH by the polymerase chain reaction (PCR) using actin primers (I) or by nucleic acid hybridization in dot-blot format (II) using the cDNA from the control sample; 1: subtraction, 2: non-subtracted cDNA, 3: control cDNA.

Identifying and isolating differentially expressed genes

Suppression subtractive hybridization (SSH) was employed for identifying and isolating genes activated during the interaction of *P. hyoscyami* f. sp. *tabacina* with *N. megalosiphon* (Figure 2). RNA from plants inoculated either with the pathogen or with water (control) was isolated and used for synthesizing cDNA at each time point. SSH was performed by mixing the cDNA obtained at these time points from *N. megalosiphon* plants inoculated either with the pathogen (treated sample) or with water (control sample) and subtracting the cDNAs of the latter from the former. This gave the first subtractive library of the interaction of *N. megalosiphon* with tobacco blue mold, composed of 182 differentially expressed cDNAs. This is the first library prepared with tobacco RNA using this method [1].

Sequencing and molecular characterization of the genes

The cDNA fragments from the SSH library were sequenced and analyzed by similarity searches with the BLAST algorithm and international sequence data banks, using the *E value* obtained as a guide for assigning putative functions based on sequence homology with known genes. Many of the transcripts identified are involved in defense mechanisms against biotic and abiotic factors (16%), cellular metabolism (20%), energy (12%), protein synthesis (8%), signal transduction (12%) and membrane transport (8%); some of them could not be assigned a defined role (24%).

Afterwards, Northern blotting was used to confirm the differential regulation of the isolated genes upon infection. A lipid transfer protein (LTP), an ethylene response transcription factor (EIL2) and glutathione synthetase were confirmed to be activated at the time points in the tobacco blue mold-resistant species; this activation was not observed in susceptible species (Figure 3A) [2]. Figure 3B shows the results of RT-PCR experiments corroborating the previous results; it can be seen that these genes are activated by the pathogen or by the treatment with ethylene and salicylic acid [3]. These validation steps allow us to eliminate spurious results from the pool of cDNA obtained after SSH, and select only the most promising candidates for the next step.

Analysis of gene function by gene silencing or gain-of-function

The last stage of the strategy, i.e. the analysis of gene function by silencing or gain-of-function experiments, is the most stringent verification of the involvement of these genes in disease resistance and provides important new information on their function. Interesting results were obtained for three of the genes of *N. megalosiphon* isolated according to their differential regulation in the interaction with *P. hyoscyami* f. sp. *tabacina*. It is important to know whether disease resistance is compromised or improved in order to include a gene or a group of genes within a plant breeding programs. Basically, there are several methods for this: turning off gene transcription (silencing) or activating it constitutively (gain-of-function), followed by the phenotypic analysis of its behavior after inoculation with the infectious agent.

Figure 4A shows the results of silencing the ethylene response transcriptional factor (EIL2), which participates in signaling the defense response to pathogens or abiotic stress factors in plants. When silencing this gene the blue mold-resistant species showed symptoms of the disease after inoculation, proving that its activation is essential for the appearance of the disease-resistant phenotype. This is the first report describing the involvement of this factor in this trait. Additionally, the constitutive expression of a lipid transfer protein (a defensin) in susceptible tobacco plants yielded clones which were highly resistant to tobacco blue mold and black shank. This result is an important step in the search for disease resistance in commercially important plants (Figures 4BI and 4BII)[4].

1. Borrás O, Thomma BPHJ, Collazo C, Chacón O, Borroto CJ, Ayra C, Portieles R, López Y, Pujol M. EIL2 transcription factor and glutathione synthetase are required for defense of tobacco against tobacco blue mold. Molecular Plant - Microbe Interaction 2006;19:399-406.

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3. Borrás O. Basic insight in plant-pathogen interaction. Biotecnología Aplicada 2004;21(1):1-4.

