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Salicylic Acid-Altering *Arabidopsis* Mutants Response to NO_2 Exposure

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Abstract Nitrogen dioxide (NO_2)-induced responses in wild type (wt) and salicylic acid (SA)-altering *Arabidopsis* mutants *snc1* (*suppressor of npr1-1, constitutive*) with high SA level, transgenic line *nahG* with low SA level, *npr1-1* (*nonexpressor of PR gene*) with SA signaling blockage and double mutant *snc1nahG* plants, were investigated. All mutant lines except *sncl* showed that NO_2 exposure at 0.25 μL L^{-1} increased chlorophyll content and biomass accumulation, elevated photosynthetic rate, and decreased MDA content compared to their respective controls. The *sncl* plants were similar to the control plants for these measured indices. NO_2 exposure at 0.5 μL L^{-1} and higher doses caused injury to wt, *nahG*, *npr1-1* and *snc1nahG* plants, whereas the *snc1* plants exhibited a stronger tolerance. To evaluate the resistance mechanism, we further investigated the changes in the mutants exposed to 1 μL L^{-1} of NO_2 in relation to endogenous SA level, antioxidant capacity and redox status. The collected data demonstrated that the NO_2 tolerance in *snc1*, with a high SA level, was tightly linked to the increased antioxidant capacity and decreased oxidative stress. This suggests that SA may play an important protective function in plant response to NO_2 stress.

Keywords Nitrogen dioxide · Lipid peroxidation · Redox homeostasis · Transgenic lines

Nitrogen dioxide (NO_2), one of the main traffic-related air pollutants and precursors of photochemical smog and ground-level ozone, is currently under intensive investigation. Although visible injury due to NO_2 is very rare in the field where NO_2 is at a ppb level (for a review, see Nouchi 2002), growing evidence shows that ambient NO_2 has caused reduction and deterioration in crop and vegetable yield and quality in some regions on the world (Maggs and Ashmore 2004; Muzika et al. 2004; Haberer et al. 2006; Han and Naeher 2006), especially in greenhouses where NO_2 at a ppm level may show visible injury symptoms in some vegetables (Nouchi 2002). To address mechanisms of plant response to NO_2 exposure, fumigation experiments under controlled conditions are used intensively with NO_2 concentrations up to ppm levels (Sabaratnam and Gupta 1988; Sandhu et al. 1990; Saxe 1994; Kondo et al. 2008). Although the effects of NO_2 on plant growth are not fully understood, evidence indicates that accumulation of nitrite (NO_2^-) resulting from atmospheric NO_2 in leaves leads to the inhibition of photosynthesis, thereby generating reactive oxygen species (ROS), which might contribute to visible injury caused by NO_2 (Shimazaki et al. 1992). To avoid ROS-caused oxidative damage, plants have evolved complex of antioxidant defense systems which are involved in the detoxification of $O_2^{\bullet -}$ and H_2O_2, respectively preventing the formation of $O\dot{H}$ radicals (Mittler 2002).

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In addition to its well-established role in plant response to pathogen attack (for a review, see Halim et al. 2006), salicylic acid (SA) has also been intensively investigated in relation to plant adaptation to various abiotic stresses (for a review, see Yuan and Lin 2008). However, to our knowledge, no information is available for SA involvement in plant response to NO_2 stress. The *snc1* (*suppressor of npr1-1, constitutive 1*) mutant, isolated in a screen to restore the inducible *PR* (pathogenesis-relative) gene expression in the *npr1-1* (*nonexpressor of PR*) background, contains high levels of SA, exhibits constitutive *PR* gene expression, and significantly enhances resistance to pathogens (Li et al. 2001). However, there is not yet any report on *snc1*-mediated abiotic stress responses. The NPR1 protein is an essential regulatory component of systemic acquired resistance (SAR), and the mutant *npr1-1* completely blocks the induction of SAR by SA, displaying little expression of *PR* genes and increased susceptibility to bacterial and fungal infections (Cao et al. 1997). The *npr1-1* mutant has been used widely to define the SA-dependent signaling pathway. The bacterial *nahG* (naphthalene hydroxylase G) gene encodes a SA-degrading enzyme, salicylate hydroxylase, and its expression in transgenic plants prevents the accumulation of SA and completely eliminates the onset of SAR (Gaffney et al. 1993). The *nahG* transgenic *Arabidopsis* lines and monocotyledonous plants have also been widely used to establish the essential role of SA in plant responses to abiotic stresses (Chini et al. 2004; Kusumi et al. 2006). To evaluate the role of endogenous SA in plants in response to NO_2 exposure, the *snc1*, *npr1-1*, *nahG* and *snc1nahG* plants were used in this experiment.

