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Salinity-induced accumulation of endogenous H_2S and NO is associated with modulation of the antioxidant and redox defense systems in *Nicotiana tabacum* L. cv. Havana

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ABSTRACT

Salinity is one of the abiotic factors that most affect crop growth and production. This study focused on the effect of high salinity on the endogenous levels of the signaling molecules hydrogen sulfite (H_2S) and nitric oxide (NO) in *Nicotiana tabacum* leaves and the extent of these for the biochemically-driven plant tolerance to such abiotic stress. The NaCl treatment for 10 days led to an expressive augment of H_2S and NO levels. This increase was correlated with the raise of L-Cys and L-Arg and the induction of L-cysteine desulfhydrase, cyanoalanine synthase, cysteine synthase, nitrate reductase and arginase, enzymes known to be involved in the biosynthesis of H_2S or NO. The enzymatic antioxidant system (superoxide dismutase and catalase activity) was boosted and the non-enzymatic antioxidant glutathione was intensively oxidized in leaves upon stress allowing plants to cope with oxidative stress. Lower stomatal conductance was observed in stressed plants in comparison with control ones. Moreover, the high activity of antioxidant enzymes and high rate of glutathione oxidation following salt stress were considerably decreased upon NO or H_2S scavenging. Thus, increment in NO and H_2S levels and their interplay, along with metabolic and physiological changes, contributed to tobacco survival to extreme salinity conditions.

1. Introduction

Approximately 7% of the world's land including the agricultural ones exhibit high salt content [1]. The increase in soil salinity can be enhanced as a result of geological events or irregular practices in agriculture, such as irrigation with salty waters and excess of fertilization [2,3]. High salinity leads to nutritional disorders, oxidative stress and consequently damage to the photosynthetic apparatus, which limit plant growth and crop yield [2,4]. Indeed, such abiotic stress is known to negatively affect crop production worldwide by over 30% [1].

One of the early events during the plant response to abiotic stresses is the occurrence of an oxidative burst, characterized by the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide anion (O_2^-) [5]. This can be detrimental to plant cells if the ROS levels are not controlled since such species are able to oxidize lipids, proteins, amino acids, nucleic acids, pigments, among others [6].

Nitric oxide (NO), a reactive nitrogen species, is a well-known gaseous signaling molecule that mediates plant responses to a variety of biotic and abiotic stresses [7–12]. With respect to salt stress, NO has been implicated in the attenuation of oxidative stress by increasing the activity of antioxidant enzymes and production of non-enzymatic antioxidants as well as the protection of photosynthetic apparatus through the control of ROS levels [13–17].

Hydrogen sulfide (H_2S) is another gaseous molecule that has been receiving considerable attention in the past few years in

Abbreviations: ACN, acetonitrile; APX, ascorbate peroxidase; BHT, butylated hydroxytoluene; CAS, cyanoalanine synthase; CAT, catalase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide; CS, cysteine synthase; DTT, dithiothreitol; ε, molar extinction coefficient; EDTA, ethylenediamine tetraacetic acid; FOX, ferrous ion oxidation in xylenol orange; GSH, reduced glutathione; g_s, stomatal conductance; GSSG, oxidized glutathione; H_2S, hydrogen sulfide; HT, hypotaurine; L/D-DES, L/D – cysteine desulfhydrase; LOOH, lipid hydroperoxides; NO, nitric oxide; NiR, nitrite reductase; NR, nitrate reductase; NTB, 2-nitro-5-thiobenzoic acid; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; SA, sodium acetate; SiR, sulfite reductase; SNP, sodium nitroprusside; SOD, superoxide dismutase; TCA, trichloroacetic acid.

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researches carried out with plants. The H_2S, a water soluble molecule that freely diffuses through cell membranes, was for many years believed to be toxic to both plants and mammals [18,19]. However, by using an H_2S donor, Zhang et al. [20] showed that H_2S not only promotes seed germination but also can mitigate Cu-induced plant stress, which indicates that the dual effect of H_2S on plant cells depends on the concentration of such molecule, similarly to the observed for NO. Later on, the use of pharmacological approaches also indicated a signaling role for H_2S on organogenesis, chloroplast biogenesis, stomatal movement and plant response to other abiotic stresses, including heating, drought and Cd-induced cell death [21–28]. Evidence points out that exogenous H_2S increases the activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) [26,29] and also induces the accumulation of the antioxidant reduced glutathione (GSH) in plants upon salt stress [30–32]. As a result, H_2S attenuated the oxidative stress in alfalfa (*Medicago sativa*), cucumber (*Cucumis sativus*), strawberry (*Fragaria* × *ananassa* cv. Camarosa) and maize (*Zea mays*) plants, which is caused by the overproduction of ROS in response to high salinity. Recently, Lai et al. [31] reported that the H_2S content in cells of alfalfa roots increased following treatment with raising concentrations of NaCl due to an increment in the activity of L-cysteine desulfhydrase (L-DES). Besides L/D-DES, cyanoalanine synthase (CAS), sulfite reductase (SiR) and cysteine synthase (CS) are also enzymes reported to contribute to H_2S production in plant cells [33].

