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Salicylic acid-mediated alleviation in NO_2 phytotoxicity correlated to increased expression levels of the genes related to photosynthesis and carbon metabolism in *Arabidopsis*

Qiu-shuang Yin[a], Xue Yuan[a], Yan-gao Jiang[b], Lin-li Huang[a], Guang-zhe Li[a,b], Lin Hao[a,b,*]

[a] College of Life Science, Shenyang Normal University, Shenyang 110034, China
[b] Liaoning Engineering Technique Research Center for Agricultural Waste Bioconversion and Regeneration, Shenyang 110034, China

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ABSTRACT

Nitrogen dioxide (NO_2) is one of the major air pollutants affecting plant growth, development and yields. Salicylic acid (SA)-mediated alleviation in NO_2-induced plant damage has been reported in *Arabidopsis*. However, the involved mechanisms are far from clear. In this study, *Arabidopsis* wild type (WT) and its SA-altering mutants, *snc1* (*suppressor of npr1-1, constitutive 1*) with high SA, *npr1-1* (*nonexpressor of PR gene*) with SA signaling blockage, and transgenic line *nahG* with low SA, were used to decipher the SA action mechanisms. NO_2 fumigation at a concentration of 10 ppm for 8 h led to more severe injuries on *nahG* and *npr1-1* plants, whereas a lesser on *snc1* plants compared with WT plants. The expressions of SA biosynthesis-related genes were up-regulated leading to more SA accumulations in all the tested plants after NO_2 treatment. The expression patterns of ethylene, oxylipin and nitric oxide biosynthesis-related genes were regularly altered among NO_2-treated genotypes, among which the expression of dioxygenase-encoding gene was dramatically increased in *snc1* plants. Total chlorophyll contents, net photosynthetic rate, maximum quantum and actual quantum efficiency of PSII were decreased, to a greater degree in *nahG* and *npr1-1* plants, whereas a lesser in *snc1* plants compared with WT plants after NO_2 treatment. We analyzed the expression patterns of the selected genes related to chlorophyll biosynthesis, photosystem I and II structure and functions, electron transport, ATP synthesis, Calvin cycle, as well as photorespiration, pentose phosphate pathway and starch degradation. The expression levels of the most genes were up-regulated in *snc1* and WT plants, whereas down-regulated in *nahG* and *npr1-1* plants after NO_2 treatment, among which the expression pattern of a photorespiration-related gene was particularly specific. The plant tolerant phenotypes were positively correlated with the gene expressions, suggesting that SA-mediated alleviation in NO_2 phytotoxicity was associated with photosynthesis and carbon metabolisms, also with other signal molecules.

1. Introduction

Nitrogen dioxide (NO_2) in air enters plant leaves mainly through stomata, and forms nitrate and nitrite in the cellular aqueous environment (Nouchi, 2002), which might impair plant photosynthesis, thereby generating reactive oxygen species (ROS), and inducing plant oxidative damage (Shimazaki et al., 1992). Therefore, plant anti-oxidative defense is a major mechanism responsible for plant tolerance to NO_2 stress. Our earlier study showed that constitutive high accumulation of endogenous salicylic acid in *Arabidopsis* mutant *snc1* effectively relieved NO_2 exposure-induced plant damage, which was associated with antioxidative defense, disulfide bond reduction, flavonoid biosynthesis, nitrate metabolism, and signaling (Qu et al., 2018). As a crucial signal molecule in plant adaptation to changing environments, salicylic acid (SA) has been extensively studied. In addition to the involvement in plant systemic acquired resistance to pathogens, SA has also been broadly implicated in plant response to various abiotic stresses (for a review see Khan et al., 2015). Although the SA action mechanisms are not fully understood, especially in molecular levels, solid evidence has demonstrated that the roles of SA are associated with

Abbreviations: Chl, chlorophyll; DOX, dioxygenase; ET, ethylene; F_v/Fm, maximum quantum of photosystem II; JA, jasmonic acid; *nahG*, *naphthalene hydroxylase G*; NO, nitric oxide; *npr1-1*, *nonexpressor of pathogenesis-related gene*; *P*n, net photosynthetic rate; PSI, photosystem I; PSII, photosystem II; SA, salicylic acid; *snc1*, *suppressor of npr1-1* constitutive 1; WT, wild type; Φ_{PSII}, actual quantum efficiency of PSII

* Corresponding author at: College of Life Science, Shenyang Normal University, Shenyang 110034, China.
E-mail address: haolinwj2001@163.com (L. Hao).

