REPORT

High protection of protein kinase NtPK against the phytopatogenic fungus Rhizoctonia solani

Orlando Borrás[1], ✍ Osmani Chacón[2], Marleny González[3], Roxana Portieles[1], Ernesto González[1], Merardo Pujol[1]

[1] Laboratorio de Genómica Funcional de Plantas, División de Plantas, Centro de Ingeniería Genética y Biotecnología, CIGB
Ave. 31 e/ 158 y 190, Cubanacán, Playa, CP 11 600, La Habana, Cuba
[2] Instituto de Investigaciones del Tabaco, IIT
Carretera Tumbadero, Km 8 $^{1}/_{2}$, San Antonio de los Baños, Artemisa, Cuba
[3] Instituto Nacional de Investigaciones en Sanidad Vegetal, Inisav
Calle 110 No. 514 e/ 5ta B y 5ta F, Playa, La Habana, Cuba
E-mail: osmani@iitabaco.co.cu

ABSTRACT

To identify *Nicotiana tabacum* genes involved in resistance and susceptibility to the phytopathogenic fungus *Rhizoctonia solani*, transcripts were generated (by subtractive libraries), which were differentially expressed in each interaction. This enabled isolation of a gene coding for a protein kinase that becomes silent during the susceptibility interaction and is activated during resistance. The expression of this gene in tobacco plants significantly increased resistance against an aggressive isolate of *R. solani*. However, silencing the gene drastically reduced resistance to a non-aggressive isolate of *R. solani*. Besides, genes such as: superoxide dismutase, *hsr203j*, chitinases and phenylalanine ammonia lyase, related with resistance to tobacco plant diseases, in which the gene coding for the protein kinase is overexpressed or silenced, were evaluated. This gene can be used to design a strategy of resistance to *R. solani* in tobacco cultures or other plants of the Solanaceae family, susceptible to this phytopathogen.

Keywords: NtPK, *Rhizoctonia solani*, fungus, *Nicotiana tabacum*, resistance, phytopathogen, gene silencing

Biotecnología Aplicada 2012;29:279-281

RESUMEN

La proteína quinasa NtPK confiere alto nivel de protección contra el hongo fitopatógeno Rhizoctonia solani. Con el objetivo de identificar los genes involucrados en la resistencia y susceptibilidad de la *Nicotiana tabacum* al hongo fitopatógeno *Rhizoctonia solani*, se generaron (a través de librerías substractivas) transcriptos que se expresan diferentemente en cada interacción. Ello permitió el aislamiento de un gen que codifica para una proteína quinasa que se silencia durante la interacción de susceptibilidad y se activa durante la resistencia. La expresión de este gen en plantas de tabaco incrementó significativamente su resistencia frente a un aislado agresivo de *R. solani*. Sin embargo, el silenciamiento del gen redujo drásticamente la resistencia a una cepa no agresiva de *R. solani*. Además se evaluaron genes como superóxido dismutasa, *hsr203j*, quitinasas y fenilalanina amonio-liasa relacionados con la resistencia a enfermedades en plantas de tabaco en las que se sobrexpresa o silencia el gen que codifica para la proteína quinasa. Este gen se puede utilizar para el diseño de una estrategia de resistencia a *R. solani* en cultivos de tabaco u otras plantas de la familia Solanácea, susceptibles a este fitopatógeno.

Palabras clave: NtPK, *Rhizoctonia solani*, hongo, *Nicotiana tabacum*, resistencia, fitopatógeno, silenciamiento de genes

Introduction

Rhizoctonia solani J. G. Kühn (teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk) is a phytopathogen that causes huge losses in plant cultures of the Solanaceae family, such as tobacco, tomato and potato. It became a problem for tobacco plantations in the 1990 decade, due, to a great extent, to the change from outside transplant production to the float systems [1]. *R. solani* causes sudden death in plantlets and rotting of the stalk in young transplants; it is a fungus of the lower part of the stalk and root [2, 3]. Transplant of infected plantlets is one of the main causes of development of this disease; however, it can also occur due to its presence in the field [1].

