Mechanical Stress Elicits Nitric Oxide Formation and DNA Fragmentation in Arabidopsis thaliana

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The effect of mechanical stress (centrifugation) on the induction of nitric oxide (NO) formation and DNA fragmentation was investigated in leaf cells of Arabidopsis thaliana. Centrifuged and non-centrifuged leaves from wild-type and nitrate reductase (NR) nia1, nia2 double mutant, defective in the assimilation of nitrate, were labelled with 4,5-diaminofluorescein diacetate (DAF-2 DA) to visualize in vivo NO production. After these treatments, DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end in situ labelling (TUNEL) method. Exposure to an NO-releasing compound, sodium nitroprusside (SNP) mimicked the cell response to centrifugation (20 g). The involvement of endogenous NO as a signal in mechanical stress and in DNA fragmentation was confirmed by inhibition of NO production using a nitric oxide synthase (NOS) inhibitor viz. Nω-monomethyl-L-arginine (l-NMMA). These results indicate that NOS-like activity was present in A. thaliana leaves and was increased by mechanical stress. The effect of leaf-wounding on nitric oxide production was identical to that of centrifugation. Experiments with A. thaliana NR mutant also showed that NO bursts were induced by mechanical and wounding stresses and that NO was not a by-product of NR activity. A positive and significant correlation between NO production and DNA fragmentation was recorded for both centrifuged and non-centrifuged cells. Our results suggest that factors other than NO contribute to DNA damage and cell death, and furthermore, that an inducible form of NOS is present in A. thaliana.

Key words: Arabidopsis thaliana, cell death, DNA fragmentation, NO, plant stress, wounding.

INTRODUCTION

Nitric oxide (NO) is an intra- and intercellular signalling molecule involved in the regulation of diverse biochemical and physiological processes (Lancaster, 1992; Stamler et al., 1992; Leshem, 1996; Kojima et al., 1998b; Van Camp et al., 1998). Recently, there has been much research into the diverse biological activities of nitric oxide (Lancaster, 1992; Leshem, 1996; Delledonne et al., 1998; Durner et al., 1998; Van Camp et al., 1998; Beligni and Lamattina, 1999a, b; Durner and Klessig, 1999; Magalhaes et al., 1999). In animals, NO is produced from L-arginine by a wide variety of cell types by both constitutive and inducible nitric oxide-synthases (NOS) (Garvin, 1999). However, in plants, NO synthesis is not confined to NOS-like activity. Rather, NO can be generated as a by-product of nitrate reductase, nitrogen fixation and/or respiration (Klepper, 1990; Noritake et al., 1996).

By itself, NO is a relatively non-toxic molecule that will not kill cells even at extremely high concentrations (Pryor and Squadrito, 1995). However, in the presence of superoxide (O2−), NO becomes a potent free radical. The reaction of NO with O2− has the fastest biochemical rate constant currently known and results in the formation of the strong oxidant, peroxynitrite (ONOO−) (Ducrocq et al., 1999).

Under normal physiological conditions, the catalytic action of cytosolic, mitochondrial and extracellular superoxide dismutase (SOD) rapidly dismutates O2− to hydrogen peroxide (H2O2) and molecular oxygen. High levels of NO may compete with this dismutation reaction and lead to the formation of peroxynitrite, which damages proteins, lipids, RNA and DNA (Yamasaki et al., 1999).

NO has been implicated in plant growth and development, senescence, ethylene emission, disease resistance and various types of defence responses to environmental stresses (Leshem et al., 1997, 1998; Millar and Day, 1997; Delledonne et al., 1998; Durner et al., 1998; Durner and Klessig, 1999; Magalhaes et al., 1999; Pedroso et al., 2000a, b). In the hypersensitive response, NO acts synergistically with reactive oxygen intermediates (ROIs) to increase oxidative cell death in soybean cells (Delledone et al., 1998). It was also shown that NO participates, directly or indirectly, in a mechanism leading to cell death in plants (Magalhaes et al., 1999; Pedroso and Durzan, 2000; Pedroso et al., 2000a, b). Cell death occurs by apoptosis or necrosis (cf. Pennell and Lamb, 1997; Gray and Johal, 1998; Mittler, 1998). In plants, apoptosis is characterized by nuclear and cytoplasmic condensation, activation of endonucleases and subsequent fragmentation of nuclear DNA (cf. Havel and Durzan, 1996; Ryerson and Heath, 1996; Wang et al., 1996; Zhou et al., 1999). Necrosis is...
characterized by random DNA cleavage and cell lysis (cf. Gray and Johal, 1998).