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plant antioxidative defense, secondary metabolite synthesis, as well as the maintenance of photosynthetic processes under stressed conditions. For the latter, the correlations between application of SA and chlorophyll contents, activity and contents of Rubisco, photosynthetic capacity, PSII photochemistry efficiency, gas exchange, ultrastructure of chloroplast, etc. have been investigated in collections of literature (Khan et al., 2015). However, little information is available about the synergic response of the whole photosynthetic process to a certain stress factor. In this study, we comprehensively analyzed the expression patterns of the genes involved in the major photosynthetic processes, including the chlorophyll biosynthesis, photosystem (PSI and PSII) structure and functions, electron transport, ATP synthesis, and Calvin cycle, also concerning the genes encoding the key enzymes functioned in photorespiration, pentose phosphate pathway and starch degradation. The aim is to explore the underlying mechanism by which SA alleviated NO_2 phytotoxicity. As the effect of adverse stress on photosynthesis is broad, it cannot be exhaustive, instead, key genes are included in this study. The names and short description of functions of the candidate genes were supplied in supplemental Table S1.

A large body of evidence has demonstrated that the biological role of SA exists a crosstalk with other phytohormones or signal molecules, such as jasmonic acid (JA), ethylene (ET), nitric oxide (NO), and so on. Either a positive or a negative interaction among these chemicals ultimately enable plants to fine-tune its defense against specific aggressors (Leon-Reyes et al., 2009; Overmyer et al., 2018), or abiotic stress factors (Tiwari et al., 2017). However, the possible interaction between SA and these signal molecules has not yet been reported in plant response to NO_2 stress, even though their positive role alone has been proposed in plant response to NO_2 stress by analyzing their respective mutant affecting biosynthesis or signaling, or by measuring the endogenous content such as of SA, JA, JA-isoleucine, 12-oxophosphodienoic acid (Kasten et al., 2016). In the present study, we analyzed the NO_2-induced expression patterns of several key genes related to the biosynthesis of SA, JA, ET and NO, with an aim to reveal the correlation of SA-mediated NO_2 tolerance with other signal molecules. In addition, the expression analysis of *α-DOX1* was also included in this study. *α-DOX1* encodes α-dioxygenase 1 which is a key enzyme in the biosynthesis of DOX pathway-derived oxylipins (Wasternack and Feussner, 2018).

Several salicylic acid-altering *Arabidopsis* mutants were used in this study, including the SA high-accumulating mutant *snc1* (*suppressor of npr1-1, constitutive*), SA-deficiency transgenic line *nahG* (*naphthalene hydroxylase G*), and SA-signal blockage mutant *npr1-1* (*nonexpressor of pathogenesis-related gene*). The *snc1* is isolated from ethyl-methanesulfonate (EMS)-mutagenized M_2 progeny on the basis of the suppression of *npr1-1* phenotype in plant resistance to pathogens (Li et al., 2001). The mature *snc1* plants have a dwarf phenotype probably due to the extremely high endogenous SA (about 15-fold over the wild type level). The transgenic line *nahG* contains a low level of SA (about 1/4 of the wild type level) due to the activity of naphthalene hydroxylase responsible for SA degradation (Gaffney et al., 1993). The *npr1-1* plant has an EMS-induced point mutation in *NPR1*, a known only positive regulator of systemic acquired resistance to function downstream of SA and regulate *PR-1* expression (Cao et al., 1994). In previous studies, we used these *Arabidopsis* genotypes to elucidate the regulatory role of SA in plant response to NO_2 stress, and found that *snc1* plants were obviously tolerant, whereas *nahG* and *npr1-1* plants were more sensitive compared with wild type plants (Qu et al., 2018). In this study, we firstly revealed that the SA-mediated tolerance to NO_2 stress was associated with the regulation of gene expressions in plant photosynthesis and carbon metabolism, also with the expressions of other signal molecule-related genes.

