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A dissection of the effects of ethylene, H_2O_2 and high irradiance on antioxidants and several genes associated with stress and senescence in tobacco leaves

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ABSTRACT

Ethylene and hydrogen peroxide are involved in the modulation of stress responses in plants, but their interrelation is not well understood. This work was designed to find differences between the actions of ethylene and H_2O_2 on antioxidants and senescence markers. Leaves of *Nicotiana tabacum* were sprayed with H_2O_2 or with ethephon (precursor of ethylene). To find the possible modulation of responses to acute abiotic stress, ethephon- and H_2O_2-sprayed leaves were further subjected to high irradiance (HL). The application of H_2O_2 strongly stimulated ethylene synthesis (ACC). Ethylene and H_2O_2, as single factors, stimulated the trolox equivalent antioxidant capacity (TEAC) and the activity of catalase (CAT), in contrast to HL alone (stimulation of nonspecific peroxidases and the total glutathione pool). However, after combined treatments (ethylene + HL and H_2O_2 + HL), the stimulatory action of H_2O_2 was related to TEAC and CAT activity, while the application of ethylene stimulated the total glutathione pool. Hydrogen peroxide enhanced the expression of the three CAT genes (*Cat1*, *Cat2* and *Cat3*), in contrast to ethylene (*Cat2* and *Cat3*) and HL (*Cat1*). In regard to the markers of senescence and pathogenesis the most pronounced difference between the actions of ethylene and H_2O_2, as single factors, was related to *NPR1*, whereas when leaf spraying was combined with HL, differences were found at *WRKY53* and *PR1a*. HL reversed the stimulatory effects of H_2O_2/ethylene-driven enhancements of the expression of several genes (*Cat1*, *Cat2*, *NPR1*, *WRKY53*). These results show that multiple stressors, as usually encountered by plants in nature, may largely change those expression patterns of genes determined in a single factor analysis. Moreover, the actions of HL (often considered the internal H_2O_2 trigger) and of exogenous H_2O_2 on gene expression are clearly different.

Introduction

Changing environmental traits trigger a plethora of stress reactions in plants. The ability of plants to orchestrate the complicated network of stress responses determines their plasticity and fitness. Stressful conditions usually lead to the enhanced generation of reactive oxygen species (ROS), which are detrimental to a cell's metabolism. This is known as oxidative stress (see: Mittler et al., 2011 for a recent review). ROS, on the other hand, play a role as secondary messengers in the regulation of stress responses in plants. Stressful conditions, mostly via the formation of ROS, influence the level of plant stress hormones (ethylene, salicylic acid, jasmonic acid and abscisic acid). The most stable among ROS, hydrogen peroxide, seems to be particularly appropriate to play this function.

The plant hormone ethylene is involved in plant stress responses, mainly as a negative regulator of growth. It is biosynthesized from methionine to S-adenosyl-L-methionine (SAM) by the enzyme Met adenosyltransferase (Yang et al., 2008). SAM is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). The activity of ACS determines the rate of ethylene production. Therefore, regulation of this enzyme is crucial to ethylene biosynthesis. The final step requires oxygen and involves the action of the enzyme ACC-oxidase (ACO). Ethylene participates in the regulation of several developmental processes, such as flowering, fruit ripening, senescence and cell death (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Wang et al., 2002; Etheridge et al., 2005; Mühlenbock et al., 2008). It is also involved in biotic and abiotic stress responses (Chamnongpol et al., 1998; Mittler et al., 2011). A growing body of data shows that the action of ROS and ethylene can be protective in different stress situations. The

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application of H_2O_2 enhances protection against high irradiance (Karpinski et al., 1999) and chilling (Yu et al., 2002). Pretreatment with ethylene modulates the activity of peroxidases (Kangasjarvi et al., 1994; Pell et al., 1997; Dat et al., 2003; Almagro et al., 2009).

Several studies indicate that ROS are located up-stream of ethylene in the stress signaling pathways (Durner and Klessig, 1996; Chamnongpol et al., 1998; Dat et al., 2003; Overmeyer et al., 2003; Yoshida et al., 2009; Mittler et al., 2011). However, a comparison of micro-array data made by Geisler et al. (2006) showed that the effects of ROS on gene expression are independent of those of ethylene. The functioning of both ethylene- and H_2O_2-signaling during stress is quite complex and may lead either to survival or to cell death, but no major molecular switch has yet been clarified.

In this work, we attempt to identify the diverse effects of ethylene and H_2O_2 at the level of several genes related to stress and senescence. Because in nature plants are usually subjected to many adverse factors at the same time, we combined the application of these two stress-related compounds with treatment with high irradiance.