Materials and Methods

Arabidopsis thaliana ecotype Col-0 (referred to as wild type, wt) and mutants were grown in pots containing a mixture of peat/perlite/vermiculite (1:1:1, v/v/v) in a growth chamber under 10 h of light (100 μM m^{-2} s^{-1}) at 22°C and 14 of darkness at 18°C. For NO_2 exposure, 3-week-old plants were transferred to a glass chamber (0.8 × 0.8 × 0.8 m) and NO_2 gas was supplied directly from cylinders, into a dilution reservoir into which charcoal filtered air was drawn. Mean concentration of the chamber NO_2 was monitored using an NO_2 analyzer (Model ML Series). Plants were fumigated during the light period for 3 h per day for 2 weeks. For the control, charcoal filtered air (<0.02 μL L^{-1} NO_2) alone was supplied. The NO_2 concentrations used in the present experiment were derived from some environmental concentrations, such as in greenhouse NO_2 concentration can be up to 20 μL L^{-1} (Nouchi 2002), and based on some information from literature using other plant species. For example, Kondo et al. (2008) exposed azalea plants to NO_2 of 4 μL L^{-1}, Saxe (1994) exposed 35 cultivars of pot plants of 20 families to NO_2 of 1 μL L^{-1}, Sabaratnam and Gupta (1988) exposed soybean to NO_2 from 0.1 to 0.5 μL L^{-1}.

Except where mentioned in the text, all the analyses in this experiment were carried out immediately following NO_2 exposure. Relative dry weight increase of plants was calculated by the formula $(W_1 - W_0)/W_0 \times 100\%$, where W_0 is the mean dry weight of plants at the beginning of NO_2 exposure and W_1 is the mean dry weight at the end of NO_2 exposure. For dry weight determination, the excised rosettes were dried for 3 days at 80°C. Photosynthesis rate and stomatal conductance were measured using a portable photosynthesis system (LI-6200) with a specific leaf chamber for Arabidopsis (LI-6400-17) at ambient climatic conditions. During the measurement the PAR over the waveband 400–700 nm was 150 μM m^{-2} s^{-1} at 23°C. Total chlorophyll content was determined following the method described by Zhang and Qu (2003). For total SA (free and conjugated forms) quantification, pooled rosette leaves from five plants receiving the same treatment were collected at the time points indicated in the text, and analyzed following the procedure described by Newman et al. (2001). Hydrogen peroxide level was determined according to a method from Mukherjee and Choudhuri (1983). The reduced glutathione (GSH) content was determined as described by Griffith and Meister (1979). GSSG content was calculated from the difference between total glutathione from DTT-treated samples and GSH from non-DTT-treated samples. SOD (EC 1.15.1.1) activity was assayed using the photochemical nitroblue tetrazolium (NBT) method as described previously (Beyer and Fridovich 1987). CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi (1983), in which the initial concentration of H_2O_2 was 0.04% (v/v) in PBS, at pH 7.0. POD (EC 1.11.1.7) activity was estimated according to Hemeda and Klein (1990). In all the enzyme preparations, protein concentration was estimated by the method of Bradford (1976) using bovine albumin as standard. The level of malondialdehyde (MDA) was determined following Shalata and Tal (1998).

