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Phosphoproteomic analysis of induced resistance reveals activation of signal transduction processes by beneficial and pathogenic interaction in grapevine

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ABSTRACT

Protein phosphorylation regulates several key processes of the plant immune system. Protein kinases and phosphatases are pivotal regulators of defense mechanisms elicited by resistance inducers. However, the phosphorylation cascades that trigger the induced resistance mechanisms in plants have not yet been deeply investigated. The beneficial fungus *Trichoderma harzianum* T39 (T39) induces resistance against grapevine downy mildew (*Plasmopara viticola*), but its efficacy could be further improved by a better understanding of the cellular regulations involved. We investigated quantitative changes in the grapevine phosphoproteome during T39-induced resistance to get an overview of regulatory mechanisms of downy mildew resistance. Immunodetection experiments revealed activation of the 45 and 49 kDa kinases by T39 treatment both before and after pathogen inoculation, and the phosphoproteomic analysis identified 103 phosphopeptides that were significantly affected by the phosphorylation cascades during T39-induced resistance. Peptides affected by T39 treatment showed comparable phosphorylation levels after *P. viticola* inoculation, indicating activation of the microbial recognition machinery before pathogen infection. Phosphorylation profiles of proteins related to photosynthetic processes and protein ubiquitination indicated a partial overlap of cellular responses in T39-treated and control plants. However, phosphorylation changes of proteins involved in response to stimuli, signal transduction, hormone signaling, gene expression regulation, and RNA metabolism were exclusively elicited by *P. viticola* inoculation in T39-treated plants. These results highlighted the relevance of phosphorylation changes during T39-induced resistance and identified key regulator candidates of the grapevine defense against downy mildew.

1. Introduction

Protein phosphorylation is an important and intensively studied post-translational modification of proteins and regulates many plant processes, including growth, development and defense (Park et al., 2012). The plant immune system recognizes exogenous microorganisms and responds by activating defense mechanisms through phosphorylation and dephosphorylation events within the proteome (Park et al., 2012). Plant defenses are triggered by pattern recognition receptors (PRRs) in response to pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), and receptor protein kinases associated with the plasma membrane are key sensors that recognize extracellular stimuli (Tena et al., 2011). Mitogen-activated protein kinase (MAPK) and phosphatase cascades are important for transducing signals generated by receptors into cellular responses through the downstream phosphorylation changes of target proteins (Meng and Zhang, 2013), and these processes are essential to the establishment of pathogen resistance (Pitzschke et al., 2009). The roles played by phosphorylation and dephosphorylation cascades in the activation of plant defenses include regulation of transcription factors and enzymatic reactions that lead to the biosynthesis of defense-related hormones and antimicrobial compounds (Tena et al., 2011).

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The defense mechanism called induced systemic resistance (ISR) (Pieterse et al., 2009) can be activated by treatments with various natural and synthetic compounds or beneficial microorganisms (Conrath et al., 2006) and increases plant resistance against various pathogens (van der Ent et al., 2009). ISR is commonly associated with enhanced defense reaction following pathogen inoculation, also known as 'priming' (Conrath et al., 2006; Verhagen et al., 2004). Priming of *Arabidopsis thaliana* is associated with accumulation of the inactive protein kinases MPK3 and MPK6, which are activated by phosphorylation upon exposure of primed plants to biotic or abiotic stresses (Beckers et al., 2009). MPK3 and MPK6 play a major role in benzodiathiazole (BTH)-induced priming (Beckers et al., 2009), while MPK6 and MPK4 are implicated in the priming activated by N-acyl-homoserine lactone (Schikora et al., 2011) and phosphite (Massoud et al., 2012), respectively. This suggests that specific MAPKs are responsible for fine regulation of plant defenses in relation to the type of resistance inducer and/or stress exposure. However, signal transduction cascades activated by MAPKs and phosphorylation changes of target proteins have not yet been deeply investigated for induced resistance mechanisms in plants. Post-translational modification of receptors and cellular regulators are particularly important to understand the regulation processes of plant interaction with beneficial microorganisms (Massart et al., 2015).

Grapevine (*Vitis vinifera*) is a major fruit crop worldwide and it can be attacked by a large number of pests and pathogens. Among those, the biotrophic oomycete *Plasmopara viticola* causes grapevine downy mildew and requires frequent application of chemical fungicides to avoid yield and quality loss (Gessler et al., 2011). Concerns about the environmental impact of the overuse of pesticides have sparked interest in developing alternative methods to chemical treatments (Gessler et al., 2011), among which resistance inducers gained increasing interest in the last years. Several molecules and beneficial microorganisms have been shown to induce grapevine resistance to downy mildew, and they represent promising alternatives to chemical fungicides (Delaunois et al., 2014). However, the efficacy of resistance inducers on grapevine is influenced by several factors, including agro-climatic conditions (Delaunois et al., 2014), cultivars (Banani et al., 2014) and abiotic stresses (Roatti et al., 2013). A better understanding of cellular regulation during plant resistance induction may help in finding ways to maximize efficacy of resistance inducers and optimize their use under field conditions (Walters et al., 2013). *Trichoderma harzianum* T39 (T39) induces resistance in grapevine against *P. viticola* with a mechanism of action based on complex reprogramming of the leaf transcriptome and proteome (Palmieri et al., 2012; Perazzolli et al., 2012). A relevant fraction of genes and proteins with significant changes in abundance after T39 treatment and *P. viticola* inoculation was assigned to the functional category of signal transduction (*e.g.*, protein kinases, receptor protein kinases and phosphatases) (Palmieri et al., 2012; Perazzolli et al., 2012), suggesting a key role of protein phosphorylation during T39-induced resistance.

