REPORT

Demonstration by RNA interference of a new molecular mechanism for resistance to an oomycete in tobacco plants

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ABSTRACT

Oomycetes constitute an important agricultural pathogen for plants of the *Solanaceae* family. Black shank, caused by *Phytophthora parasitica* var. *nicotianae*, is one of the most important diseases affecting tobacco plantations in Cuba and worldwide, constituting an occasional cause of major economic losses. We have constructed a subtractive library with the aim of identifying tobacco genes involved in the resistance to this oomycete. The analysis by real-time PCR confirmed, for the first time, the patterns of differential expression between resistance and susceptibility reactions for the identified genes. This constitutes the first report demonstrating the pattern of expression of the glutathione-S-transferase (GST) gene in varieties and species independently of the degree of resistance or susceptibility to the pathogen. The gene was cloned in a plant transformation vector for gene silencing, and transgenic *N. tabacum* plants with silenced GST were obtained. They significantly displayed increased resistance to the disease, in highly susceptible plants, in comparison to control plants. This work constitutes the first report on the role of GST as a negative regulator of the defensive response to oomycetes, as well as the first instance where RNA interference technology has ever been used to obtain resistance to diseases produced by Oomycetes.

*I*ntroduction

Black shank, caused by *Phytophthora parasitica* var. *nicotianae*, is one of the most important agricultural pathogens for tobacco (*Nicotiana tabacum*) worldwide [1]. This pathogen attacks the roots, stem and leaves in all developmental stages, producing root necrosis, withering and shriveling, chlorosis, stem lesions, atrophy and, ultimately, death. The disease progresses rapidly under conditions of high humidity and temperature [2]. Annually, agricultural losses due to this mold are significant, resulting in important financial costs [1].

The disease is usually managed with an integrated approach that includes specific cultural practices, the application of fungicides and the use of resistant cultivars. However, the use of resistant cultivars constitutes by far the most effective measure [3]. Both *N. longiflora* and *N. plumbaginifolia* have been shown to be resistant to race 0 of *P. parasitica* var. *nicotianae* [4], and cultivars inheriting this resistance are immune (*i.e.* no growth or reproduction of the pathogen takes place).

Nicotiana megalosiphon is a wild tobacco species commonly used in genetic improvement programs for tobacco [5]. *N. megalosiphon* is highly resistant to oomycetes *Phytophthora parasitica* var. *nicotianae* and *Peronospora hyoscyami* f. sp. *tabacina* [6]. However, this species is highly susceptible to viral pathogens such as virus A of potato and TMTV [7].

Several studies have approached the tobacco - *P. parasitica* var. *nicotianae* interaction from a molecular perspective. The activity of lipoxygenase (LOX) and the expression of the *lox* gene upon infection with *P. parasitica* var. *nicotianae*, for instance, have been studied in intact tobacco plants, observing a rapid and temporary accumulation of LOX after the inoculation with *P. parasitica* var. *nicotianae* zoospores. This result suggests an important role for the *lox* gene in mediating resistance to the pathogen and establishing a defensive state in the host plant [8]. The defensive state against *P. parasitica* var. *nicotianae* has also been associated with an extracellular cytotoxic activity originated from the accumulation of salicylic acid-dependant PR-1 proteins in the apoplasm, as well as a salicylic acid-independent cytotoxic activity which has not been detected in leaves in which a systemic acquired resistance has been induced [9].

On the other hand, the transition from the vegetative to the reproductive state also produces a transition from susceptibility to resistance to the causal agent of black shank. This resistance manifests as it decreases in infection efficiency and hyphal expansion, and has also been found to be associated to the accumulation of an extracellular cytotoxic activity. Microarray analyses and ribonucleic acid hybridization revealed that during late developmental stages, leaves are characterized by a coordinated regulation of genes involved in the acquired systemic response and the induction of structural proteins of oxidation in the cell wall [10].

Additionally, the infection of plant cells by a pathogen usually elicits a battery of disease response genes, which can result in the production of several toxic products that include reactive oxygen species [11]. One of such responses observed in plants after infection is the increased expression of genes coding for glutathione-S-transferase (GST) [12].

