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Research article

Reactions of tobacco genotypes with different antioxidant capacities to powdery mildew and *Tobacco mosaic virus* infections

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ABSTRACT

The interactions of powdery mildew (*Golovinomyces orontii*) and *Tobacco mosaic virus* (TMV) with tobacco lines having down or upregulated antioxidants were investigated. Xanthi-nc, its salicylic acid-deficient *NahG* mutant, a paraquat-sensitive Samsun (PS) and its paraquat tolerant (PT) mutant were used. Cell membrane damage caused by H_2O_2 was significantly higher in *NahG* than Xanthi, whereas it was lower in PT than in PS. Leakage of ions from PT was reduced by the powdery mildew infection. On the other hand TMV inoculation led to a 6-fold and 2-fold elevation of ion leakage from hypersensitive resistant *NahG* and Xanthi leaves, respectively, whereas ion leakage increased slightly from susceptible PS leaves. *G. orontii* infection induced ribonuclease (RNase) enzyme activity in extracts from Xanthi and *NahG* (about 200–250% increase) and weakly (about 20–30% increase) from PS and PT lines. Pretreatment with protein kinase inhibitor staurosporine or protein phosphatase inhibitor okadaic acid very strongly inhibited mildew development on tobacco lines. Our experiments suggest that protein kinases inhibited by staurosporine seem to be important factors, while protein phosphatases inhibited by okadaic acid play less significant role in TMV-induced lesion development. Both powdery mildew and TMV infections up-regulated the expression of *PR-1b*, *PR-1c* and *WRKY12* genes in all tobacco lines to various extents.

1. Introduction

Following the perception of microbial pathogens in host plant cells, signals are transmitted to the nucleus leading to the rapid and extensive reprogramming of plant gene expression patterns. The reprogramming of the transcriptome is regulated by a complex, multilayered regulatory network, in which reactive oxygen species (ROS), defense-related plant proteins, hormones and various transcription factors play critical roles (Rushton et al., 2010; Moore et al., 2011; Pieterse et al., 2012). The key role of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) in plant immunity has been known for a long time (Pieterse et al., 2012; Alazem and Lin, 2015). It is generally accepted that SA signaling induces defense reactions against biotrophic, whereas JA/ET signaling against necrotrophic pathogens (Glazebrook, 2005; Barna et al., 2012). However, many data have supported recently that auxins, cytokinins, gibberellins, abscisic acid and brassinosteroids have important functions in the plant immune responses as well (Pieterse et al., 2009; Denance et al., 2013; Alazem and Lin, 2015; Opara and Amarachi, 2015; Dziurka et al., 2016).

One of the earliest reactions of plants to pathogen attacks is the accumulation of reactive oxygen species (ROS). ROS are harmful not only to the plant cell and to the pathogen, but can serve as signal molecules as well. One of the first targets of ROS is the plant cell membrane, and the damage of it can lead to cell death. Earlier we found that an *in vitro* selected paraquat-tolerant (PT) Samsun tobacco genotype (Furusawa et al., 1984) showed elevated cytokinin levels (Székács et al., 2000) and higher protective antioxidant capacity as compared to the wild-type paraquat-sensitive (PS) Samsun line (Barna et al., 1993, 1995). The bipyridylium herbicide paraquat (1,1'-dimethyl-4,4′-bipyridylium) acts in the chloroplast in the light through the generation of ROS (Donahue et al., 1997). In addition, we also found that this PT tobacco genotype showed enhanced tolerance to necrotrophic pathogens and to various abiotic stresses (Barna et al., 1993, 1995). On the contrary, a SA-

Abbreviations: dpi, days post-inoculation; hpi, hours post-inoculation; HR, hypersensitive response; PR, pathogenesis-related; PS, paraquat sensitive Samsun tobacco; PT, paraquat tolerant cytokinin overproducing tobacco; RNase, ribonuclease enzyme; SA, salicylic acid; TMV, *Tobacco mosaic virus*.

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deficient *NahG* tobacco mutant of Xanthi-nc tobacco with the *N* resistance gene to TMV was more susceptible to a number of pathogens (Friedrich et al., 1995). In these transgenic tobacco plants expressing a bacterial *NahG* gene encoding salicylate hydroxylase (which transforms SA to catechol), little or no SA accumulation was detected after TMV infection (Gaffney et al., 1993). We found that enzymatic and non-enzymatic antioxidants were down-regulated after TMV infection in *NahG* plants as compared to its Xanthi control line (Fodor et al., 1997; Király et al., 2002).

Due to the transcriptional reprogramming in infected plants a large array of genes can be induced, among them those encoding various pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999; Sels et al., 2008). PR-1 proteins were initially identified as pathogen-inducible proteins in tobacco leaves after *Tobacco mosaic virus* (TMV) infection. Despite many years of intensive research the biological function of the PR-1 protein family has remained unclear but these proteins have been often investigated as useful resistance markers (Van Loon and Van Strien, 1999; Gamir et al., 2017). Transgenic tobacco plants constitutively over-expressing the *PR-1c* gene did not display increased resistance against TMV infection (Cutt et al., 1989). In barley leaves the PR-1b protein was shown to contribute to the penetration resistance against barley powdery mildew (Schultheiss et al., 2003). Limited information is available about the role of PR-1 proteins in powdery mildew-infected tobacco. Currently promising research targets are the plant-specific WRKY transcription factors because of their up-regulation of *PR*-genes (Chen and Chen, 2000; Rushton et al., 2010; Yamamoto et al., 2004). The up-regulation of the *WRKY12* gene was observed in TMV-infected tobacco plants. The WRKY12 protein participates in the up-regulation of *PR-1a* gene in TMV-inoculated tobacco leaves (Van Verk et al., 2008). The potential role of *WRKY* genes in powdery mildew-infected tobacco leaves is still far from understood (Marchive et al., 2007).

