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Role of dioxygenase α-DOX2 and SA in basal response and in hexanoic acid-induced resistance of tomato (*Solanum lycopersicum*) plants against *Botrytis cinerea*

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ABSTRACT

Resistance of tomato (*Solanum Lycopersicum*) to the fungal pathogen *Botrytis cinerea* requires complex interplay between hormonal signalling. In this study, we explored the involvement of new oxylipins in the tomato basal and induced response to this necrotroph through the functional analysis of the tomato α-dioxygenase2 (α-DOX2)-deficient mutant *divaricata*. We also investigated the role of SA in the defence response against this necrotrophic fungus using SA-deficient tomato *nahG* plants. The plants lacking dioxigenase α-DOX2, which catalyses oxylipins production from fatty acids, were more susceptible to *Botrytis*, and hexanoic acid-induced resistance (Hx-IR) was impaired; hence α-DOX2 is required for both tomato defence and the enhanced protection conferred by natural inducer hexanoic acid (Hx) against *B. cinerea*. The *divaricata* plants accumulated less pathogen-induced callose and presented lower levels of jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA) upon infection if compared to the wild type. Glutathion-S-transferase (*GST*) gene expression decreased and ROS production significantly increased in *Botrytis*-infected *divaricata* plants. These results indicate that absence of α-DOX2 influences the hormonal changes, oxidative burst and callose deposition that occur upon *Botrytis* infection in tomato. The study of SA-deficient *nahG* tomato plants showed that the plants with low SA levels displayed increased resistance to *Botrytis*, but were unable to display Hx-IR. This supports the involvement of SA in Hx-IR. *NaghG* plants displayed reduced callose and ROS accumulation upon infection and an increased *GST* expression. This reflects a positive relationship between SA and these defensive mechanisms in tomato. Finally, Hx boosted the pathogen-induced callose in *nahG* plants, suggesting that this priming mechanism is SA-independent. Our results support the involvement of the oxylipins pathway and SA in tomato response to *Botrytis*, probably through complex crosstalk of the hormonal balance with callose and ROS accumulation, and reinforce the role of the oxidative stress in the outcome of the plant-*Botrytis* interaction.

Introduction

Plants respond to pathogens by activating basal resistance mechanisms to prevent disease. This process is organised through a complex hormonal network that involves mainly jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), gibberellic acid, nitric oxide and auxins. The fine equilibrium and timing of hormonal changes coordinate plant metabolism and determine the outcome of a stress situation (Robert-Seilaniantz et al., 2011). SA is generally considered the main part of the defence responses against biotrophic pathogens, whereas JA participates in activating responses against necrotrophs (Beckers and Spoel, 2006). The crosstalk between SA and JA creates a flexible signalling

Abbreviations: DAB, 3,3′-diaminobencidine; α-DOX2, α-dioxygenase2; ABA, abscisic acid; ET, ethylene; Hx, hexanoic acid; Hx-IR, hexanoic acid-induced resistance; hpi, hours post-inoculation; H_2O_2, hydrogen peroxide; HR, hypersensitive response; IR, induced resistance; ISR, induced systemic resistance; JA, jasmonic acid; LSD, least significant difference; MM, Moneymaker; NBT, nitroblue tetrazolium; OPDA, *powdery mildew resistant 4* (*pmr4*) oxylipin 12-oxo-phytodienoic acid; ROS, reactive oxygen species; SA, salicylic acid; O_2^-, superoxide ion; SAR, systemic acquired resistance; WT, wild type.

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network that leads to the expression of different responses in accordance with the challenging pathogen, including the hypersensitive response (HR), cell wall strengthening, oxidative burst and the expression of various defence-related genes (Bostock, 2005; Glazebrook, 2005). To prevent infection, attacked cells respond by the local reinforcement of the cell wall beneath the site of the penetration attempt by forming a papilla. This process involves callose deposition in addition to the accumulation of H_2O_2 and phenolic compounds, as well as increased amounts of proteins and glycoproteins with hydrolytic and antifungal properties (Osbourn et al., 1996). H_2O_2, together with other reactive oxygen species (ROS), plays a major role in plant-pathogen interactions. The oxidative burst may contribute to the killing of biotrophic pathogens and/or the activation of further defence reactions. Nevertheless, ROS scavenging systems are crucial for the suppression of toxic ROS levels in cells and must be regulated very tightly (Lamb and Dixon, 1997).

In response to the first pathogen attack, plants become more resistant against further challenges, what is known as induced resistance (IR). In addition, IR can also be prompted by other non-pathogenic microorganisms or by treatment with natural or synthetic compounds known as defence inducers (Beckers and Conrath, 2007). Several IR processes are associated with the enhanced capacity to express specific defence responses upon pathogen attack, which is called priming (Conrath, 2011). Epigenetic changes have emerged as a putative priming mechanism that can involve modifications in DNA activity by methylation, histone modification or chromatin remodelling with no alteration to the nucleotide sequence (van den Burg and Takken, 2009; Slaughter et al., 2012). We demonstrated that root treatment with natural compound hexanoic acid (Hx) primed a set of defence responses that protect tomato plants against *Botrytis cinerea* (Leyva et al., 2008; Vicedo et al., 2009). Functional analyses evidenced that the JA-pathway is involved and required for hexanoic acid-induced resistance (Hx-IR) (Vicedo et al., 2009).