Although the involvement of GST of *N. tabacum* on the response to cold, salt stress and aluminum-induced toxicity is established [13], little is known about its role during pathogenic infection. Using binding primers to preserved regions of *N. tabacum* GST, four different genes for this enzyme were amplified from cDNA of *Nicotiana benthamiana* leaves infected with *Colletotrichum destructivum* [14]. After cloning each of these genes on the PVX vector for

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virus-induced gene silencing and obtaining the corresponding transgenic plants, a significant increase in susceptibility to the infection was observed only in individuals bearing a silenced *NbGSTU1* gene. These plants showed a larger number of lesions (130%) and more extensive *C. orbiculare* colonization (67%) than the controls [14], proving that the different GST variants differently respond to fungal infections, and demonstrating the involvement of at least one of them in the development of the disease [14].

Taking into account the importance of dissecting the molecular components in *Nicotiana tabacum* responsible for establishing a state of resistance or susceptibility to *P. parasitica* var. *nicotianae*, as well as the need for exploring new strategies for harnessing disease resistance, the objectives of the present work were: To obtain a cDNA library formed by transcripts derived from genes induced during the resistance interaction with *P. parasitica* var. *nicotianae*; to characterize by realtime PCR the expression of genes involved in resistance to the disease; to evaluate the role of resistance- or susceptibility-related genes by using RNA interference technology, and to characterize the expression of a set of defense genes in the tobacco with RNA interference, using realtime PCR.

Results and discussion

Obtention of a cDNA library formed by transcripts derived from genes induced during the resistance interaction with *P. parasitica* var. *nicotianae*

This work identified a set of genes involved in the response against *P. parasitica* var. *nicotianae* in *N. tabacum* which had not been previously shown to participate in this phenomenon. Although the functional assignments for these genes were made based only on sequence homologies to known orthologs -and therefore demand a stricter experimental verification-, this step offers, however, a clue about the genetic diversity of this response.

A total of 65 clones were obtained, of which 48 were sequenced. For nearly 40% of the clones no known orthologs were found; these were classified, therefore, as having an unknown function. The genes that did have significant sequence homologies to known orthologs fell into six primary functional categories. Most of these genes (15%) were defense-related, followed by those others involved in signal transduction, metabolism and protein synthesis (10%). Genes involved in energy constituted 8% of the cDNA collection, and those involved with transport constituted 7% of the sequences [15].

Characterization by realtime PCR of the expression of genes involved in resistance to the disease

Based on the homologies detected in public sequence databases, 11 genes with a significant E-value in BLAST searches were selected for further analysis of their expression patterns (Table 1). Quantitative PCR assays were performed on RNA extracted from resistant (*N. megalosiphon*) or susceptible (*N. tabacum* var. Sumatra) plants at 0, 3, 7 and 10 days post-inoculation with *P. parasitica* var. *nicotianae*. This analysis indicated that the levels of expression of the transcripts from all 11 genes were generally higher during the resistance interaction and lower during the susceptible interaction [15].

Table 1. Summary of the identified clones and BLAST search results

Clones	Sequence homologs by BLASTN / BLASTX	Accession number	E-value*
NmP5	*Zea mays* superoxide dismutase	GO247697	6e-29
NmP8	*Arabidopsis thaliana* pdi23 gene for PDI-like protein	GO247698	3e-40
NmP11	*Nicotiana tabacum* glutathione S transferase	GO247699	4e-51
NmP14	Potato mRNA for light inducible tissue-specific	GO247700	2e-24
NmP18	*Arabidopsis thaliana* dehydration-responsive protein	GO247701	2e-48
NmP21	*Nicotiana tabacum* hrs203J gene	GO247702	2e-20
NmP23	*Solanum lycopersicum* osmotin-like protein	GO247703	2e-67
NmP26	*Arabidopsis thaliana* RING finger family protein	GO247704	3e-26
NmP33	*Nicotiana rustica* cathepsin B-like Cys proteinase	GO247705	6e-40
NmP37	*Medicago sativa* carbamoyl-phosphate synthase	GO247706	4e-13
NmP52	*Nicotiana tabacum* mitogen-activated protein kinase	GO247707	2e-39

*E-value: Probability for the occurrence of an equivalent match by chance alone.