Tobacco leaf spot, caused by the sexual phase of this fungus, emerged in the United States for the first time in the 1980 decade and today accounts for economic losses in tobacco production [1, 4]. It appears due to the infection by *T. cucumeris* basidiospores that produce hymenium, which form on the ground surface or on infected tissue [5]. Symptoms start as small aqueous lesions on the leaves, which can spread and form large circular spots of concentric rings. They appear on tobacco plants in environments with greenhouse effect and in general after the leaves have grown enough to form a canopy and create a very humid environment that favors disease development. In extreme cases, this phytopathogen can grow from the leaf tissue to the stalk and cause plant death [1]. *R. solani* isolates causing stalk and root rot are classified in anastomosis groups AG-1, AG-2-2 and AG-4, while the one causing leaf spots belongs to group AG-3 [6].

Resistance of tobacco cultures to R. *solani* could be determinant; but previous examinations of tobacco germplasm have shown lack of resistance sources. By the contrary, assessment and identification of resistance in other cultures, such as peanuts, sorghum,

✍ Corresponding author

1. Elliot PE, Lewis RS, Shew HD, Gutierrez WA, Nicholson JS. Evaluation of tobacco germplasm for seedling resistance to stem rot and target spot caused by Thanatephorus cucumeris. Plant Dis. 2008;92(3):425-30.

2. Lucas GB. Diseases of Tobacco. 3rd ed. Raleigh, NC: Biological Consulting Associates; 1975.

3. Sneh B, Jabaji-Hare S, Neate S, Dijst G, editors. Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology, and Disease Control. Dordrecht: Kluwer Academic Publishers; 1996.

4. Shew HD. Target spot. In: Shew HD, Lucas GB. Compendium of Tobacco Diseases. St. Paul, MN: The American Phytopathological Society; 1991. p. 15.

beans, rice and sugar beet have been successful [7-11]. Recently, several tobacco genotypes and species were evaluated to detect resistance to *R. solani* [1]; but significant differences were observed only at a low pressure incidence of the disease between genotypes, with no disease resistance at high disease pressure [1].

Several factors have been associated with *R. solani* resistance, including the thickness of the epicuticular wax [12], cuticle thickness [13] and calcium content in the cell wall [14, 15]. Cuticle and epicuticular wax prevent the action of enzymes or toxins released by *Rhizoctonia* spp., which participate in phytopathogen tolerance [16]. Besides, induced resistance mechanisms, such as the hypersensitivity response [12] or increase in the production of pathogenesis-related proteins may also be involved in resistance [17].

However, little is known about the molecular events associated with *R. solani* resistance in tobacco. It has been recently described a decrease in resistance to an *R. solani* compatible strain in a type of tobacco lines, in which calmodulin was silenced. In those lines, expression of jasmonic acid or ethylene acid were not affected, suggesting that calmodulin is probably involved in the defense against the necrotrophic phytopathogen at basal levels, independent of the jasmonic or ethylene acid [18].

To understand the molecular components of *Nicotiana tabacum* responsible for susceptibility and resistance to *R. solani*, the subtractive hybridization method was used to generate cDNA libraries, containing fragments of gene transcripts derived from *N. tabacum*, either induced or repressed during a compatible or non-compatible interaction with *R. solani*. Candidate genes were evaluated using overexpression or silencing strategies. This procedure enabled identification of a gene that codes for an *N. tabacum* protein kinase (NtPK), which might confer high resistance to *R. solani*. This work was awarded the Prize of the Cuban Academy of Sciences in 2011.

Results and discussion

During their evolution, plants developed mechanisms to recognize invading pathogens and trigger an effective defense response. At the same time, phytopathogenic organisms create mechanisms to evade and suppress those plant defense responses. Under such selective pressure, plants improve their defense mechanisms, so the phytopathogenic organism succeeds in causing the disease as an exception, rather than a regularity.

Plants are resistant to most pathogenic microorganisms, due to a basal defense mechanism called innate immune system. This immune response is activated after the recognition of pathogen-associated molecular patterns (PAMPs), described as general inductors, which comprise proteins, peptides, carbohydrates and lipids. There are two pathways for activation of the plant immune system: the first uses pattern recognition receptors, as that recognizing flagellin, which slowly respond to PAMPs. The second acts mainly within the cell and uses polymorphic nucleotide-binding site leucine-rich repeat proteins coded by most resistance genes.