Each result shown in Table 1 and the figures was the mean of three replicated treatments. Data were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute, Cary NC) and expressed as means ± SD.

Results and Discussion

Table 1 shows that NO_2 exposure at 0.25 μL L^{-1} increased chlorophyll content and biomass accumulation, elevated

Table 1 Effect of NO_2 on total chlorophyll content, plant growth, photosynthetic rate, stomatal conductance and malondialdehyde (MDA) content

Index	Genotype	NO_2 concentrations ($\mu L\ L^{-1}$)				
		Control (<0.02)	0.25	0.5	1.0	2.0
Total chlorophyll content (% of control)	wt	100b	112 ± 6.2a	86 ± 5.4c	72 ± 4.2d	58 ± 3.1e
	sncl	100a	98 ± 5.6ax	92 ± 5.6a	83 ± 4.6bx	71 ± 4.2cx
	snclnahG	100b	110 ± 6.3a	86 ± 5.7c	75 ± 4.3d	56 ± 3.2c
	nahG	100b	114 ± 6.8a	88 ± 6.5c	69 ± 3.8d	52 ± 2.8ex
	nprl-l	100b	116 ± 7.2a	87 ± 6.6c	68 ± 3.6d	51 ± 2.4ex
Relative biomass increase (%)	wt	80 ± 6.6b	91 ± 7.5a	72 ± 6.2c	51 ± 3.8d	36 ± 2.4e
	sncl	58 ± 4.1ax	61 ± 4.6ax	56 ± 4.8ax	48 ± 3.2b	42 ± 2.7cx
	snclnahG	78 ± 6.1b	89 ± 7.2a	70 ± 4.4c	52 ± 3.6d	37 ± 2.6e
	nahG	80 ± 5.8b	91 ± 7.9a	68 ± 4.5c	44 ± 2.9dx	31 ± 2.1ex
	nprl-l	82 ± 7.3b	90 ± 7.1a	70 ± 4.3c	42 ± 2.8dx	30 ± 1.8ex
Photosynthetic rate ($\mu mol\ CO_2\ m^{-2}s^{-1}$)	wt	14.6 ± 0.86b	16.7 ± 0.92a	12.2 ± 0.65c	8.3 ± 0.45d	5.6 ± 0.28e
	sncl	8.5 ± 0.32ax	8.6 ± 0.43ax	8.4 ± 0.42ax	7.7 ± 0.39b	7.1 ± 0.32cx
	snclnahG	14.3 ± 0.81b	15.1 ± 0.93a	12.3 ± 0.86c	7.9 ± 0.53d	5.5 ± 0.34e
	nahG	14.3 ± 0.76b	16.2 ± 0.84a	11.6 ± 0.56c	7.6 ± 0.42d	4.8 ± 0.23ex
	nprl-l	14.5 ± 0.82b	15.9 ± 0.81a	11.9 ± 0.61c	7.8 ± 0.36d	5.2 ± 0.24e
Stomata conductance ($mol\ H_2O\ m^{-2}s^{-1}$)	wt	0.25 ± 0.01a	0.25 ± 0.01a	0.18 ± 0.01b	0.17 ± 0.01b	0.14 ± 0.01c
	sncl	0.17 ± 0.01ax	0.18 ± 0.01ax	0.14 ± 0.01bx	0.14 ± 0.01b	0.13 ± 0.01b
	snclnahG	0.22 ± 0.02a	0.21 ± 0.01a	0.18 ± 0.01b	0.15 ± 0.01c	0.15 ± 0.01c
	nahG	0.24 ± 0.01a	0.25 ± 0.01a	0.18 ± 0.01b	0.18 ± 0.02b	0.13 ± 0.01c
	nprl-l	0.25 ± 0.02a	0.25 ± 0.02a	0.19 ± 0.01b	0.16 ± 0.02c	0.14 ± 0.01d
MDA content ($nmol\ g^{-1}$ FW)	wt	1.4 ± 0.06d	1.1 ± 0.05e	1.7 ± 0.08c	2.5 ± 0.09b	2.8 ± 0.16a
	sncl	1.8 ± 0.07bx	1.8 ± 0.07bx	1.9 ± 0.08b	2.2 ± 0.11a	2.2 ± 0.9ax
	snclnahG	1.4 ± 0.07d	1.2 ± 0.06e	1.7 ± 0.07c	2.6 ± 0.12b	2.9 ± 0.18a
	nahG	1.4 ± 0.07d	1.1 ± 0.05e	1.8 ± 0.07c	2.7 ± 0.13b	3.2 ± 0.18ax
	nprl-l	1.5 ± 0.06c	1.0 ± 0.05d	1.6 ± 0.06c	2.6 ± 0.11b	3.1 ± 0.16ax

