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Prolonged dark period modulates the oxidative burst and enzymatic antioxidant systems in the leaves of salicylic acid-treated tomato

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ABSTRACT

Salicylic acid (SA) is an important plant growth regulator playing a role in the hypersensitive reaction (HR) and the induction of systemic acquired resistance. Since the SA-mediated signalling pathways and the formation of reactive oxygen species (ROS) are light-dependent, the time- and concentration-specific induction of oxidative stress was investigated in leaves of tomato plants kept under light and dark conditions after treatments with 0.1 mM and 1 mM SA. The application of exogenous SA induced early superoxide- and H_2O_2 production in the leaves, which was different in the absence or presence of light and showed time- and concentration-dependent changes. 1 mM SA, which induced HR-like cell death resulted in two peaks in the H_2O_2 production in the light but the first, priming peak was not detected in the dark. Unlike 0.1 mM SA, 1 mM SA application induced NADPH oxidase activity leading to increased superoxide production in the first hours of SA treatments in the light. Moreover, SA treatments inhibited catalase (CAT) activity and caused a transient decline in ascorbate peroxidase (APX), the two main enzymes responsible for H_2O_2 degradation, which led to a fast H_2O_2 burst in the light. Their activity as well as the expression of some isoenzymes of SOD and APX increased only from the 12th h in the illuminated samples. The activity of NADPH oxidase and expression *SlRBOH1* gene encoding a NADPH oxidase subunit was much lower in the dark. In spite of low CAT and APX activity after SA treatments in the dark, the activation of guaiacol-dependent peroxidase (POD) could partially substitute H_2O_2 scavenging activity of these enzymes in the dark, which reduced the ROS burst and development of lesion formation in the leaves.

1. Introduction

Light is the most important energy source for biomass production and it is required for growth and developmental processes in plant kingdom (Janda et al., 2014). Light may also control the plant defence mechanisms and excess of light energy under stress conditions leads to oxidative stress, which may contribute to the initiation of cell death in tissues (Karpinski et al., 2003; Kangasjärvi et al., 2012; Ballaré, 2014). Plants are able to sense the amount of photons, the intensity and quality of light as well as the changes in light/dark cycles (Chen et al., 2004). Hence, the absence of light (i.e. prolonged darkness) can alter the light-dependent activation of plant responses and can turn up new signalling and regulation pathways of defence.

Salicylic acid (SA) is a natural phenolic compound, which accumulates under abiotic and biotic stress and controls physiological and biochemical functions in plants (Khan et al., 2015). The effect of SA depends on plant species and developmental stage, on the mode of application and concentration of SA or on environmental conditions (Raskin, 1992; Horváth et al., 2007; Hayat et al., 2010; Khan et al., 2013).

The amount of endogenous SA in unstressed plant tissues may change from 10 to 200 ng g^{-1} fresh mass to as high as 37.19 μg g^{-1} g fresh weight (FW), the latter was detected in rice (Silverman et al., 1995). Changes in free and glucosylated SA (bound SA) can be detected upon exposure of plants to abiotic stresses. Increases in free SA were found in mustard plants (from ~2 to ~5 μg g^{-1} FW) under heat stress (Dat et al., 1998) and in tobacco leaves exposed to UV-C light (from ~300 ng to 4 μg g^{-1} FW) (Catinot et al., 2008). It was found that upon ozone-exposure SA accumulated in the leaf tissues of wild type Col-0, an O_3-sensitive, Cvi-0 *Arabidopsis* genotypes, while NahG plants

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; HR, hypersensitive response; ROS, reactive oxygen species; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NBT, nitro blue tetrazolium; PM, plasma membrane; POD, guaiacol-dependent peroxidase; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase

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expressing salicylate hydroxylase gene failed to increase SA concentrations. The accumulation of reactive oxygen species (ROS), defence gene expression and severity of lesion formation correlated with SA levels in these genotypes upon ozone exposure (Rao and Davis, 1999).

SA-induced defence reactions can also protect plants against many pathogens, including fungi, bacteria and viruses. Pathogens can be classified into biotrophs, hemibiotrophs and nectrotrophs on the basis of their lifestyle, which may predict the response of plants to the infection and hormone signalling pathways initiated by the pathogen invasion. Thus the salicylate response is involved in resistance to many biotrophs (*Pseudomonas syringae*, *Xanthomonas campestris*) and some necrotrophs (*Botrytis cinerea*) in tomato (Thaler et al., 2004).

SA plays a role in the formation of local and systemic acquired resistance (SAR) and in the initiation of cell death in the tissues surrounding the invading pathogens (Li et al., 2014). SA levels can increase more than 40-fold, up to 75 μg g^{-1} FW after tobacco mosaic virus (TMV) infection in the immediate vicinity of hypersensitive response (HR), at the site of pathogen penetration and up to 1.5 μg g^{-1} FW in non-infected leaves (Enyedi et al., 1992). Interestingly, 10–15 day after infection SA accumulation proved to be an important component of susceptible disease response of tomato to *Xanthomonas campestris* cv. *vesicatoria* infection. In this work the authors found 22.06 μg g^{-1} FW SA in the leaves of UC828 tomato genotype, this SA accumulation led to an enhanced necrotic cell death in the infected tissues (O'Donnell et al., 2001).

The application of exogenous SA may enhance the resistance to abiotic and to biotic stresses and the development of the resistance may depend on the illumination since the accumulation of SA and the initiation of SA-mediated signalling pathways are light-dependent (Genoud et al., 2002; Chandra-Shekara et al., 2006). Plants infected in the dark showed reduced lesion formation in response to an avirulent pathogen (Zeier et al., 2004). In addition, SAR development in response to the infection is completely lost in the absence of light (Karpinski et al., 2003). Nevertheless, light availability is particularly important during the early stages of the plant-pathogen interaction (Griebel and Zeier, 2008).

