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Research article

Response of phytohormones and correlation of SAR signal pathway genes to the different resistance levels of grapevine against *Plasmopara viticola* infection

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A B S T R A C T

Phytohormones play an important role in the process of disease resistance in plants. Here, we investigated which among salicylic acid, jasmonic acid, and abscisic acid performs a key role in plant defense after *Plasmopara viticola* infection in grapevine. We used grapevines possessing different resistance levels against *P. viticola* infection to study the relationship between the expression of key genes in the related resistance signaling pathways and the level of resistance. We performed high-performance liquid chromatography–mass spectrometry to estimate the phytohormone contents in grape leaves at different time points after the infection. Furthermore, we performed quantitative analyses of key genes such as *EDS1, PAD4, ICS2, PAL, NPR1, TGA1,* and *PR1* in the systemic acquired resistance pathway by quantitative reverse transcription-polymerase chain reaction. The results showed an increased variation in the SA content, which was maintained at high levels, after *P. viticola* infection in plant species exhibiting stronger resistance to the pathogen; this finding highlights the importance of SA in plant defense mechanisms. Moreover, *EDS1* and *PAD4* expression did not show a positive correlation with disease resistance in grape; however, higher expression of other genes that were analyzed was observed in highly resistant grape varieties. Our results provide insights into the role of phytohormone regulation in the induction and maintenance of plant defense response to pathogens.

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1. Introduction

Grapevine (*Vitis* spp.), an economically important and one of the most extensively grown plants worldwide, is susceptible to attacks by pathogens, including downy mildew. Downy mildew, which is caused by an obligate biotrophic oomycete, *Plasmopara viticola* (Berk. and Curtis) Berl & De Toni, is a common grapevine disease, widespread throughout the world. The economic and negative environmental impact of the disease necessitates formulation of alternative strategies, involving activation of plant's innate defense system, against it. In recent years, detailed resistance mechanisms have been described in a few model species (Pieterse and Dicke, 2007). These mechanisms often involve a signal transduction cascade triggered by infection, which induces the resistance response. In one of these mechanisms, grapevine resistance is triggered by elicitation of its innate immunity.

Upon infection by pathogens, plants identify pathogen-associated molecular patterns (PAMP) through pattern recognition receptors (PRR) present on the plasma membrane, resulting in a PAMP-triggered immunity (PTI) (Badel et al., 2002). Strong pathogens release effectors that can weaken the PTI in plant cells. This results in a resistance (R) protein-mediated activation of an effector-triggered immunity (ETI), which can promote the initiation of a hypersensitive response (HR) and generation of reactive oxygen species (ROS), as well as the expression of pathogenesis-related (PR) genes (Shamsul et al., 2012). Plant hormones play important

Abbreviations used: SA, salicylic acid; ABA, abscisic acid; ETI, effector-triggered immunity; *EDS1*, enhanced disease susceptibility 1; HR, hypersensitive response; *ICS2*, isochorismate synthase 2; JA, jasmonic acid; *NPR1*, non-expresser of pathogenesis related gene 1; *PAD4*, phytoalexin deficient 4; *PAL*, phenylalanine ammonia-lyase; PAMP, Pathogen-associated molecular patterns; *PR1*, pathogenesis-related 1; PTI, PAMP-triggered immunity; SAR, systemic acquired resistance.

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roles as signaling molecules, during disease resistance. Several reports have confirmed the existence of plant immune responses as well as the involvement of salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) in such responses (Vlot et al., 2009; Bari and Jones, 2009). Different plant hormone signals can trigger a series of physiological and metabolic processes in cells by regulating resistance-related genes and by initiating the corresponding immune responses (Koornneef and Pieterse, 2008).

Plant resistance to biotrophic pathogens has been classically thought to be mediated through the SA signaling pathway (Loake and Grant, 2007). SA accumulation, as well as the coordinated expression of PR genes encoding small proteins with antimicrobial activity, is also necessary for the onset of Systemic Acquired Resistance (SAR) in plants. SAR is a plant immune response that establishes broad-spectrum resistance in tissues distant from the site of primary infection (Dong, 2004). When infected with pathogens, the SA levels in plants drastically increase, leading to the expression of PR genes. This increase in SA levels usually occurs through two pathways, which include the catalysis of chorismate by isochorismate synthase (ICS) and that of phenylalanine by phenylalanine ammonia lyase (PAL). *EDS1* gene, which generally participates downstream of the R gene, can induce the initial accumulation of SA and elementary development of HR. Thereafter, it can function along with *PAD4* (phytoalexin deficient 4) to induce further accumulation of SA (Tsuda et al., 2009). The accumulation of SA induces the activation of NPR1 (non-expresser of pathogenesis related gene 1), which is transferred inside the nucleus from the cytoplasm. NPR1 interacts with TGA, a basic leucine zipper (bZIP) transcription factor in the nucleus, to induce downstream expression of the PR gene (Loake and Grant, 2007).

