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Arabidopsis Nitric Oxide Synthase1 Is Targeted to Mitochondria and Protects against Oxidative Damage and Dark-Induced Senescence

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The *Arabidopsis thaliana* protein nitric oxide synthase1 (NOS1) is needed for nitric oxide (NO) synthesis and signaling during defense responses, hormonal signaling, and flowering. The cellular localization of NOS1 was examined because it is predicted to be a mitochondrial protein. NOS1–green fluorescent protein fusions were localized by confocal microscopy to mitochondria in roots. Isolated mitochondria from leaves of wild-type plants supported Arg-stimulated NO synthesis that could be inhibited by NOS inhibitors and quenched by a NO scavenger; this NOS activity is absent in mitochondria isolated from *nos1* mutant plants. Because mitochondria are a source of reactive oxygen species (ROS), which participate in senescence and programmed cell death, these parameters were examined in the *nos1* mutant. Dark-induced senescence of detached leaves and intact plants progressed more rapidly in the mutant compared with the wild type. Hydrogen peroxide, superoxide anion, oxidized lipid, and oxidized protein levels were all higher in the mutant. These results demonstrate that NOS1 is a mitochondrial NOS that reduces ROS levels, mitigates oxidative damage, and acts as an antisenescence agent.

INTRODUCTION

Nitric oxide (NO) is a central signaling molecule in plants and animals (reviewed in Wendehenne et al., 2001, 2004; Lamattina et al., 2003; Neill et al., 2003; del Rio et al., 2004; Romero-Puertas et al., 2004; Crawford and Guo, 2005; Delledonne, 2005; Lamotte et al., 2005). Its signaling function was first recognized in animals when it was discovered that NO is the endothelium-derived relaxation factor produced in response to vasodilators (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Palmer et al., 1987). In plants, early work showed that NO could be emitted by plants (Klepper, 1979) and acted as a growth regulator (reviewed in Beligni and Lamattina, 2001a, 2001b). Subsequently, it was shown to serve as a signal in defense and programmed cell death (PCD), hormone responses, abiotic stress, root and xylem development, germination, iron homeostasis, and flowering (reviewed in Lamattina et al., 2003; Neill et al., 2003; Wendehenne et al., 2004; Crawford and Guo, 2005; Delledonne, 2005; Lamotte et al., 2005; Simpson, 2005). For example, NO mediates abscisic acid–induced stomatal closing (reviewed in Desikan et al., 2004) and auxin-induced lateral and adventitious root growth (Pagnussat et al., 2002, 2003).

Studies on the mechanisms of NO synthesis have shown that plants and animals rely on different enzymes. Animals primarily use large heme flavoproteins (130 to 160 kD) that are found in three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible (iNOS) (Alderton et al., 2001). These enzymes catalyze the conversion of Arg to citrulline and NO in the presence of NADPH and oxygen and are activated by calcium and calmodulin. nNOS and eNOS are classified as constitutive NOSs because they are rapidly activated by increases in calcium levels and show limited transcriptional regulation. By contrast, iNOS constitutively binds calcium and calmodulin and is regulated primarily at the transcriptional level. eNOS and nNOS produce NO at much lower rates than iNOS and thus serve primarily to generate NO for signaling. iNOS makes large amounts of NO as a cytotoxic agent in immune responses. A mitochondrial NOS (mtNOS) activity has been characterized in mammals, but the identification of the protein(s) responsible for this activity has been controversial (Brookes, 2004; Lacza et al., 2004; Ghafourifar and Cadenas, 2005). All three isoforms of NOS have at one time or another been implicated as mtNOS.

Plants produce NO using different enzymes. The two known substrates for NO synthesis in plants are nitrite and Arg. It has long been known that nitrate reductase can reduce nitrite to NO (Dean and Harper, 1986, 1988; Klepper, 1990; Yamasaki and Sakihama, 2000; Rockel et al., 2002). Recently, it was shown that mitochondria also support nitrite-dependent NO synthesis (Tischner et al., 2004; Planchet et al., 2005). NO emission from wild-type plants or intact mitochondria is very weak in air and requires anaerobic conditions to detect strong signals. Other mechanisms of nitrite-dependent NO production include nonenzymatic reactions (Bethke et al., 2004) and a Ni–NO reductase activity detected in roots (Stohr et al., 2001). Arg-dependent nitric NOS characteristic of animal systems has also been reported in plants (reviewed in Neill et al., 2003; del Rio et al., 2004). Plant NOS activity can be inhibited by animal NOS inhibitors that act as Arg analogs, such as L-NAME and L-NMMA;

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however, no gene or protein with similarity to the full animal NOS proteins has been found.