Disease caused by the fungus *R. solani* in crops of economic importance, like potato, tomato, tobacco and rice, is a main limitation at the different stages of plant growth and severely affects production yields. Despite research to genetically improve these cultures, there are no varieties showing stable resistance to this phytopathogen.

cDNA libraries with fragments of gene transcripts derived from *N. tabacum*, either induced or repressed during compatible or incompatible interaction with *R. solani*, were obtained by subtractive hybridization. Through overexpression and silencing, a gene coding for a *N. tabacum* protein kinase, called NtPK that could provide high resistance to *R. solani* was identified.

Quantification of *R. solani* biomass in infected tobacco plants

The first symptom of infection by the aggressive *R. solani* isolate was a small lesion soaked in water on the stalk, close to the ground line that rapidly became silver brown. The lesion continued to grow in the stalk and the leaves became brown until they died. Chlorosis in foliar areas was not evidenced until two weeks after inoculation. The fungal biomass gradually increased, while the fungus colonized the roots during compatible interaction, as quantified by real-time polymerase chain reaction (PCR). After inoculation of the non-aggressive *R. solani* isolate, no disease symptom was observed; while real-time PCR did not show any significant increase of fungal biomass in the compatible interaction.

Molecular identification and characterization of the tobacco NtPK gene

Four cDNA libraries were made, which contained the genes expressed during a compatible and an incompatible interaction, after inoculation of the corresponding *R. solani* isolates. The sequences of the 122 differentially-expressed clones were used to search for homologies in international databases by the BLASTX (version 5.1) and BLASTN (version 4.3) programs. Six genes were identified for further analysis, based on homology and expression during compatible and incompatible interactions.

These were only present in cDNA gene libraries expressed during incompatible interaction and, at the same time, they were in the cDNA gene library silenced during compatible interaction. There was no coincidence of other genes with those of the analyzed libraries. This was the main criterion for selection. Of these, only one coded for a protein kinase (NtPK) and showed a high level of expression in the incompatible interaction with respect to the compatible one in a separate experiment.

Expression was induced for genes coding for cytochrome P450, manganese superoxide dismutase, hydrolase, protein activated by mitogen and phospholipase B, in comparison to non-infected controls. Nevertheless, expression levels were similar when compatible and incompatible interactions were compared at the same analyzed times. For this reason, the gene coding for the protein kinase NtPK was selected for a more detailed study of its function.

In a comparison of amino acid sequences, NtPK was found to be highly homologous to a protein ki-

5. Shew HD, Main CE. Infection and development of target spot of flue-cured tobacco caused by *Thanatephorus cucumeris*. Plant Dis. 1990;74(12):1009-13.

6. Stevens JS, Jones RK, Shew HD, Carling DE. Characterization of populations of *Rhizoctonia solani* AG-3 from potato and tobacco. Phytopathol. 1993;83(8):854-8.

7. Francke MD, Brenneman TB, Holbrook CC. Identification of resistance to *Rhizoctonia* limb rot in a core collection of peanut germplasm. Plant Dis. 1999; 83(10):944-8.

8. Montoya CA, Beaver JS, Rodriguez R, Miklas PN, Godoy-Lutz G. Heritability of resistance of web blight in five common bean populations. Crop Sci. 1997; 37(3):780-3.

9. Pan XB, Rush MC, Sha XY, Xie QJ, Linscombe SD, Stetina SR, *et al*. Major gene, nonallelic sheath blight resistance from the rice cultivars Jasmine 85 and Tequing. Crop Sci. 1999;39:338-46.

10. Pascual CB, Raymundo AD, Hyakumachi M. Resistance of sorghum line CS 621 to *Rhizoctonia solani* AG1-IA and other sorghum pathogens. J Gen Plant Pathol. 2000;66(1):23-9.

11. Scholten OE, Panella LW, De Bock TSM, Lange W. A greenhouse test for screening sugar beet (*Beta vulgaris*) for resistance to *Rhizoctonia solani*. Eur J Plant Pathol. 2001;107(2):161-6.

12. Marshall DS, Rush MC. Infection cushion formation on rice sheaths by *Rhizoctonia solani*. Phytopathol. 1980;70(10):947-50.

13. Reddy MN. Studies on groundnut hypocotyl exudates and the behavior of *Rhizoctonia solani* influencing the disease. Plant Soil. 1980;55(3):445-54.