Although the role of H_2S or NO by itself has been studied using pharmacological approaches, the relationship between such molecules during plant responses to salt stress deserves further investigation as up to date very few reports have addressed this question [26,34,35]. Germination of salt-stressed alfalfa seeds in the presence of H_2S donor increased the NO content in the corresponding seedlings that, in turn, led to the accumulation of SOD, CAT and APX transcripts and increment of the activity of the corresponding enzymes [26]. Under the same experimental conditions, alfalfa roots exhibited higher K^+/Na^+ ratio. The NO content in salt-stressed barley (*Hordeum vulgare*) seedlings also increased by the treatment with an H_2S donor, in which higher expression levels of a high-affinity K^+ uptake system and an inward-rectifying potassium channel were observed, with subsequent increment of salt tolerance by plants [35].

To expand our knowledge on signaling processes in plant cells during salt stress, we investigated the extent of H_2S and NO production in tobacco leaves, its interplay and implication to the antioxidant response of plants to severe stress. For this purpose, we determined the endogenous content of H_2S and NO in stressed plants and correlated it with the levels of reducing molecules, activity of antioxidant enzymes, free amino acid contents, stomatal conductance and ROS production. The activity of enzymes involved in both H_2S and NO biosynthesis in salt-stressed plants was also addressed. The relevance of H_2S and NO production during tobacco response to high salinity was checked from the use of scavengers of these signaling molecules.

2. Materials and methods

2.1. Plant material and experimental conditions

Tobacco plants, which exhibit fast growth rate, were chosen as a model to better understand the extent of H_2S and NO production and its correlation to metabolic changes in plants upon high salinity.

Tobacco (*Nicotiana tabacum* L. cv Havana) seeds were germinated in Plantmax™ substrate imbibed with KNO_3 0.2% (w/v) to improve seed germination. The germinated seedlings were transferred to 5-L plastic pots (one seedling per pot) containing the same substrate. Plants were daily irrigated for 30 days with a half strength Hoagland's solution [36] to the point of runoff. The experiments were arranged in a completely randomized design in which plants were submitted to salt stress by treatment with Hoagland's solution supplemented with NaCl at 0, 300 or 600 mM. The chosen salt concentrations, known to induce stress, were based on previous reports [37,38]. Each treatment was applied to 6 individual pots (unless otherwise stated in the Figure legend), in which each one was considered a biological replicate. The third completely expanded leaf (from top to bottom) of each plant was harvested 10-day post salt stress imposition [38] and kept in liquid N_2 (−196 °C) for further analysis, unless otherwise stated. All analyses were carried out using 4 technical replicates from each biological one, except for the quantification of the reactive/low stable H_2S, NO and H_2O_2 performed with a single technical replicate due to the relatively long time necessary to stabilize the equipment baseline for the upcoming measurements.

A possible interaction between H_2S and NO during tobacco response to salt stress was investigated using hypotaurine (HT; H_2S scavenger) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (cPTIO; NO scavenger) at concentrations (300 and 200 μM, respectively) usually reported in the literature. The third completely expanded leaf (from top to bottom) of each plant was carefully infiltrated with HT or cPTIO every day at 9 am during the 10 days of salt treatment using an insulin syringe. Control plants were infiltrated with ultrapure H_2O, instead. Leaves were harvested at the end of the experiment and used immediately for quantification of H_2S and NO or kept in liquid N_2 (−196 °C) for further analysis of GSH and GSSG levels and the activity of antioxidant enzymes.

2.2. Quantification of endogenous H_2S and NO

Tobacco leaves were harvested 10 days post salt treatment for the quantification of H_2S and NO from leaf homogenates obtained using 50 mM phosphate buffer (pH 6.8). The levels of H_2S and NO were quantified in one volume of leaf homogenate supplemented with 4 vols of 100 mM phosphate buffered saline (PBS) or 100 mM $CuCl_2$, respectively. The measurements were carried out in a TBR4100 Free Radical Analyzer (WPI, USA) equipped with the amperometric sensors ISO-H_2S-2 and ISO-NOP, which allows the quantification of H_2S and NO at detection limits lower than 5 nM and 1 nM, respectively. Standard curves of Na_2S and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were plotted to quantify H_2S and NO, respectively, according to the manufacturer instructions as it follows: $H_2S = [Na_2S]/3.04173977$ and NO = SNAP × 0.6.

2.3. Activity of nitrate reductase (NR) and arginase

The activity of nitrate reductase (EC 1.7.1.1) was assessed according to Modolo et al. [9], with some modifications. Leaf proteins were extracted with 1 mL of 50 mM MOPS buffer (pH 7.5) containing protease inhibitor cocktail (Sigma, USA). Homogenate was centrifuged at 13,000 × *g* and 4 °C for 10 min and the supernatant collected for the analysis. One volume of the supernatant was incubated for 30 min with an equal volume of 50 mM MOPS (pH 7.5) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 10 mM KNO_3 and 1 mM NADH. Each reaction was stopped by addition of 0.1 vol of 1 M zinc acetate followed by centrifugation at 10,000 *x g* for 5 min. Then, one volume of a mixture constituted of 0.5% (w/v) sulfanilamide in 1.25% (v/v) H_3PO_4 and 0.5% (w/v) naphthylethylene-diamine dihydrochloride was added to an equal volume of the resulting supernatant and analyzed at 540 nm for determining NO_2^- formation.