The role of protein phosphorylation in the activation of grapevine defense mechanisms was demonstrated in methyl jasmonate (MeJA)-induced resistance (Faurie et al., 2009) and in cyclodextrin-elicited resveratrol production (Belchi-Navarro et al., 2013) by application of phosphatase inhibitors to cell cultures. Defense reactions were mediated by MAPK activation in grapevine (Dubreuil-Maurizi et al., 2010; Poinssot et al., 2003; Vandelle et al., 2006) and the MAPKs of 45 and 49 kDa were activated by laminarin and protein hydrolysates in the induction of resistance against downy mildew (Gauthier et al., 2014; Lachhab et al., 2014). The expression profiles of MAPKs and calcium-dependent protein kinases indicated specific functionalization in defense- and growth-related processes in grapevine (Chen et al., 2013; Kiselev et al., 2013; Wang et al., 2014), but their exact role in the signal transduction and phosphorylation of the target proteins through signaling cascades has remained elusive so far. Global changes of protein phosphorylation state in grapevine were only recently analyzed in response to *Lobesia botrana* (Melo-Braga et al., 2012) and Flavescence dorée phytoplasma infections (Margaria et al., 2013), where complex changes in the occupancy of several phosphorylation sites were demonstrated.

In this study, MAPK phosphorylation and quantitative changes in the phosphoproteome were analyzed in grapevine leaves during T39-induced resistance and in response to *P. viticola* inoculation. Our aim was to identify MAPK target proteins that are phosphorylated and dephosphorylated during grapevine response to the pathogen (*P. viticola*) and the beneficial microorganism (T39) in order to get an overview of proteins and mechanisms implicated in T39-induced resistance.

2. Materials and methods

2.1. Resistance induction and pathogen inoculation

Two-year-old plants of the susceptible grapevine *V. vinifera* cv. Pinot noir plants grafted onto Kober 5BB were grown for two months under greenhouse conditions at 25 ± 1 °C with a photoperiod of 16 h light and relative humidity (RH) of 60 ± 10%. A commercial product based on *T. harzianum* T39 (Trichodex, Makhteshim Agan Industries Ltd., Israel) was applied at a concentration of 8 g/L in water according to the manufacturer's dosage instruction, corresponding to a conidia suspension of 10^5 colony forming units/mL. All leaves of grapevine plants were treated with water (H_2O) or T39 using a compressed-air hand sprayer (20–30 mL per plant). Treatments were carried out three times: three, two and one day before pathogen inoculation to induce the highest level of phenotypic resistance activation (Perazzolli et al., 2008). A fresh suspension of *P. viticola* sporangia (10^5 sporangia/mL) was prepared as previously described (Perazzolli et al., 2011) and sprayed onto the abaxial leaf surfaces of grapevine leaves using a compressed air hand sprayer (20–30 mL per plant). Inoculated plants were incubated overnight in the dark at 25 ± 1 °C with 99–100% RH and kept under greenhouse conditions. Ten days after inoculation, disease severity was assessed visually as percentage of abaxial leaf area covered by white sporulation of *P. viticola* (EPPO, 2001). All experiments were carried out in triplicate and one representative experiment is presented in the results.

2.2. Sample collection and protein extraction

Leaf samples were collected immediately before inoculation (0 h), at 6 h and at 24 h post inoculation (6 and 24 hpi) with *P. viticola* from H_2O- and T39-treated plants. For each treatment, leaf samples from three different plants (replicates) were collected at each time point with a total of nine plants for each treatment. Protein extraction and quantification was carried out as described by Palmieri et al. (2012). Briefly, ground leaf samples were suspended in 10 volumes of a trichloroacetic acid/acetone solution (10% w/v trichloroacetic acid and 0.07% w/v 2-mercaptoethanol in acetone) for overnight precipitation and, after centrifugation, precipitated proteins were washed twice with three volumes of cold (−20 °C) 100% acetone. The air-dried protein pellet was suspended in the solubilization buffer (6 M urea, 2 M thiourea, 1% CHAPS, 2 mM dithiothreitol) supplemented with protease inhibitor cocktail (Complete Tablet, Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined with a Bio-Rad Protein Assay reagent (Bio-Rad).