Recent completion of *Vitis vinifera* genome sequencing in a highly homozygous genotype and in a heterozygous grapevine variety has led to the identification of putative resistance genes and defense signaling elements (Borie et al., 2004). Transcriptomic analysis indicates that downy mildew resistance is mainly a post-infection phenomenon (Polesani et al., 2010), and emphasizes the importance of transcriptional reprogramming in both the resistant and susceptible genotypes in response to *P. viticola* inoculation (Polesani et al., 2010; Legay et al., 2011; Malacarne et al., 2011). Transcriptional changes associated with *P. viticola* infection of susceptible grapevines have been related to a weak defense response (Polesani et al., 2010) and to the establishment of a compatible interaction (Hayes et al., 2010; Gamm et al., 2011). The response of resistant genotypes has been characterized by strong and rapid transcriptional reprogramming of processes related to defense, signal transduction, and secondary metabolism, which are either not induced or induced to a lesser extent, in susceptible grapevines (Kortekamp, 2006; Figueiredo et al., 2012). In particular, downy mildew resistance has been correlated with enhanced expression of genes encoding PR proteins and enzymes of phenylpropanoid biosynthesis, and with specific modulation of signal transduction components and markers of HR in resistant grapevines (Malacarne et al., 2011; Figueiredo et al., 2012).

Given the pivotal role of SAR in plant defense, and to systematically analyze the changes in the process of the grapevine resistance to *P. viticola*, we chose six wild grapevine species with different resistance levels for our investigations. Since all *V. vinifera* cultivars are susceptible to *P. viticola*, the resistance needs to be introduced from other *Vitis* species. It is generally considered that *Muscadine rotundifolia* (a subgenus of *Vitis*) is completely immune to downy mildew, Chinese *Vitis amurensis* has considerable resistance, whereas *V. vinifera* has poor resistance. In the present study, we estimated the phytohormone content in different grape species with varying susceptibility to infection and resistance levels, to decipher their functions in resistance of the vines against downy mildew and to determine the phytohormone that is most important in plant immunity. To achieve this, we quantitatively analyzed the differential expression of pivotal genes in the SAR process in the above-mentioned grape species. Our study, therefore, provides a broad overview of the molecular events underlying the changes induced by *P. viticola* infection in susceptible and resistant grapevine species and will provide valuable candidate genes that could be used to develop commercial mildew-resistant grapevine plants.

2. Materials and methods

2.1. Plant material and *P. viticola* inoculation

One-year-old grapevines with different levels of resistance against downy mildew, including *V. vinifera*, *V. amurensis*, and *M. rotundifolia*, were maintained in a greenhouse under a 16-h light/8-h dark photoperiod at 25 °C and 85% relative humidity. Two cultivars of each of the three species were used, namely *V. vinifera* Chardonnay and Cabernet, *V. amurensis* Shuanghong and Zuoshanyi, and *M. rotundifolia* Noble and Carlos.

A mix culture of *P. viticola* was obtained from a natural field population in the grape cultivation base of China Agricultural University (Beijing, China). The excised leaves were rinsed with distilled water and carefully dried on filter paper. Leaf discs (11 mm in diameter) were punched out using a cork borer and placed (bottom side up) on water agar (0.8% wt/vol) in petri dishes. Sixty discs of each genotype were inoculated. Inoculation was done by applying 35 μL droplets of the sporangial suspension (10^5 sporangia/ml of deionized water) onto the discs. The leaf discs in the petri dishes were maintained in a culture chamber at 25/22 °C with a photoperiod of 16/8 h (light/dark, respectively). Deionized water was applied as control. Disease severity, disease incidence, and sporulation density on leaf discs were quantified 8 days post inoculation (dpi). Disease incidences were quantified by determining the number of discs with sporulation per total number of discs, as described by Boso and Kassemeyer (Boso and Kassemeyer, 2008). Magnitude of sporulation density per individual was concomitantly rated by a visual index, namely the OIV452 descriptor recommended by the Office International de la Vigne et du Vin (Anonymous, 2001).

The third-to fifth-unfolded leaf surfaces were sprayed with freshly collected sporangia re-suspended in water at approximately 10^5 spores/mL. After *P. viticola* treatment, leaf from the shoot apex was harvested from each of the three vines, and the three leaves from each cultivar were combined to represent one replicate. Three independent replicates were collected for each sample. Infected leaves were collected at 0, 2, 6, 12, and 16 h post-infection (hpi), and 1, 3, 5, and 8 days post-infection (dpi). After the collection, the leaves were immediately frozen in liquid nitrogen and stored at −80 °C. The same genotypes were used in the studies for RT-PCR and hormone quantification. Control samples were harvested from water-treated leaves, incubated under the same conditions.

2.2. Observation of *P. viticola* development

Leaf discs were stained with lactophenol-trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue, dissolved in 10 mL distilled water), as described by Keogh et al. (Keogh et al., 1980). The discs were then boiled for approximately 30 min in the stain solution and decolorized in chloral hydrate (2.5 g chloral hydrate dissolved in 1 mL distilled water) for at least 30 min. After multiple rounds of decoloration, the background of the samples was reduced. Finally, the samples were equilibrated with 50% glycerol and observed under a light microscope (Olympus, Japan).

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