Based on the relationships previously demonstrated between NO, cell death, and plant stresses (Magalhaes et al., 1999; Pedroso et al., 2000a, b), we propose that NO is produced in response to mechanical stress and/or wounding, and leads to DNA fragmentation. Using A. thaliana wild-type and nitrate reductase (NR) nia1, nia2 double mutant (G/4-3) we investigated: (a) how mechanical stress (centrifugation) and wounding affect the production of nitric oxide and DNA fragmentation; and (b) if NO production is elicited by NOS-like activity and/or as a by-product of NR activity.

MATERIALS AND METHODS

Plant material

The Arabidopsis thaliana NR mutant seeds designated G/4-3 (a nia1, nia2 double mutant line defective in the assimilation of nitrate) (Wilkinson and Crawford, 1993) were a gift from Nigel M. Crawford (University of California, San Diego, CA, USA). Seeds of wild-type (Columbia) and NR G/4-3 mutant were surface sterilized for 2 min in 70% ethanol, followed by immersion for 15 min in domestic bleach. Then seeds were then washed five times in sterile water and cultured in darkness in Petri dishes on half-strength Murashige and Skoog (1962) basal medium, with 5 mg l⁻¹ dithiothreitol, 25 g l⁻¹ d-glucose, without glycine and growth regulators (MS/2 medium); pH was adjusted to 5.5 prior to agar addition (7.5 g l⁻¹) and medium sterilization (10⁻⁵ Pa, 120 °C, for 20 min). For mutant seeds the medium was further supplemented with 1-9 g l⁻¹ ammonium sulfate. After 3 d, seedlings were transferred to a controlled environment (16 h photoperiod, at 23 ± 2 °C). Entire excised leaves from 21-d-old plantlets were used as explants.

Nitric oxide assays

The effect of mechanical stress on the production of NO was investigated using sodium nitroprusside (SNP) (CALBIOCHEM, La Jolla, CA, USA) as a non-enzymatic NO donor and N⁶-monomethyl-L-arginine (l-NMMA) (CALBIOCHEM) as a nitric oxide synthase (NOS) inhibitor. l-NMMA is an arginine analogue that acts as a competitive inhibitor of all three isoforms of NOS.

Isolated wild-type leaves were incubated under aseptic conditions for 3 h at 1 g (non-centrifuged) or 20 g (centrifuged), in 15 ml centrifuge tubes (three leaves per tube) with 2 ml of liquid modified MS/2 medium with the following compounds: 10⁻⁶ M SNP; 10⁻⁴ M SNP; 0.5 mM l-NMMA; or 1 mM l-NMMA. SNP and l-NMMA were sterilized by filtration and added to the autoclaved basal culture media. Several controls were performed: wild-type leaves were incubated in distilled water; in MS/2 medium; in media containing the N⁶-monomethyl-D-arginine (d-NMMA, 1 mM), enantiomer of l-NMMA (CALBIOCHEM); in media containing an NO scavenger, 2-(4-carboxyxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide, potassium salt (carboxy-PTIO, 50 mM) (Molecular Probes, Inc., Eugene, OR, USA); or in media lacking NO donors or inhibitors. d-NMMA, which does not have any significant effect on NOS, served as a negative control for non-specific inhibitors. NO was investigated using sodium nitroprusside (SNP) and L-NMMA were performed without either compound. Leaves were stained with DAF-2 DA for NO visualization and with TUNEL, for in situ detection of DNA fragmentation. Values are the mean of six independent experiments and results expressed in % (standard errors did not exceed 7% of mean values).

A. thaliana G/4-3 mutant leaves were also used to verify whether NO production originated from NOS-like activity and/or as a by-product of NR activity after mechanical stress. Leaves of the G/4-3 mutant were exposed to the same assays as wild-type leaves, except that SNP treatments were not performed because previous results had shown that exposure to SNP mimicked cell response to centrifugation independently of plant species (Pedroso et al., 2000a).
To study the effect of leaf-wounding on NO production, wild-type and G4-3 mutant leaves were wounded with forceps and subjected to the same treatments described above. Our aim was to investigate (a) whether physical wounding induced NO production; and (b) whether a saturated solution of L-NMMA (5 mM) blocked NO production in drastically wounded leaves and, once blocked, whether it could be restored by removing L-NMMA. For this purpose, non-centrifuged wild-type and G4-3 mutant leaves were wounded in MS/2 medium with 5 mM L-NMMA, incubated for 3 h and stained with DAF-2 DA. Some of these leaves were rinsed with water (three times) and wounded a second time. Leaves were then mounted in water and observed for NO visualization. All experiments were repeated six times with three leaves per treatment.