The different letters (a, b, etc.) in the same line indicate a significant difference at $p \leq 0.05$ and x in the same column indicates a significant difference at $p \leq 0.05$ between the mutants and wt

photosynthetic rate, and decreased MDA content of wt, *nahG*, *npr1-1* and *snc1nahG* plants when compared to their respective controls. This is in agreement with other reports in which low concentrations of NO_2 have been shown to increase plant growth or net photosynthetic rate (Sabaratnam and Gupta 1988; Takahashi et al. 2005). No major change was observed in stomatal conductance, suggesting that the stomatal factor may be excluded from the plant response at this concentration of NO_2. However, the *snc1* plant seemed to be insensitive to the low NO_2, as shown by no significant difference in the above indices from the control (Table 1). NO_2 exposure at 0.5 $\mu L\ L^{-1}$ and higher levels caused injury to wt, *nahG*, *npr1-1* and *snc1nahG* plants, as indicated by significantly decreased chlorophyll content and relative biomass increase, impaired photosynthetic rate, and elevated MDA level, with *nahG* and *npr1-1* plants being most severe. The stomatal conductance also was obviously lowered in these plants. However, the *snc1* plants exhibited a stronger tolerance to the high doses of NO_2 exposure than other plant types, receiving significantly negative effects in above-mentioned parameters only at 1 $\mu L\ L^{-1}$ and higher concentrations, and even then, the difference from the control was the least of all analysed lines (Table 1). Together with the observation that a similar response occurred in *snc1nahG* plants and the more susceptive *nahG* and *npr1-1* plants when compared to the wt plants, the data implied that endogenous SA levels and/or signaling may play an important role in plants in response to NO_2 stress. To address the response mechanism, we further investigated the changes of the SA-altering mutants exposed to 1 $\mu L\ L^{-1}$ of NO_2 in endogenous SA level, antioxidant capacity and redox status.

Under control conditions, total SA in *snc1* and *npr1-1* were 7- and 1.9-fold higher than that in wt, respectively, but only 25% of the wt in *nahG*. The expression of *nahG* in *snc1nahG* plants decreased SA concentration to the wt level. Increased SA levels were observed in *snc1* and *npr1-1* after 6 h of NO_2 exposure and reached a maximum value

at 12 h, then declined (Fig. 1). An increased SA accumulation in wt was detected at 12 h of NO_2 exposure, then reduced gradually to control level at 24 h. No major change in SA level was observed in *nahG* and *snc1nahG* plants due to the nahG activity (Fig. 1).

The *snc1* plants had higher SOD activity than other lines under unstressed conditions (Fig. 2A), providing additional evidence of an increased oxidative stress occurring in this line as shown by elevated MDA content (Table 1). Although the NO_2 exposure suppressed SOD activity in all lines tested, a greater reduction occurred only in *nahG* and *npr1-1* (Fig. 2A), suggesting that the SA level or signaling may be involved in the protection of SOD activity against NO_2 stress. Likewise, the *snc1* had highest POD activity relative to other lines in both unstressed and stressed conditions (Fig. 2B). However, the CAT activity was the lowest in *snc1* compared to other lines under unstressed conditions (Fig. 2C), indicating that the high levels of SA may inhibit the CAT activity.