Recently, a gene (*AtNOS1*; referred to hereafter as *NOS1*) was identified that is needed for NO production in *Arabidopsis thaliana* (Guo et al., 2003). NOS1 has no sequence similarity to animal-type NOS isoforms yet catalyzes NO synthesis in vitro, indicating that NOS1 is a novel NOS. NOS1 was first identified by its similarity to a snail protein that was implicated in NO synthesis in an unknown way (Huang et al., 1997). In *Arabidopsis*, NOS1 is needed for efficient germination, root and shoot growth, seed fertility, and abscisic acid–induced stomatal closure (Guo et al., 2003) and participates in the control of flower timing (He et al., 2004). NOS1 is also needed for defense responses; *nos1* mutants are more susceptible to a bacterial pathogen and show almost no response to lipopolysaccharide treatment by microarray analysis (Zeidler et al., 2004). Approximately 80% of NOS activity is eliminated in *nos1* mutants (Guo et al., 2003; He et al., 2004; Zeidler et al., 2004), indicating that NOS1 accounts for most but not all NOS activity. The biochemical properties of NOS1 are more similar to eNOS and nNOS than to iNOS, because NOS1 purified 80 times by affinity chromatography supports Arg-dependent NO production that has a low V_{max} similar to that of eNOS and is calcium- and calmodulin-dependent (Guo et al., 2003). In addition, abscisic acid does not affect the level of *NOS1* mRNA levels, indicating that NOS1 is constitutive, like the signaling eNOS and nNOS enzymes.

The identification of a novel NOS protein in plants raises many questions. Where is it located in the cell, and what role does it play in reactive oxygen species (ROS) production and oxidative stress? As described below, computational analyses predict that NOS1 is targeted to the mitochondria. Such localization would place this NOS enzyme in a key location to control cellular processes such as ROS production and PCD or senescence. It would also provide a new candidate for the long-sought mtNOS enzyme. The results of experiments performed to address these questions are presented below.

RESULTS

NOS1 Is Targeted to the Mitochondria, Where It Is Required for Arg-Dependent NO Synthesis

Computational analysis of the NOS1 protein sequence revealed that NOS1 has a high probability of being targeted to the mitochondria (MitoProtII, 95%; PSORT, 80% [in matrix]; TargetP, 78%). To test this prediction, transgenic *Arabidopsis* plants were generated that carried a *green fluorescent protein* (*GFP*) reporter fused in frame to the C terminus of the *NOS1* coding region driven by a 35S promoter. Seedlings (the T2 generation) were grown vertically on agarose plates, and then roots of 5-d-old transgenic plants were examined by confocal microscopy for *GFP* expression. Strong GFP fluorescence was observed in a punctate pattern in the mature part of roots (Figure 1A) and in root hairs (Figure 1B), indicating that the NOS1-GFP fusion protein was localized to an organelle. To determine whether these sites of GFP accumulation are mitochondria, the pattern of GFP fluorescence was compared with that from MitoTracker Red, a mitochondria-specific stain (Poot et al., 1996). Fluorescence signals from NOS1-GFP (Figure 1C) colocalized with those from MitoTracker in root hairs (Figures 1D and 1E). These results indicate that NOS1 is targeted to the mitochondria.

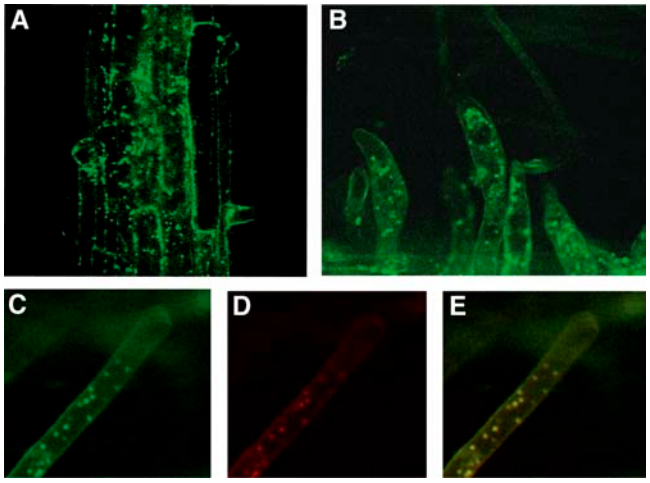

Figure 1. Mitochondrial Localization of NOS1.