**Visualization of NO**

Entire wild-type and G4-3 mutant fresh leaves were stained immediately after each treatment using 4,5-diamino-fluorescein diacetate (DAF-2 DA) as a specific indicator of NO. The DAF-2 DA (diacetate form) enters the cells and is hydrolysed by cytosolic esterases releasing DAF-2 (4,5-diaminofluorescein). In the presence of NO and oxygen, a fluorescent DAF-2 triazole (DAF-2T) product is formed (Kojima et al., 1998a, b). Centrifuged and non-centrifuged leaves were incubated for 1 h at 25 °C, with 10 μM DAF-2 DA (CALBIOCHEM) (Kojima et al., 1998a, b; Pedroso et al., 2000a, b). After staining, samples were washed in water and mounted in Vectashield (Vector Laboratories, Inc, Burlingame, CA, USA) for NO visualization. DAF-2 DA-positive cells stain bright green at 495–515 nm. Guard
cells producing NO were counted in the whole leaves. Data are the percentage of the average number of DAF-2 DA positive guard cells.

TUNEL assay

"In situ" DNA fragmentation was detected by the TUNEL method. In this method the DNA fragments formed are detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) of DNA 3'-OH groups in cells (Gavrieli et al., 1992). The protocol (Pedroso, 1998) was as follows: entire Arabidopsis wild-type and G4-3 mutant leaves from all assays were fixed in 4 % (v/v) formaldehyde. Leaves were washed five times in PBS (10 mM NaH2PO4 × H2O, 150 mM sodium chloride, pH 7.2) for 1 h at 25 °C and incubated for 30 min in 4 % pectinase, 2 % cellulase. Leaves were then immersed in TDT buffer (30 mM Trisma base, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7-2) for 2 min at 37 °C. Leaves were immediately incubated at 37 °C for 1 h with 45 μl of TUNEL label (fluorescein-dUTP and dNTP) and 5 μl of TUNEL enzyme (terminal deoxynucleotidyl transferase) (Boehringer Mannheim, Mannheim, Germany) per slide. After washing for 15 min in TB buffer (300 mM sodium chloride, 30 mM sodium citrate, pH 8-5) at 37 °C to terminate the reaction, leaves were rinsed in PBS and counterstained for 10 min with DAPI (4,6-diamino-2-phenylindole dihydrochloride). Negative controls were carried out as described above but adding only the TUNEL label. Positive controls were incubated with 1 mg ml⁻¹ DNase I (Boehringer Mannheim) for 10 min. TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450–490 nm, whereas DAPI-positive nuclei (intact DNA) fluoresced blue at 360 nm. To further confirm the "in situ" DNA fragmentation in A. thaliana after mechanical stress, centrifuged and non-centrifuged G4-3 mutant leaves were stained for TUNEL as described above for wild-type leaves.

TUNEL-positive nuclei vs. TUNEL-negative ones were counted in whole leaves. Data are the percentage of the average number of TUNEL-positive nuclei recorded per leaf.

Fluorescence and confocal microscopy

DAF-2 DA- and TUNEL-labelled leaves were observed with an epi-fluorescence microscope, equipped with DAPI (excitation at 360 nm; emission ≥ 420 nm) and FITC filters (excitation at 450–490 nm; emission ≥ 520 nm), and Nikon camera with 400 ASA Elite Chrome Kodak film. Confocal images were acquired with a Leica TCS-NT laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany), equipped with Argon/Krypton and UV lasers, a Leitz DM IRBE epi-fluorescence microscope with an objective UV 40 × 1NA oil PL FLUOTA, and Leica TCS-NT software (TCS-NT version 1.5.451). Profiles from each acquired image were obtained using this software and further processed by Microsoft Excel. Leica files were imported to a Silicon Graphics (SIG) workstation with version 3.2 of Molecular Dynamics Image Space software (Molecular Dynamics, Inc., Sunnyvale, CA, USA), further processed using Photoshop® (Adobe Systems, Inc., Mountain View, CA, USA), and printed on a Kodak ds 8650P Colour Printer (Eastman Kodak Company, Rochester, NY, USA).
RESULTS