2. Materials and methods

2.1. Plant material and treatment

Seeds of *Arabidopsis thaliana* (L.) Heydn. (ecotype Columbia) including wild type and its mutants *snc1* and *npr1-1*, and transgenic line *nahG*, were graciously provided by Prof. Dong (Duke University). Briefly, vernalized seeds were sown in pots containing a mixture of peat/perlite/vermiculite (1:1:1, v/v/v), and grown in a culture room under 10 h of light with 100 μmol photons m^{-2} s^{-1} at 22 °C and 14 h of dark at 18 °C, and 70% relative humidity. Before 3 d of NO_2 fumigation, the matrix-grown plants were not watered. Twenty growth-uniform plants for each genotype were used to NO_2 treatment or as control, respectively. For NO_2 fumigation, 4-week-old plants (with about six fully developed leaves) with matrix were transferred to a self-made air-tight chamber, and charcoal-filtered air-diluted NO_2 gas was drawn according to the method described by Qu et al. (2018). Briefly, NO_2 gas was supplied directly from a cylinder into a dilution reservoir into which charcoal filtered air was drawn. The NO_2 gas is purchased from Dalian Special Gases Co., LTD (Dalian, China) with an original concentration of 2% prepared by charcoal filtered compressed air. The diluted gas was delivered to fumigation chamber equipped with inlet (at the upper portion) and outlet (at the lower portion) tubings. The NO_2 concentrations in the chamber were controlled by adjusting the NO_2 flow rate (about 20 ml min^{-1}) using a mass flow controller (D07-7C; Sevenstar Electronics, Beijing, China), and continuously monitored using a gas analyzer (SWG 300-1; MRU, Germany). The outlet was connected to three glass bottles in tandem containing NaOH solution trap for exhaust gas. In our experimental conditions, the NO_2 recovery rate can reach to 95%. Plants were fumigated using 10 ± 1 ppm of NO_2 for 8 h under continuous illumination (100 μmol m^{-2} s^{-1}) and 22 °C. The tested dose of NO_2 in the present experiment was derived from previous studies (Kasten et al., 2016; Qu et al., 2018). Although NO_2 concentrations up to ppm levels are not generally found in the outdoor environments, higher concentrations of NO_2 are present in special areas, such as 20 ppm in greenhouse (Nouchi, 2002). In addition, a short-term exposure experiment normally used high concentrations of NO_2, such as 20 ppm for 1 h leading to slight curl and yellowing of some leaves of *Arabidopsis* plants, but an obvious increase of ion leakage (Kasten et al., 2016). In this study, fumigation with 10 ppm of NO_2 for 8 h just induced a minor visible injury to plant leaves. At the immediate end of fumigation, leaves were randomly collected and pooled from 10 plants, and stored in liquid nitrogen to be used for the analyses of physiological parameters, and gene expressions. The control plants were just exposed to charcoal-filtered air.

2.2. Detection of physiological parameters

For total SA quantification, collected rosette leaves were ground in liquid N_2, and SA was extracted using 90% methanol followed by ethyl acetate extraction as described in Newman et al. (2001). SA content was analyzed using HPLC equipped with a Shim-pack GIST 5-μm reverse phase C18 column (150 × 4.6 mm) (Shimadzu, LC-20 A, Kyoto, Japan). Net photosynthetic rate was measured using a portable system (LI-6200, LI-COR, Lincoln, NE, USA) with a specific *Arabidopsis* leaf chamber (LI-6400-17) at ambient climatic conditions, irradiance of 150 μmol m^{-2} s^{-1}, and 22 °C as described by the manufacturer's instructions. Chlorophyll fluorescence parameters were detected by a portable fluorometer (Handy-PEA, Hansatech, Norfolk, UK). Briefly, plants were placed in darkness for 15 min to assay minimum fluorescence F_0 under a weak irradiance from a light-emitting diode, then by an 1.0-s saturating pulse (1500 μmol m^{-2} s^{-1}) to determine maximum fluorescence (F_m), and by 15-min actinic light (30 μmol m^{-2} s^{-1}) to detect steady state fluorescence (F_s). Maximum quantum of photosystem II [$F_v/F_m = (F_m - F_0)/F_m$] and actual quantum efficiency of PSII [$\Phi_{PSII} = (F_m - F_s)/F_m$] were calculated as described by Genty et al.