Roots and root hairs from *Arabidopsis* plants carrying *p35S-NOS1cDNA-GFP* constructs were examined by confocal microscopy. NOS1-GFP fluorescence (green) is shown in mature root **(A)**, root hairs **(B)**, and a single root hair **(C)**. **(D)** shows MitoTracker fluorescence (red) in the same root hair shown in **(C)** stained with 500 nM MitoTracker Red 580 (Molecular Probes), and **(E)** shows a composite of NOS1-GFP and MitoTracker signals. Yellow indicates an overlap in NOS1-GFP and MitoTracker fluorescence. More than 30 seedlings were examined, and representative tissues are shown.

These findings led us to test the role of NOS1 in Arg-dependent NO synthesis in mitochondria. The NO-reactive dye 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) was used to detect NO production by mitochondria. A similar approach has been used in animal cells using the related DAF dye 4,5-diaminofluorescein diacetate (Lopez-Figueroa et al., 2000, 2002). In intact mammalian cells, part of the overall NO-dependent fluorescence colocalized with a subpopulation of mitochondria that stained with MitoTracker Red. In our study, NO production was assayed in isolated mitochondria from leaves of wild-type and *nos1* mutant plants. The mitochondrial preparation showed a punctate pattern of DAF-FM fluorescence without the addition of any substrate (Figure 2A). When simultaneously stained with MitoTracker, the majority of strong DAF-FM signals could be seen to overlap with the MitoTracker signal (Figures 2A to 2C), indicating that most of the strong DAF-FM fluorescence could be assigned to mitochondria. Next, the effect of the substrate Arg and various inhibitors on NO synthesis was examined. Preincubating the mitochondrial preparation with 2.5 mM Arg significantly increased the level of DAF-FM fluorescence (Figures 2D and 2E). This strong fluorescence overlapped with MitoTracker fluorescence, indicating that the DAF-FM fluorescence was from mitochondria (data not shown). Increased DAF-FM fluorescence was not observed with 2.5 mM citrulline (Figure 2F) and was severely inhibited by 500 μM 2-4-carboxyphenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) (Figure 2G), a NO scavenger, and by 5 mM L-NAME (Figure 2H) or L-NMMA

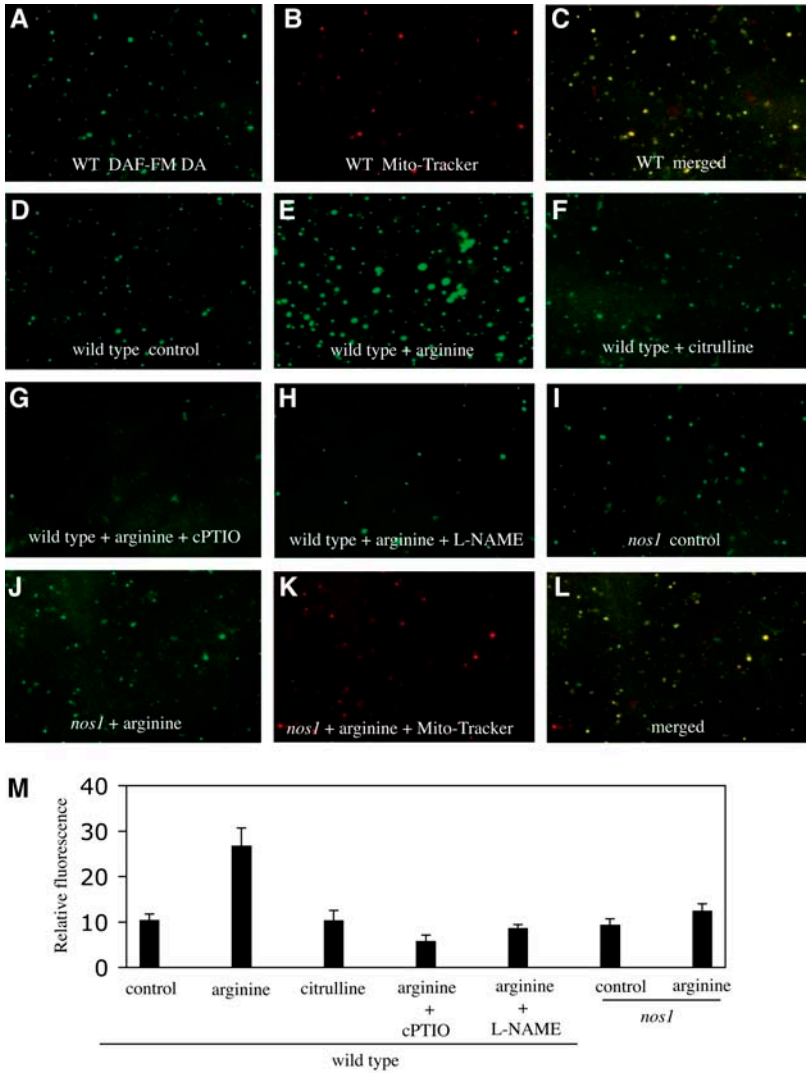

Figure 2. NO Production in Isolated Mitochondria.

