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Research article

The involvement of ROS producing aldehyde oxidase in plant response to Tombusvirus infection

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ABSTRACT

The influence of *Tomato bushy stunt virus* (TBSV) infection on the activity and isoformic composition of aldehyde oxidase and catalase in *Nicotiana benthamiana* plants was investigated. It was shown that the infection of plants with TBSV results in enhancement of leaf aldehyde oxidase (AO) isoforms AO2 and AO3. Significantly enhanced levels of superoxide radical producing activity of AO isoforms were also detected. This is the first demonstration of involvement of plant AO in defense mechanisms against viral infection. In addition, the infection caused an increased accumulation of hydrogen peroxide, compared to mock-inoculated plants. The virus infection resulted in increased activity of catalase (CAT) and superoxide dismutase (SOD) in roots and leaves of *N. benthamiana*. Moreover, activation of two additional CAT isoforms was observed in the leaves of plants after virus inoculation. Our findings indicate that the virus infection significantly affects enzymes responsible for the balance of ROS accumulation in plant tissue in response to pathogen attack.

1. Introduction

Plants are constantly forced to confront numerous of challenges, caused by various biotic and abiotic factors such as salinity, drought and pathogen invasions. To overcome stress factors plants evolved different defense strategies. It is well known that reactive oxygen species (ROS) signaling network is involved in the regulation of numerous biological processes, including resistance to pathogens (Mittler et al., 2011). One of the earliest plant responses to pathogen invasion is a significant increase of ROS production, called oxidative (respiratory) burst (Baxter et al., 2014). During the oxidative burst hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and superoxide radical (O_2^-) are rapidly produced (Riedle-Bauer and Bauer, 2000). These molecules are usually presented in plants at very low concentrations, but under the influence of different stress factors the production of ROS significantly increases. ROS are highly reactive molecules and may be toxic for organism at large quantities, leading to the oxidative damage of nucleic acids, lipids and proteins. Because of the toxic effect of excessive ROS accumulation, plants evolved non-enzymatic and enzymatic systems to protect from their damaging effects (Mehdy, 1994). The oxidative burst also highly associated with the hypersensitive response (HR) which is one of the specific plant resistance mechanisms (Durner et al., 1997). Plants can form necrotic lesions around the site of a pathogen attack in order to prevent further spread of the infection (Richael and Gilchrist, 1999). After pathogen invasion and its recognition by the host, the production of ROS in cells increases and this may trigger HR in infected tissues (Otulak and Garbaczewska, 2010). H_2O_2 is involved in spread of ROS-mediated long-distance

Abbreviations: TBSV, *Tomato bushy stunt virus*; *N. benthamiana*, *Nicotiana benthamiana*; CAT, catalase; ROS, reactive oxygen species; H2O2, hydrogen peroxide; $_1O^2$, singlet oxygen; O^{2-}, superoxide radical; HR, hypersensitive response; AO, aldehyde oxidase; ABA, abscisic acid; PAGE, polyacrylamide gel electrophoresis; DAB, 3,3′-diaminobenzidine tetrahydrochloride; SA, salicylic acid.

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signal between cells, and may act as a trigger and one of the main factors of the oxidative burst in response to various stress factors (Mittler et al., 2011). High production of H_2O_2 plays an important role in plant resistance to viruses. H_2O_2 triggers HR activation in response to virus infection and results in strengthening of cell walls, preventing the spread of virus infection from cell to cell (Baebler et al., 2014; Jovel et al., 2011). However, it was reported that some viruses evolved the ability to overcome HR in plants and this mechanism may not be sufficient for pathogen restriction (Coll et al., 2010; Hussain et al., 2007). Interestingly, it has been recently shown, that the increased content of H_2O_2 in virus infected plants promote the spread of silencing signal between different cells, contributing to the activation of RNA interference (RNAi) in the whole organism (Liang et al., 2014).

Aldehyde oxidase (AO) is a cytosolic enzyme with molecular mass of 300 kDa, containing FAD, iron and molybdenum cofactor (Moco) as prosthetic groups and belongs to the family of molybdenum-containing hydroxylases (Koshiba et al., 1996). AO catalyzes the oxidative hydroxylation of different aromatic and non-aromatic aldehydes into corresponding carboxylic acids, and is also responsible for the heterocycles hydroxylation in plants and animals (Mendel and Bittner, 2006). It was reported that AO directly participates in the synthesis of phytohormones. The enzyme catalyzes oxidative conversion of abscisic aldehyde to abscisic acid (ABA), and oxidizes indole-3-acetaldehyde into indole-3-acetic acid (Koshiba et al., 1996; Omarov et al., 1998; Seo et al., 2000; Walker-Simmons et al., 1989). The involvement of AO in plant adaptation to abiotic stress factors was previously studied (Omarov et al., 1998; Sagi et al., 1998; Zdunek-Zastocka et al., 2004). The importance of AO during growth in conditions of high salinity was also recently confirmed by exogenous Mo-treatment of *Agropyron cristatum* plants, triggering activation of AO and thereby increasing adaptation of treated plants to salinity stress (Babenko et al., 2015). The functional interactions between ABA pathway and viral infections are still poorly understood. Earlier studies indicate that ABA synthesis is influenced by viral infections. For example, it was shown that the infection with *Tobacco mosaic virus* induced accumulation of ABA in *Nicotiana tabacum* (Fraser and Whenham, 1989). Recent studies showed that AO3 mutant of Arabidopsis (*aao3*), with reduced level of ABA content, accumulated higher titers of *Bamboo mosaic virus* (BaMV) (Alazem et al., 2014). Thus, it was suggested that ABA pathway may at least partly be involved in the defense against BaMV.