There is close correlation between ROS production and changes in SA content (Xu and Brosche, 2014). SA-induced biotic stress tolerance depends on the accumulation of superoxide radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2), that are essential mediators of the HR and cell death induction and they could contribute to cellular redox homeostasis through the regulation of the expression and activity of antioxidant enzymes (Saruhan et al., 2012; Janda and Ruelland, 2015). To overcome oxidative stress, plants have fast acting defence systems such as non-enzymatic antioxidants and antioxidant enzymes to alleviate the oxidative damage (Mittler et al., 2011).

Several studies reported that SA treatments induced the accumulation of ROS by modulating the activity of some antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Khan et al., 2014). SODs belong to a group of metallo-enzymes, which are the main scavengers of superoxide by catalyzing the conversion of $\cdot O_2^-$ to H_2O_2. Mn-SOD is localized to mitochondria and peroxisomes, Fe-SODs function inside the chloroplast peroxisomes and isoforms of Cu/Zn-SOD can be found in the chloroplasts, in the cytosol as well as in peroxisomes and apoplast (Fernández-Ocaña et al., 2011). It was also found that SA induced the activity of Cu/Zn-SOD in tobacco plants (Horváth et al., 2007). Most of H_2O_2 in plant cells is eliminated by CAT and APX. SA can bind directly to the CAT enzyme and it can inhibit the activity of certain isoenzymes (Horváth et al., 2002). At the same time SA induces the activity of APXs and guaiacol-peroxidases (POD), which also catalyze the decomposition of H_2O_2 to water (Horváth et al., 2007; Rivas-San Vicente and Plasencia, 2011; Tari et al., 2015; Chen et al., 2016). However, the timing and the role of the early activation/inactivation of various isoforms of these antioxidant enzymes under SA-induced ROS wave are less known.

Formation of ROS is strictly coupled to light-driven electron transport chain in the chloroplasts under abiotic- or biotic stress (Bailey-Serres and Mittler, 2006; Xing et al., 2013). However, a flavoenzyme NADPH oxidase, localized to the plasma membrane (PM), also known as respiratory burst oxidase homologue (RBOH), can translocate electrons from cytosolic NADPH to oxygen, leading to the generation of $\cdot O_2^-$ in the apoplast (Sagi and Fluhr, 2006). The activation of NADPH oxidases mediates the progression of ROS signals from cell to cell under pathogen infection (Miller et al., 2009; Dubiella et al., 2012) and the enzyme is also activated by SA treatment (Kawano et al., 2004). Two RBOH genes, *SlRBOH1* and *SlWFI1*, were identified in tomato and it was found that *SlRBOH1* plays a role in apoplastic H_2O_2 production and in the induction of stomatal closure (Li et al., 2015). The apoplastic ROS burst mediated by NADPH oxidase can also communicate with ROS production in chloroplasts, because under high light conditions chloroplasts adopt a position adjacent to the PM (Shapiguzov et al., 2012). Moreover, the activity of NADPH oxidase can contribute to the first, priming oxidative burst after pathogen infection (Jiménez-Quesada et al., 2016).

Thus, the regulation of defence mechanisms and cell death in plant tissues seems to be different in light and dark conditions, although many pathogens are active in the dark. It was also found in many cases that HR was suppressed or delayed after pathogen infection in the dark (Chandra-Shekara et al., 2006; Grimmer et al., 2012), but the role of SA and SA induced signalling in this process remained unclear. There is also little information about the effects of different concentrations of SA on the expression and activity of antioxidant enzymes in the light or in the dark, especially of those, which participate in $\cdot O_2^-$ and H_2O_2 metabolism.

The aim of this study was to elucidate the time- and concentration-specific induction of oxidative defence responses elicited by SA in tomato leaves under normal photoperiod and extended dark conditions. The question is whether the SA-induced $\cdot O_2^-$ and H_2O_2 metabolism are mediated by different enzymes in the presence or absence of light. In this study we would like to reveal the changes in the activity of most important ROS producing and ROS-scavenging enzymes as a function of time in plants grown under normal photoperiod or under prolonged darkness. These results broaden our knowledge about the SA generated oxidative stress and defence in the first particularly important 24 h after SA accumulation in the presence or absence of light.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated at 26 °C for three days in the dark, and the seedlings were subsequently transferred to perlite for two weeks. The plants were then placed into plastic boxes (40 cm length, 30 cm width, 20 cm depth, 12 seedlings per box) filled with 20 L of modified Hoagland nutrient solution as described earlier (Poór et al., 2011). The culture medium was changed twice a week. Plants were grown in a controlled environment condition under 200 μmol m^{-2} s^{-1} photon flux density (F36W/GRO lamps, OSRAM SYLVANIA, Danvers, MA, USA), with 12/12-h light/dark period, a day/night temperatures of 24/22 °C and a relative humidity of 55–60% for eight weeks.

Tomatoes were treated with 0.1 mM or 1 mM salicylic acid (SA) supplied in the nutrient solution, which was presumed to adjust SA content to the levels that found in systemically induced leaves or in tissues exposed directly to pathogen infection. Half of the plants remained for 24 h under the growing light/dark cycle (light samples) or half of them were put into prolonged darkness (dark samples) at 25 °C. The experiments were conducted from 9 a.m. and were repeated three times. The samples were prepared from the second, fully expanded young leaves in three replicates 1; 3; 6; 12; 24 h after the different SA treatments.