Materials and methods

Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. SR1) were grown in a glasshouse supplied with additional illumination by HS2000 lamps at an average intensity of 450 μmol quanta m^{-2} s^{-1}. The temperature in the light period varied between 20 and 30 °C depending on the weather conditions. During the dark period, the temperature was adjusted to 18 °C. Plants with approximately 5 leaves (8 weeks from sowing) were divided into three groups of 6 plants each: (1) plants irrigated with water (control), (2) plants sprayed with 6.6 mM ethephon, and (3) plants sprayed with 20 mM H_2O_2. Leaf spraying was done 2 h after the onset of light. Half of the control, ethephon- and H_2O_2-treated plants were kept at growth light conditions (GL), while the other half were illuminated with high irradiance (HL, 1000 μmol quantum m^{-2} s^{-1}) for 8 h. Immediately after the HL treatment, the maximal PSII quantum yield (F_v/F_m) was measured on fully expanded tobacco leaves after 20 min dark adaptation. Fluorescence measurements were done with PAM 2000 (Heinz Walz GmbH, Effeltrich, Germany). Leaves were collected and stored at −80 °C for further use.

Level of thiobarbituric acid reactive substances (TBARS)

Leaves (0.1 g FW) were homogenized in 1 ml of 0.1% trichloroacetic acid (TCA). Insoluble material was removed by centrifugation (5 min × 10,000 × *g*) and further steps were performed as described previously (Gawrońska et al., 2013).

Protein extraction

For the extraction of soluble proteins, leaves (0.1 g FW) were ground in 1 ml of 0.05 M phosphate buffer pH 7.5, containing 1 M dithiothreitol, 3 mM ethylenediaminetetraacetic acid (EDTA) and 2% polyvinylpolypyrrolidone. Insoluble material was removed by centrifugation for 5 min × 3000 × *g*. Protein concentration was determined with Bio-Rad Protein Assay (BioRad, Hercules, USA).

Trolox equivalent antioxidant capacity

The total antioxidant capacity was determined using the antioxidant assay kit (CS0790, Sigma–Aldrich Co.) according to the manufacturer's instructions. Briefly, leaf material (0.02 g FW) was ground in 1 ml of assay buffer, and centrifuged at 15 min × 10,000 × *g* at 4 °C to remove insoluble material. The antioxidant capacity of the samples was estimated by their ability to scavenge the ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) cation radical which absorbs at 405 nm. Trolox, a water-soluble vitamin E analog, was used as a standard.

Catalase activity

Catalase activity was measured as described previously (Niewiadomska and Miszalski, 2008) by monitoring H_2O_2 decomposition at 240 nm during a 2 min reaction run.

Peroxidase activity

The nonspecific peroxidase activity was assayed according to Luck (1963) by following the H_2O_2-dependent p-phenylenediamine (pPD) decomposition at 460 nm for 2 min. The extinction coefficient of 1.545×10^3 M^{-1} cm^{-1} was used according to (Allgood and Perry, 1985).

Determination of glutathione level

The total glutathione content was measured according to Luwe et al. (1993). The reaction mixture consisted of 132 mM phosphate buffer pH 7.5, containing 6.3 mM ethylenediaminetetraacetic acid (EDTA), 0.21 mM NADPH and 0.6 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The reaction was initiated by the addition of glutathione reductase (0.3 U) into the cuvette (1 mL) and the increase in absorbance at 412 nm, which was linear for 200 s and compared with GSH standard. Control rates in the absence of extract were subtracted.

ACC level

ACC was determined by LC/MS after derivatization with phenyl isothiocyanate (PITC) basically according to Chauvaux et al. (1997) with several modifications. Briefly, frozen samples were extracted two times with 80% (v/v) methanol. After centrifugation (15 min, 34,000 × *g*) both supernatants were combined and subjected to solid phase extraction on C18 columns. The effluent was evaporated to dryness under vacuum at 40 °C. The residue was dissolved in a mixture containing ethanol–water–triethylamine–PITC (2:1:1:1, v/v) and the reaction proceeded at room temperature for 20 min. After drying, 40% trifluoroacetic acid was added and the samples were heated at 90 °C for 1 h. Standard samples were obtained in the same way by derivatizing 5 nmol of ACC. LC/MS was carried out using the API LC/MS/MS system (Applera, USA) with an electrospray ionization (ESI) source equipped with a Dionex (Germany) HPLC system. Separation of the phenylthiohydantoin compound of ACC (PTH-ACC) was achieved on a Hypersil GOLD RP column (C18, 2.1 mm × 150 mm, 5 μm) at 30 °C, at a flow rate of 0.4 mL/min, using the following gradient composed of methanol (A) and 0.01 M ammonium acetate (B): 0–5 min 40% solvent A; 5–10 min 80% solvent A; 10–13 min equilibration under the initial conditions. Detection was performed in negative ion mode with conditions set as follows: drying gas (N_2) 11.0 L/min, temperature 350 °C, nitrogen nebulizer pressure 40 psi, capillary voltage 4.5 kW, a detector gain of 1600 V, fragmentation voltage 100 V and full scan range from 50 to 300 *m*/*z*. Selective ion monitoring at *m*/*z* 219 corresponding to the protonated molecule $[MH]^+$ of PTH-ACC was used for quantification.

Isolation of RNA and quantitative RT-PCR

Total RNA was isolated using an RNA isolation kit (A&A Biotechnology, Gdynia, Poland). The first-strand cDNA was synthesized from 1 μg of RNA using the Revert Aid™ HMinus