Gene *hrs203J* and the one coding for the RING finger protein displayed a rapid and intense induction during the resistance interaction, but were undetectable during susceptibility interaction. This indicates that they might be important candidates for the evaluation of disease-resistant genotypes within the framework of crop improvement programs (Table 2). Significantly, a clone homologous to *GST* showed the highest induction during the resistance and susceptibility interaction in all evaluated time points, generating a hypothesis about its possible involvement in resistance and susceptibility that was later tested through a functional analysis examining its potential role as negative resistance regulator.

Evaluation of the role of genes implicated in resistance and susceptibility by using RNA interference technology

This study analyzed the biological relevance of the GST gene during the susceptibility interaction between *P. parasitica* var. *nicotianae* - tobacco by silencing this gene in transgenic tobacco plants. Surprisingly, the plants with silenced GST displayed higher levels of resistance to the pathogen, with decreased withering of the stem (Figure 1). One of the possible reasons for this result is that the expression of GST during the susceptibility interaction might be limiting the hypersensitive response (HR), according to the

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Table 2. Relative expression of three genes in different species of *Nicotiana* infected with *P. parasitica* var. *nicotianae* 3 days post-inoculation (dpi)

Species of *Nicotiana*	Disease phenotype[a]	Relative expression[b]		
		GST	*hrs203J*	*RING finger protein*
KY 14	S	4.1	0.1 d	0.1 c
N. rustica	S	4.2	0.3 d	0.2 c
N. tabacum Sumatra	S	5.3	0.1 d	0.1 c
N. alata	S	4.1	0.1 d	0.1 c
N. longiflora	S	5.1	1.5 c	0.1 c
N. megalosiphon	R	5.5	5.2 a	1.8 b
N. excelsior	S	6.4	0.3 d	0.2 c
N. debneyi	R	6.5	3.4 b	6.8 a

[a] Disease phenotype was determined according to Li *et al*. [16].
[b] The relative expression of each gene was compared to the constitutive expression of the 26S rRNA used as endogenous control. Each value represents the mean and standard deviation from 5 replicates. The experiments were repeated twice. The values on a column followed by the same letter did not have statistically significant differences according to Fisher's Protected LSD ($P < 0.05$).

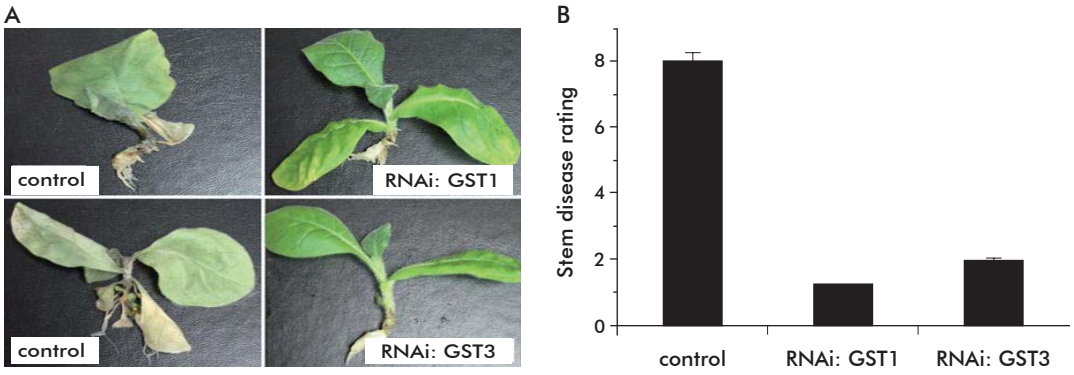

Figure 1. Greenhouse evaluation of tobacco plants bearing a silenced *GST* gene and inoculated with *P. parasitica* var. *nicotianae*. Phenotype (A) and stem disease rating (B) of control and transgenic tobacco plants with suppressed *GST* expression in interaction with *P. parasitica* var. *nicotianae* at 7 days post-inoculation (dpi). The stem disease scoring scheme of Csinos [17] was used. N=50; +/- SD.

expression of some genes involved in this type os response such as SOD and Hsr203J [18].