4. Portieles R, Ayra C, Borrás O. Basic insight on plant defensins. Biotecnología Aplicada 2006;23(2):1-4.

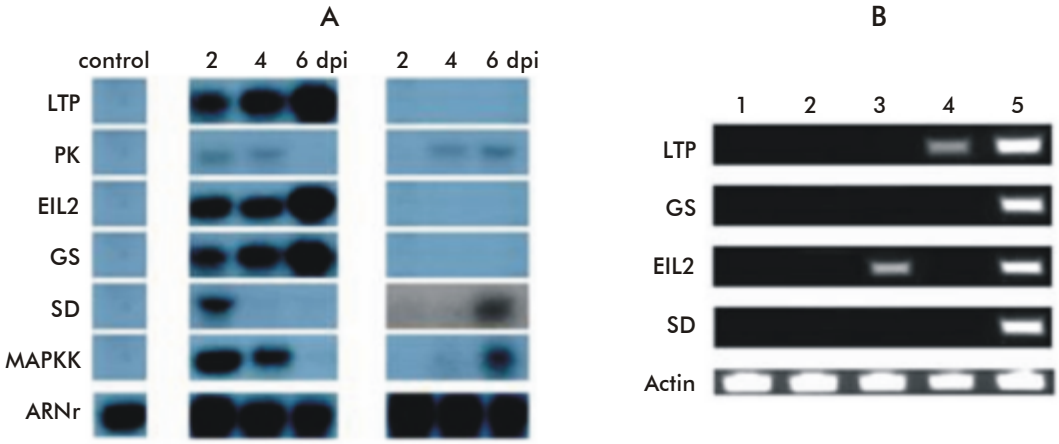

Figure 3. Methods for the molecular characterization of the genes identified after SSH. A: Analysis by Northern blot of the expression of the isolated genes during the interaction of *N. megalosiphon* and *P. hyoscyami f. sp. tabacina* in varieties that are resistant (R) or susceptible (S) to tobacco blue mold. B: Analysis of the transcriptional behavior of some of the identified genes in plants treated with water (1), jasmonic acid (2), ethylene (3), salicylic acid (4) or the pathogen (5).

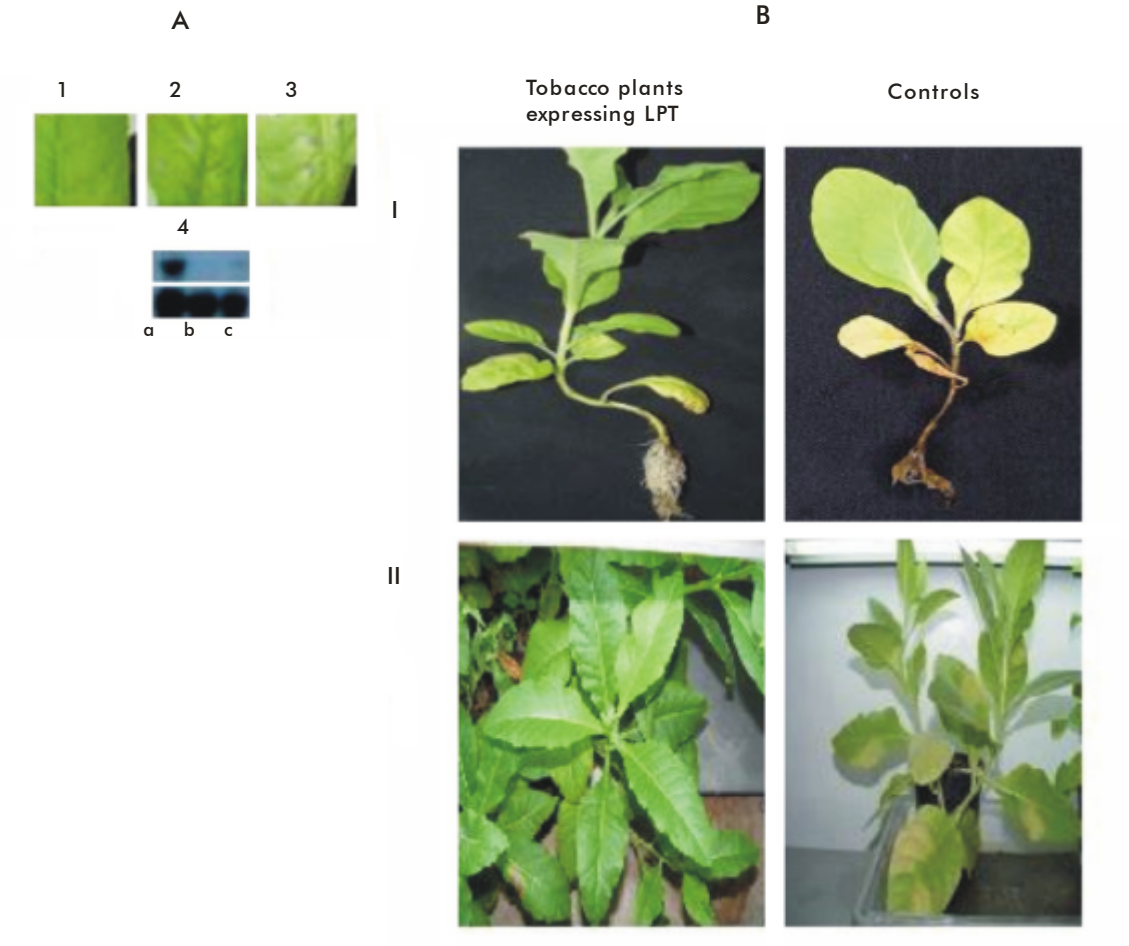

Figure 4. Virus-induced gene silencing (A) and gain-of-function (B) to analyze gene function. A1: Inoculated *N. megalosiphon* leaf, EIL2 is not silenced, A2: Inoculated *N. megalosiphon* leaf, EIL2 is silenced, A3: Inoculated *N. tabacum* var. «Sumatra» leaf, A4: Northern blotting of non-silenced (a) or silenced (b) EIL2 in *N. megalosiphon* and *N. tabacum* (c). B: Tobacco plants expressing LTP (defensin) constitutively, inoculated with black shank (I) or tobacco blue mold (II). These plants show higher resistance to these diseases.