14. Bateman DF. An induced mechanism of tissue resistance to polygalacturonase in *Rhizoctonia*-infected hypocotyls of bean. Phytopathol. 1964;54(4):438-5.

15. Bateman DF, Lumsden RD. Relation of calcium content and nature of the pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of Rhizoctonia solani. Phytopathol. 1965;55(7):734-8.

16. Kenning LA, Hanchey P. Ultrastructure of lesion formation in *Rhizoctonia* infected bean hypocotyls. Phytopathol. 1980;70(10):998-1004.

17. Anuratha CS, Zen KC, Cole KC, Mew T, Muthukrishnan S. Induction of chitinases and beta-1,3-glucanases in *Rhizoctonia solani*-infected rice plants: Isolation of an infection-related chitinase ADNc clone. Physiol Plant. 1996;97(1):39-46.

18. Takabatake R, Karita E, Seo S, Mitsuhara I, Kuchitsu K, Ohashi Y. Pathogen-induced calmodulin isoforms in basal resistance against bacterial and fungal pathogens in tobacco. Plant Cell Physiol. 2007;48(3): 414-23.

nase of *N. tabacum* (Q40547). Some regions of this protein were preserved between protein kinases of the analyzed plants. However, the N and C terminals of the studied proteins were not preserved between the examined species.

Functional analysis of the tobacco NtPK gene for resistance to *R. solani*

The NtPK gene was expressed or silenced in tobacco plants, and the resulting transformant lines were treated with aggressive and non-aggressive *R. solani* isolates. In wild tobacco plants, this gene was induced or repressed during incompatible or compatible interactions with *R. solani*, respectively. This confirmed the results of subtractive hybridization. High and low levels of gene transcription were observed in plants over-expressing NtPK or silencing it, respectively.

When plants over-expressing NtPK were inoculated with an aggressive *R. solani* isolate, resistance drastically increased, demonstrating the relevance of this gene's function for resistance against the phytopathogen. Curiously, disease symptoms caused by a non-aggressive isolate were not severe in plants with the silenced NtPK gene, as compared to control plants inoculated with the same isolate and there was a higher percentage of disease incidence.

Plants with silenced *ntpk* gene and inoculated with an aggressive isolate showed a higher percentage of disease. Conversely, transformed plants that overexpress the NtPK gene were highly resistant to the aggressive *R. solani* isolate, as compared to the control and showing lower disease incidence.

Real-time PCR evidenced that the fungal biomass gradually increased in plant tissues of tobacco plants with a silenced *ntpk* gene and inoculated with a non-aggressive isolate. With this technique, it was also possible to confirm that there was no significant increase of the fungal biomass in tobacco plants over-expressing the *ntpk* gene and inoculated with an aggressive isolate.

Relative expression of genes related with disease resistance

The relative expression of several genes associated to disease resistance was evaluated in tobacco plants either overexpressing or silencing the *ntpk* gene. In the former, manganese superoxide dismutase, *hsr203j* and chitinase genes were highly and rapidly induced a week after inoculation. However, expression of β-1, 3 glucanase and phenylalanine ammonia lyase genes reached maximum expression two weeks after inoculation. All analyzed genes were differentially induced in plants overexpressing NtPK. The expression of the β-1, 3 glucanase genes was very similar in the two samples analyzed. Regulation of these genes involved in plant defense was also evaluated in interfering RNA (iRNA) lines with a silenced *ntpk* gene.

The genes coding for manganese superoxide dismutase, *hsr203j*, chitinases and phenylalanine ammonia lyase were significantly induced in comparison to iRNA lines, during the incompatible interaction, with delayed or low expression levels. Expression of β-1, 3 glucanase was similar for iRNA lines and during incompatible interactions. The genes coding for manganese superoxide dismutase, *hsr203j*, chitinases and phenylalanine ammonia lyase could contribute to the disease resistance phenotype in *N. tabacum* plants, due to its fast and high level induction in tobacco plants over-expressing the *ntpk* gene and the lack of induction in iRNA lines.

In summary, the NtPk gene identified is involved in the defensive response of tobacco plants to *R. solani*. Therefore, it could be advantageously used in strategies aimed at developing long-lasting resistance in cultured tobacco plants, either by marker-assisted selection or other biotechnological methods.