B. cinerea is a pathogen with a broad host range. Although the SA, JA, ET and ABA signalling pathways are known to be implicated in the interaction by complex interplays, tomato defence mechanisms against this pathogen are still unclear (Asselbergh et al., 2007; Diaz et al., 2002; Flors et al., 2007). ROS plays an important role in all fungus-plant interactions, especially for their signalling function. In plant-*Botrytis* interactions, oxidative imbalance takes place upon infection. The fungus can produce ROS and contribute to enhance this imbalance in plants by taking advantage of this host's response (Heller and Tudzinsky, 2011). We recently reported that the basal and Hx-IR of tomato plants to *B. cinerea* were associated with the differential expression of many genes involved in hormone and metabolites synthesis, signalling pathways, ROS and oxidative stress response (Finiti et al., 2014).

In this work, we further studied the tomato basal and Hx induced-defence response to *Botrytis* by investigating the role of a set of oxylipins. The term oxylipin covers a broad spectrum of compounds formed from unsaturated fatty acids in a cascade of reactions, most of which include at least one step of mono- or dioxygen-catalysed oxygenation (Vellosillo et al., 2007). α-dioxygenases initiate the synthesis of oxylipins by catalysing the incorporation of molecular oxygen at the α-methylene carbon atom of fatty acids, which leads to the production of aldehydes and hydroxides (Tirajoh et al., 2005). In *Arabidopsis*, α-DOX1 was shown to be involved in plant defence against the hemibiotrophic bacterium *Pseudomonas syringae*, probably by generating lipid-derived molecules for a process that protects plant tissues from oxidative stress and cell death (de León et al., 2002). More recently, Vicente et al. (2012) investigated the role of 9-lipoxygenase and α-dioxygenase oxylipin pathways in local and systemic defence in *Arabidopsis*. It has also been demonstrated that *Slα-DOX1* contributes to basal resistance to aphids in tomato and Arabidopsis plants (Avila et al., 2013), and that *Naα-DOX1* functions in anti-herbivore defence in *Nicotiana attenuata* (Steppuhn et al., 2010). No mutant line has been developed for tomato α-*DOX1* gene, but the tomato mutant *divaricata* described by van der Biezen et al. (1996) is deficient for *α-DOX2* and was used in this work to perform a functional analysis in response to *B. cinerea*. Bannenberg et al. (2009) demonstrated that *divaricata* was a null allele of *Sl*α-DOX2, which results in both defects in plant development and accumulation of anthocyanins, which supports that this dioxygenase is critical for normal plant development. In the present work, we found the involvement of tomato α-DOX2 in resistance against *B. cinerea*, which supports the relevance of oxylipins in the plant response to this necrotrophic fungus.

Classically, it is considered that resistance against necrotrophic pathogens requires the activation of the JA-signalling pathway, although SA can antagonise JA-signalling, and vice versa (Mur et al., 2006; Truman et al., 2007). The role of SA signalling in plant response to *B. cinerea* is complex. It contributes to the resistance of *Arabidopsis* against *B. cinerea*, but does not play a key role (Ferrari et al., 2003; Glazebrook, 2005). On the contrary, it has been recently shown in tomato that SA-deficient *nahG* plants expressing a SA hydroxylase that degrades SA are less susceptible to *B. cinerea* (El Oirdi et al., 2011). These authors also showed that *B. cinerea* manipulates the SA- and JA-pathways to promote disease development. In this work, we analysed the involvement of SA in the basal and Hx-IR by using tomato *nahG* plants. The data reported herein study confirm the involvement of this hormone in tomato response to *B. cinerea* and show that is also required for Hx priming. Finally, we analysed callose deposition, ROS accumulation and hormone balance in *divaricata* and *nahG* plants upon fungal infection, which provides data for possible interesting interactions among different defence mechanisms in tomato.

Material and methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum*) plants were grown in jiffy pots for 4 weeks in a greenhouse with 16 h of daylight. Moneymaker WT, Moneymaker transgenic *nahG* (Brading et al., 2000), Condine Red WT and Condine Red mutant *divaricata* plants (Bannenberg et al., 2009) were kindly provided by Dr. Carmen Castresana (CNB-CSIC, Spain).

Microbial strains and growth conditions

Botrytis cinerea CECT2100 (Spanish collection of type cultures) was cultured on potato dextrose agar as described by Flors et al. (2007).

Plant treatment and pathogen inoculation on tomato plants

Four-week-old tomato plants were adapted at 20 °C, 16/8 h day/night and 70% relative humidity in climatic chambers for 2 days prior to the assays. Hexanoic acid treatment (Sigma-Aldrich, U.S.A.) was prepared in Hoagland solution at 8 mM, adjusted to pH 6 and applied to plants by watering 2 days prior to the infection. The final Hx concentration in the jiffy pots was 0.8 mM. Treated and/or infected plants were kept in climatic chambers. Spore collection of *B. cinerea* and inoculation on plants were done as described by Flors et al. (2007). Disease symptoms were estimated 72 h post-inoculation (hpi) by determining the average lesion diameter on the third and fourth inoculated leaves.