Mechanical stress increases NO production

The number of NO-producing guard cells was positively and significantly correlated with the concentration of SNP, an NO donor, in both non-centrifuged and centrifuged leaves (Fig. 1A, B). Addition of $10^{-4}$ M SNP to non-centrifuged samples increased (eight-fold) the number of DAF-2 DA-positive cells (green fluorescence) compared to the control (Fig. 1A). No significant differences were recorded when $10^{-6}$ SNP was added to the medium compared to the control. Centrifugation plus $10^{-4}$ M SNP resulted in a significant increase in NO production (approx. 2.7-fold) compared to centrifuged controls (Figs 1A, 2E). However, centrifugation alone led to an increase in NO production (Figs 1A, 2D). Furthermore, SNP mimicked centrifugation effects (Figs 1A, 2B). NO production occurred preferentially in the chloroplasts of guard cells (Fig. 2A and B) and in the cytosol of epidermal (Fig. 2B, D and E) and subepidermal cells (Fig. 2D1 and E1 inserts).
Fluorescence was visibly brighter in the chloroplasts of guard cells (Fig. 2A1 and B1 inserts) and at the cytosol level of subepidermal cells (Fig. 2D1 and E1 inserts), in both non-centrifuged and centrifuged leaves. The NOS inhibitor, L-NMMA, inhibited NO synthesis in non-centrifuged and centrifuged wild-type cells at both concentrations (0.5 and 1 mM) (Fig. 1A, B, Fig. 2C, F). NO profiles complemented these observations for both centrifuged and non-centrifuged samples (Fig. 2i and vi). To test whether NO bursts, produced after mechanical or wounding stresses, originated from NOS-like activity and/or as a by-product of NR activity, experiments with A. thaliana G^4-3 mutant, defective in the assimilation of nitrate, were performed. Fig. 2U shows that A. thaliana mutant leaves were able to produce NO after centrifugation treatment. As expected, NO production was not detected in unstained centrifuged and wounded wild-type and mutant leaves incubated in water or MS/2 medium (negative controls) (Fig. 3C, D, H and I), confirming that primary fluorescence was not interfering with our observations. Centrifuged leaves with D-NMMA, the enantiomer of L-NMMA, which has no significant effect on NOS activity, showed significant NO production in both wild-type and mutant leaves (Fig. 3M and R), compared with centrifuged leaves incubated with L-NMMA (Fig. 3L and Q). This confirmed the specificity of L-NMMA. Furthermore, NO staining was not observed in either wild-type or mutant leaves centrifuged with carboxy-PTIO (Fig. 3K and P).

Wounding plus centrifugation led to a significant increase in NO production in both wild-type and G^4-3 mutant leaves (Fig. 3E and J, respectively). NO production was observed in the cytosol of both epidermal and subepidermal leaf cells. Incubation of non-centrifuged-wounded leaves with 5 mM L-NMMA inhibited NO production in both the wild-type (Fig. 3N, arrowheads) and mutant (Fig. 3S, arrowheads). In contrast, a drastic increase in NO production was detected in the cytosol of parenchyma cells when wild-type and mutant leaves were wounded, after L-NMMA removal (Fig. 3O and T, respectively), showing that the inhibitor was effective in suppressing NO production following drastic wounding, and suggesting the presence of an inducible form of NOS in A. thaliana.

In situ DNA fragmentation

The effect of NO and centrifugation on DNA fragmentation was studied in centrifuged and non-centrifuged Arabidopsis wild-type and G^4-3 mutant leaves using the TUNEL method. Chloroplast DNA fragmentation occurred in both centrifuged and non-centrifuged controls (without SNP or L-NMMA) (Fig. 1A, B). DNA fragmentation was significantly higher in centrifuged-control leaves (59%) compared to non-centrifuged ones (21%). The same results were obtained for mutant leaves (data not shown). NO generated by SNP (10^-4 M) significantly increased DNA fragmentation in centrifuged (96%) and non-centrifuged (50%) leaves, compared with control leaves incubated without SNP. Chloroplast and nuclear DNA fragmentation was observed in both centrifuged wild-type and G^4-3 mutant parenchyma cells (Fig. 3W and Z, respectively). Non-centrifuged cells did not show nuclear DNA fragmentation (Fig. 3Y). To investigate whether NO was the only cause of DNA fragmentation, we incubated Arabidopsis leaves with L-NMMA, an inhibitor of NOS. Both 0.5 and 1 mM L-NMMA reduced DNA fragmentation in non-centrifuged whole leaves (approx. 2.1-fold and 5.25-fold, respectively) (Fig. 1A). However, the higher concentration (1 mM) was less effective than the lower concentration (0.5 mM) for the centrifuged leaves (approx. 1.79-fold and 2.68-fold DNA decrease, respectively) (Fig. 1B).

To test if a problem in the uptake of L-NMMA solution was the reason for these results, the same experiments were performed with Arabidopsis leaf sections; identical results were obtained (data not shown).