The GSH content was much higher in *snc1* plants than in wt under unstressed conditions (Fig. 3A), whereas it was at a comparable level in *snc1nahG* relative to wt, suggesting that SA may promote glutathione biosynthesis, which has been described previously (Mateo et al. 2006). The NO_2 exposure increased GSH accumulation in all lines compared to their unstressed parallels. Glutathione exists in both a reduced (GSH) and oxidised form (glutathione disulphide; GSSG), and its influence on cellular redox status depends on both the GSH/GSSG ratio and GSH concentration (Schaffer and Buettner 2001). In comparison with other lines, the GSH/GSSG ratio was the lowest in *snc1* under unstressed conditions, whereas it was the highest in the *snc1* under NO_2 exposure, even though the

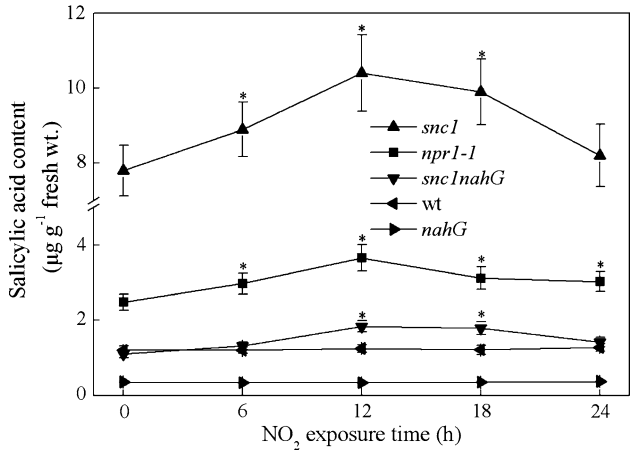

Fig. 1 Nitrogen dioxide-induced accumulation of total SA in *Arabidopsis thaliana*. Plants were exposed to 1 μL L^{-1} NO_2 for different times as indicated in the figure. The data shown here from three replicated experiments and represent the mean ± SD. "*" indicates significant difference at $p \leq 0.05$

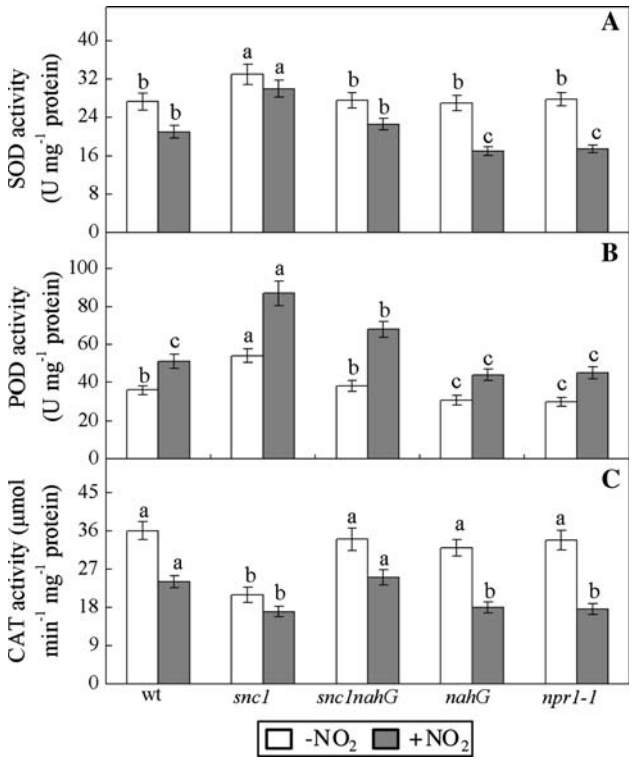

Fig. 2 Effect of NO_2 exposure at 1 μL L^{-1} on the activity of superoxide dismutase (**A**), peroxidase (**B**) and catalase (**C**). The data shown here from three replicated experiments and represent the mean ± SD. The different letters (a, b, etc.) in the same series indicate significant difference at $p \leq 0.05$

NO_2 exposure reduced the ratio in all analysed lines (Fig. 3B). The remarkable decrease in H_2O_2 level in *snc1* exposed to NO_2 (Fig. 3C) indicated that the effective control of ROS by the promoted antioxidant capacity may contribute the *snc1* plant tolerance to NO_2 stress.