We wanted to test the effects of pre-treatment with the protein kinase inhibitor staurosporine and the protein phosphatase inhibitor okadaic acid on the infection of tobacco with powdery mildew or TMV. Since a superfamily of barley powdery mildew effector candidates showed structural similarities to ribonucleases (RNases) (Pedersen et al., 2012), the RNase pattern of extracts from powdery mildew infected and control tobacco leaves were also analyzed. Considering that PR-proteins and some WRKY transcription factors are thought to be important players in plant–pathogen interactions, and the information on the effect of powdery mildew infection on these gene expressions in tobacco is very limited, changes of the expression of *PR-1b*, *PR-1c* and *WRKY12* genes were compared in leaves of all tobacco lines following powdery mildew or TMV infection as well.

2. Materials and methods

2.1. Plants and pathogens

Nicotiana tabacum L. cv. Xanthi-nc carrying the hypersensitive *N* resistance gene against TMV, and its SA-deficient *NahG* transgenic line (Gaffney et al., 1993), as well as an *in vitro* selected paraquat-tolerant (PT) *Nicotiana tabacum* L. cv. Samsun and its control, a paraquat-sensitive (PS) Samsun tobacco line (Furusawa et al., 1984) were grown under standard greenhouse conditions (18–23 °C; about 16 h daylight with 160 μmol m^{-2} s^{-1} supplemental light for 8 h per day; relative humidity: 75–80%). To compare incompatible to compatible tobacco - powdery mildew interactions, *Nicotiana glutinosa* plants were also grown under the same conditions.

For all experiments the fourth, fifth and sixth true leaves of 60- to 70-day-old tobaccos were used. Leaves of all tobacco lines were inoculated with powdery mildew (*Golovinomyces orontii* isolate BP-1TOB) (Vági et al., 2007). Conidia from heavily infected Samsun tobacco leaves were dusted equally on tobacco plants to be tested. The formation of symptoms on leaves was evaluated every day after infections. In separate experiments tobacco leaves were inoculated with a suspension of the U_1 strain of TMV as described earlier (Fodor et al., 1997). Briefly, the virus was maintained in *N. tabacum* cv. Samsun plants carrying no *N* resistance gene. Leaves of infected plants showing typical disease symptoms of TMV were ground (1 g in 10 ml of 10 mM sodium phosphate buffer, pH 7.0) in a mortar and the homogenate was used for inoculation. The inoculated and control plants were incubated in growth chambers (23 °C, 63% humidity, 150 μmol m^{-2} s^{-1} light exposure with 16-h light period).

2.2. Conductivity measurements

Plant membrane damage was detected by measuring ion leakage from inoculated and control tobacco leaves with a conductivity meter as described earlier by Harrach et al. (2008). Briefly, 5 leaf discs (each 1 cm of diameter) were cut from healthy and infected tobacco leaves 24 h post-inoculation (hpi), and floated on 10 ml distilled water or in separate experiments in 20 mM H_2O_2 in Petri dishes. Samples were randomly taken from the fourth (older), fifth and sixth (younger) true leaves of tobaccos. Changes in conductivity of distilled water or H_2O_2 were measured by a conductivity bridge (Radelkis OK-102/10, Budapest, Hungary).

2.3. Effect of pre-treatment with staurosporine and okadaic acid on the visible symptoms of powdery mildew or TMV inoculations

The effects of pre-treatment with the protein kinase inhibitor staurosporine and the protein phosphatase inhibitor okadaic acid on powdery mildew and TMV infections on tobacco lines were investigated as well. In each experiment staurosporine (5 μM), okadaic acid (0.2 μM) or distilled water as control was brushed on both surface of fully developed 4th to 6th leaves of four tobacco plants 3 h before inoculation with powdery mildew or TMV. In separate experiments, 3 h after pre-treatment with the inhibitors, the treated leaves were washed off with distilled water, blotted dry with paper towel and inoculated with powdery mildew or TMV. Water pre-treated leaves were handled by the same way.

2.4. Ribonuclease activity measurements

Patterns of ribonuclease (RNase) isoenzymes in control and powdery mildew infected tobacco leaf extracts were analyzed. Briefly, 0.5 g leaf tissue was homogenized at 0–4 °C in 3 ml of TRIS–HCl buffer (50 mM, pH 7.8) containing 1 mM EDTA-Na_2 and 7.5% (w/v) soluble polyvinylpyrrolidone. Homogenates were centrifuged (12,000 *g*, 20 min, 4 °C) and the proteins of these total extracts were separated with 10% native polyacrylamide gel electrophoresis (PAGE) and analyzed by specific negative staining with 1% low molecular mass RNA and 0.1% toluidine blue as described earlier (Barna et al., 1989). Total RNase activity of tobacco leaf extracts was determined by densitometric analysis of all RNase bands on PAGE gels (Multiplex Band Analysis, AlphaView SA, version 3.4.0).

2.5. RNA extraction and gene expression analysis by RT-PCR

To analyze the expression of *PR-1b*, *PR-1c* and *WRKY12* genes a reverse transcription - polymerase chain reaction (RT-PCR) procedure was applied as described earlier (Rys et al., 2014). Total RNA was extracted from 0.1 g infected or control tobacco leaf material ground under liquid nitrogen with a Total RNA Miniprep kit (Viogene, Sunnyvale, CA, USA). Reverse transcription (RT) of 2.5 μg total