A number of studies on the HR in a variety of experimental systems have demonstrated the involvement of reactive oxygen species such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) as important signals or effectors of programmed cell death. O_2^- can promote the HR through the production of H_2O_2 or repress it through the elimination of nitric oxide or the induction of antioxidants. In this scheme, SOD is an important mediator to compensate these functions.

Characterization of the expression of a set of defense genes in tobacco plants with RNA interference using realtime PCR

Relative expression levels of several genes associated to disease resistance in plants were evaluated in transgenic tobacco plants where *GST* had been silenced. The genes coding for beta-1,3-glucanase, superoxide dismutase (SOD), mitogen-activated protein kinase (MAPK) and the cell-death associated protein from *N. tabacum* (Hsr203J) displayed a higher level of expression in the plants bearing RNAi construction compared to the control plants, where their expression was lower or delayed in time. On the other hand, the expression of the gene for phenylalanine-ammonia lyase (PAL) was repressed in the RNAi-transformed lines, but induced in the controls. Results showed that silencing the *GST* gene contributes to the disease-resistant phenotype as negative regulator of innate immunity to black shank in susceptible plants [18].

Silencing *GST* led to reduced expression of the *PAL* gene, which codes for a key enzyme in the phytoalexin and salicylic acid synthesis pathways [18]. Also, as mentioned above, the plants with silenced *GST* displayed increased expression not only of the genes coding for Hsr203J, SOD and MAPK, but also for beta-1,3-glucanase, a well-known gene coding for a protein involved in pathogenesis. Possibly, the resistant phenotype observed in this study as a result of *GST* silencing is actually the outcome from the combined action of several gene products under direct or indirect control by *GST* [17].

Finally, it should be pointed out that during the compatible (susceptible) interaction the pathogen induces what is known as a hypersensitive response in the host plant through the production of reactive oxygen species and nitric oxide. However, there is a strong activation of GST, which is a key enzyme in the synthesis of glutathione, a strong antioxidant. GST participates in the detoxification of reactive oxygen species in order to avoid cell death induced by free radicals produced during the hypersensitive response of the susceptibility interaction [18], explaining the successful invasion of the pathogen into the cells of the host. In plants where *GST* is silenced, however, hypersensitivity response is active throughout the entire range of evaluated time points; and as a result, the plants display a strong resistance to the pathogen (Figure 2).

The dissection of new molecular mechanisms for pest resistance will allow the modification of highly susceptible plants into disease-resistant cultivars, taking advantage of the activation of more efficient resistance mechanisms. It will, additionally, provide genes that can be used as molecular markers within ongoing genetic crop improvement programs.

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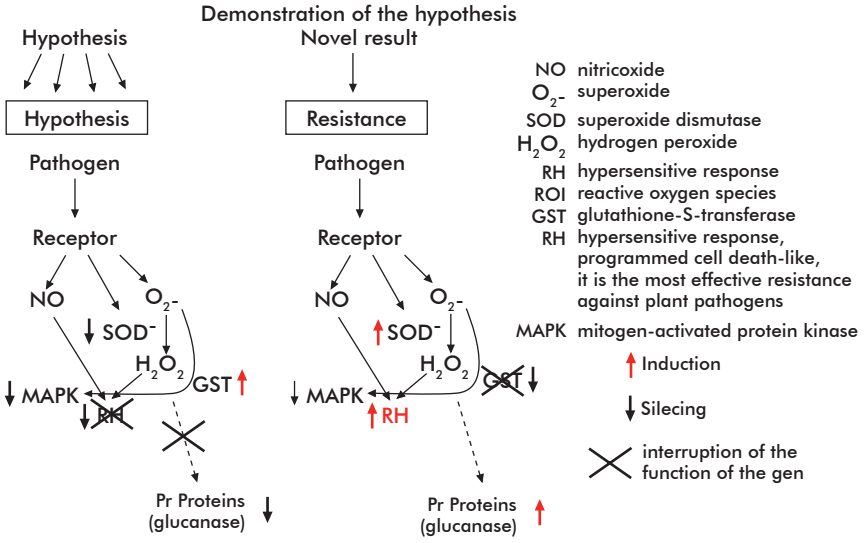

Figure 2. Scheme showing the integration of the hypothesis and the obtained results.