SA has been extensively implicated in plant response to biotic and abiotic stresses, but the mechanism of SA function remains to be elucidated. Increasing evidence indicates that SA-improved plant resistance to biotic and abiotic stresses is associated with the maintenance of reducing conditions or redox homeostasis in cells (Mou et al. 2003; Mateo et al. 2006), which is dependent on the balance between ROS formation and removal. In this experiment, SOD and POD activities were much higher in *snc1* than in other lines in both unstressed and NO_2 stressed conditions (Fig. 2A and B), suggesting that high level of SA was favorable to the SOD and POD activity, thus ameliorating the ROS-induced oxidative injury. In fact, there has been considerable evidence to show that exogenous SA can activate antioxidant enzymes in plants in response to different abiotic stresses such as paraquat (Ananieva et al. 2004), heavy metals (Popova et al. 2009), and salinity (Xu et al. 2008). However, the lower CAT activity in *snc1* relative to wt suggested that CAT may be not a major contributor to ROS removal in this

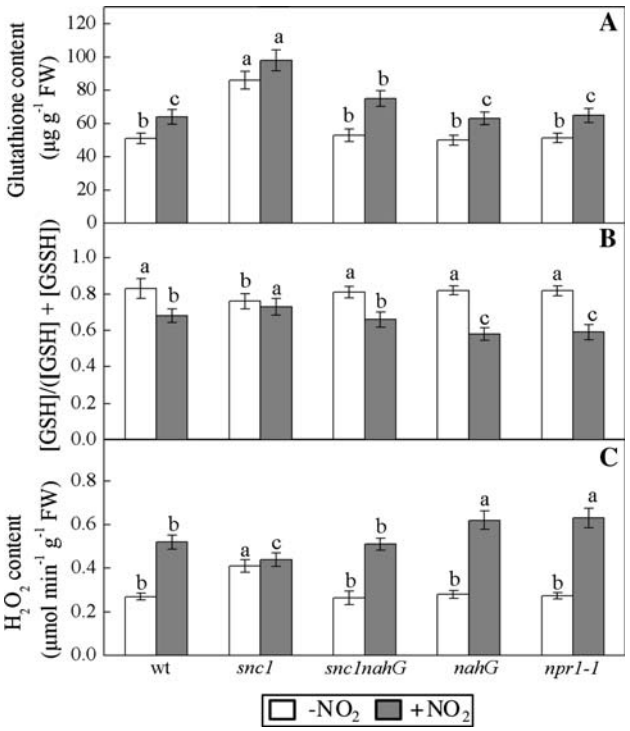

Fig. 3 Effect of NO_2 exposure at 1 μL L^{-1} on reduced glutathione content (**A**), GSH/GSSG ratio (**B**) and H_2O_2 level (**C**). The data shown here from three replicated experiments and represent the mean ± SD. The different letters (a, b, etc.) in the same series indicate significant difference at $p \leq 0.05$

experiment. The inhibition of CAT activity by SA has been described in many studies and a typical report is Chen et al. (1993). In addition, the high GSH level and GSH/GSSG ratio in *snc1* exposed to NO_2 also indicated that SA may play an important role in the maintenance of reducing conditions in cells.

In summary, through the comparative studies, this experiment shows that the high accumulation of SA in *snc1* plants appears to has dual attributes in the regulation of cellular redox status that on one hand may exert oxidant stress through initiating ROS generation under unstressed conditions, but on the other hand may contribute to a protective function through enhancing antioxidant capacity under NO_2 stress.

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