DISCUSSION

NO production and DNA fragmentation were identical for Arabidopsis wild-type and the nitrate reductase (NR) nia1, nia2 double mutant (G^4-3) exposed to wounding and centrifugation treatments. NO production increased significantly following centrifugation and wounding, and was also increased by exposure to an NO generator (SNP) and inhibited by an NO-synthase inhibitor (L-NMMA). These results were consistent for all the experiments performed. NO was visualized in the chloroplasts of guard cells and in the cytosol of epidermal and subepidermal cells. Localization of NO production in chloroplasts and in the cytosol was also reported for Taxus brevifolia and Kalanchōe daigremontiana cells (Magalhaes et al., 1999; Pedroso and Durzan, 2000; Pedroso et al., 2000a, b).

The addition of D-NMMA (L-NMMA enantiomer) did not prevent NO production, showing that NOS inhibition by L-NMMA was stereospecific. DAF-2 DA-positive cells were not observed when carboxy-PTIO was included in the medium, confirming that DAF-2 DA is a specific indicator of NO.

Although G^4-3 mutant plants are known to have low residual activity for NR (0-5% of the wild-type control) (Wilkinson and Crawford, 1993), NO production was not detected in 1 g mutant controls. Furthermore, in wild-type and G^4-3 mutant leaves, L-NMMA completely inhibited NO bursts elicited by centrifugation stress and wounding. These results indicated that in Arabidopsis thaliana, NO production originated from NOS-like activity and not as a by-product of NR. Our results reaffirm the work reported by Barroso et al. (1999) and Ribeiro et al. (1999), in which NOS was localized in the cytosol, nucleus, peroxisomes and chloroplasts of Pisum sativum and Zea mays cells. A DNA sequence with similarity to NOS of Rattus norvegicus was recently identified on chromosome 4 of A. thaliana (Mayer et al., 1999). A protein that inhibits constitutive animal neuronal NOS (nNOS) was also identified as an EST from Arabidopsis (Jaffrey and Snyder, 1996). Taken together, these findings indicate the occurrence of mammalian-type NOS activity in plants.

Our results show that the triggers for the increased NO bursts were the mechanical stressful treatments since: (a) NO production was not detected or was reduced in the controls; and (b) NO levels were significantly increased by...
centrifugation and wounding. These results are consistent with previous reports in plants, where increased NO production was reported following other forms of plant stress (Haramaty and Leshem, 1997; Leshem et al., 1997, 1998; Delledonne et al., 1998; Durner et al., 1998; Beligni and Lamattina, 1999a; Magalhaes et al., 1999; Pedrosa et al., 2000a, b).

Whether NO is cytoprotective or cytotoxic depends on specific conditions (Wink and Mitchell, 1998). It has a positive role in plant defence responses (Inzé and Montagu, 1995; Noritake et al., 1996; Delledonne et al., 1998; Beligni and Lamattina, 1999a) and in growth and development (Leshem, 1996; Leshem and Haramaty, 1996; Leshem et al., 1998; Pedrosa and Durzan, 1999a). However, NO can act synergistically with reactive oxygen species (ROS) to induce oxidative cell death (OCD) in plants (Miller and Day, 1997; Delledonne et al., 1998). These authors also reported that an oxidative burst requires NO for maximal killing. Our study shows that SNP and centrifugation led to an NO burst, and to a significant increase in the number of cells with DNA fragmentation, compared to controls (see Fig. 1A, B). DNA fragmentation was observed in the nucleus and chloroplasts of wild-type and G4-3 mutant parenchyma cells. Nucleoid DNA fragmentation following an NO burst was previously reported for chloroplasts of guard and parenchyma cells in Kalanchoe daigremontiana (Pedroso and Durzan, 1999b, 2000). As in Kalanchoe, nucleoid DNA fragmentation in Arabidopsis cells was more frequently observed than nuclear DNA fragmentation, suggesting that NO-induced-nucleoid DNA fragmentation preceded nuclear DNA fragmentation and eventually cell death. When 0.5 mM l-NMMA was added to both centrifugation and non-centrifugation assays, it significantly reduced DNA fragmentation compared to controls. Identical results with 0.5 mM l-NMMA were reported for callus cells and foliar tissues of Kalanchoe daigremontiana and Taxus brevifolia (Pedroso et al., 2000a, b). When Arabidopsis cells were centrifuged with 1 mM l-NMMA, an unexpected increase (11%) in DNA fragmentation was recorded (see Fig. 1A, B). These experiments were repeated six times with identical results for both centrifuged-whole leaves and leaf sections, suggesting that factors other than NO and related to l-NMMA somehow contributed to DNA damage and cell death.

The present results support a role for NO as a signalling molecule in the response to mechanical stimulation and wounding.

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