The involvement of a member of AO in production of H_2O_2 in plants was previously reported (Yesbergenova et al., 2005). It was further demonstrated that heterologously expressed two members of the AO family from *Arabidopsis thaliana* generate hydrogen peroxide and superoxide anions by transferring aldehyde-derived electrons to molecular oxygen. Moreover, it was shown that AO enzyme isoforms are directly involved not only in the generation of H_2O_2, but also in production of one another ROS - O_2^- in Arabidopsis, using NADH as a substrate (Zarepour et al., 2012). To avoid the negative effect of O_2^- high concentration in tissues the molecule is converted to H_2O_2 by superoxide dismutase (SOD) – one of the key enzymes in the antioxidant defense (Bowler et al., 1992). In earlier studies it was shown that *Pepper mild mottle virus* infection in *N. benthamiana* plants trigger activation of SOD isoforms (Hakmaoui et al., 2012). Thus, the activation of both AO and SOD activities may lead to increased levels of H_2O_2 accumulation.

Catalase (CAT) plays an important role in regulation of cellular ROS accumulation. The enzyme catalyzes decomposition of H_2O_2 to water and oxygen and involved in various biological processes including plant resistance to pathogens (Radwan et al., 2010; Riedle-Bauer and Bauer, 2000). The most important function of CAT activity in plants is to prevent damaging of tissues when concentration of H_2O_2 is drastically increased (Willekens et al., 1997). Catalases represent a class of tetrameric heme-containing enzymes that converts two molecules of H_2O_2 into water and oxygen (Chelikani et al., 2004).

Tomato bushy stunt virus (TBSV) belongs to Tombusviridae family and has a single stranded positive sense RNA genome with length of about 4800 nt, which is wrapped by 180 subunits of capsid protein (Olson et al., 1983). The virus contains 5 different open reading frames: p33 and p92 for replicase, which translates from genomic RNA; capsid protein P41 is translated from subgenomic RNA_1, whereas P22 and P19 from subgenomic RNA_2 (Hayes et al., 1988; Hillman et al., 1989). P19 is a major viral pathogenicity determinant and functions as a suppressor of defensive RNAi (Omarov et al., 2006; Scholthof et al., 1995a,b; Scholthof, 2007).

To date very limited information is available regarding functional relationship between AO and CAT activities upon biotic stress factors. The role of AO in response to pathogen invasion has not yet been studied. The aim of this work was to investigate the influence of viral infection on AO and CAT activities in infected *N. benthamiana.* Our findings indicate that inoculation of plants with TBSV results in significant enhancement of AO2 and AO3 isoforms, whereas the activity of AO1 somewhat decreased. It is also shown that superoxide radical producing activity of AO isoforms increased upon infection. Moreover, virus infection resulted in increased accumulation of H_2O_2, in comparison with healthy plants. The results of this study reveal an important role of Moco-containing AO in defensive response to pathogen attack. In addition, here we describe a new method for rapid determination of TBSV virions in infected tissues.

2. Material and methods

2.1. Plant material

N. benthamiana plants were grown in growth room in conditions of long-day photoperiod (16-h light/8-h dark) and 75–80% relative humidity. Temperatures fluctuated from 20 to 27 °C and the average temperature during the day was 25 °C and 22 °C at night. For lighting of growth room lamps with 2700 K and 6400 K spectrum were used.

2.2. Plants inoculation by TBSV RNA transcripts

For plants inoculation in vitro generated transcripts of full-length TBSV cDNAs were used (Knorr et al., 1991; Scholthof et al., 1993; Scholthof et al., 1995a,b). For this, plasmids containing the inserts were linearized at the 3′-end of the viral cDNA sequence by restriction *Sma1* enzyme digest. Transcripts were synthesized using T7 RNA polymerase, and these transcripts were used for inoculation of plants as previously described (Hearne et al., 1990; Scholthof et al., 1993). Control plants were mock-inoculated by using phosphate buffer without viral RNA. Healthy and infected plants were grown separately in the same conditions.

2.3. Western blot analysis

Upper non-inoculated leaves were analyzed for the presence of P19 expression. For each experiment samples were combined from three different plants. Protein samples extracted from mock-inoculated and TBSV infected plants were separated by 12% SDS - polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Osmonics, Westborough, MA). After transfer the membranes were stained with Ponceau S (Sigma, St. Louis, MO) for verification of protein transfer efficiency. The resulting membrane was incubated with diluted primary