NO production was detected by confocal microscopy in isolated mitochondria stained with DAF-FM DA (**[A]**, **[C]** to **[J]**, and **[L]**) or with MitoTracker (**[B]**, **[C]**, **[K]**, and **[L]**). Mitochondria were treated as follows.

(A) to **(C)** Wild-type mitochondria showing fluorescence from DAF-FM **(A)**, MitoTracker **(B)**, and both DAF-FM and MitoTracker as a merged composite **(C)**.

(D) to **(H)** Wild-type mitochondria stained with DAF-FM and incubated with no Arg **(D)**, with 2.5 mM Arg **(E)**, with 2.5 mM citrulline **(F)**, with 2.5 mM Arg and 500 μM cPTIO **(G)**, and with 2.5 mM Arg and 5 mM L-NAME **(H)**.

(I) to **(L)** *nos1* mutant mitochondria showing fluorescence from DAF-FM (**[I]** and **[J]**), from MitoTracker **(K)**, and from both as a merged composite **(L)**. *nos1* mitochondria were incubated with no Arg **(I)** or with 2.5 mM Arg (**[J]** to **[L]**).

(M) Average relative DAF-FM fluorescence signal densities are shown for isolated mitochondria corresponding to **(D)** to **(J)**. Error bars indicate SD.

(data not shown), two mammalian NOS inhibitors. The amount of fluorescence was quantified and is shown in Figure 2M. These results indicate that mitochondria purified from *Arabidopsis* leaves support Arg-dependent NO synthesis.

These experiments allowed us to determine whether NOS1 is required for NO synthesis in mitochondria. Arg-stimulated NO synthesis was examined in mitochondria prepared from *nos1* mutant plants. The results showed that Arg has little effect on DAF-FM fluorescence in *nos1* mitochondria compared with the untreated control (Figures 2I to 2K). The level of fluorescence for *nos1* mitochondria in the presence or absence of Arg is approximately the same as for wild-type mitochondria in the absence of Arg (Figure 2A). In addition, the MitoTracker fluorescence from *nos1* mitochondria (Figure 2K) was approximately the same as for wild-type mitochondria (Figure 2B), indicating that both preparations had an equivalent concentration of intact mitochondria. We conclude that NOS1 is a mtNOS required for Arg-dependent NO production in mitochondria of plant cells.

NOS1 Regulates Dark-Induced Leaf Senescence

Leaf senescence is a developmentally controlled, degenerative process induced by exogenous signals such as light and water deficits and regulated by endogenous factors such as ethylene and cytokinin (Dangl et al., 2000; Buchanan-Wollaston et al., 2003; Lim et al., 2003; Yoshida, 2003; Lin and Wu, 2004). NO has been implicated as an antisenescence signal in that NO treatments extend the postharvest life of fruits and vegetables (Leshem et al., 1998) and reverse the loss of protein in abscisic acid–, methyl jasmonate–, and H_2O_2-treated rice (*Oryza sativa*) (Hung and Kao, 2003, 2004, 2005). Also, NO levels are downregulated during the natural senescence of pea (*Pisum sativum*) leaves (Corpas et al., 2004) and are negatively correlated with ethylene levels (Leshem et al., 1998; Leshem and Pinchasov, 2000).

We investigated the role of NOS1 in leaf senescence. Senescence was induced by dark treatment of detached leaves, a procedure that is commonly used to artificially induce senescence (Weaver and Amasino, 2001; del Rio et al., 2003; Oh et al., 2003; Chrost et al., 2004). Under these conditions, *nos1* mutant leaves senesce more rapidly than do wild-type leaves. Individual leaves from wild-type plants became pale green after 3 to 4 d of dark treatment, whereas leaves from the *nos1* mutant showed severe yellowing and cell death (Figure 3A). Measurements of chlorophyll content showed that chlorophyll was lost more quickly in *nos1* mutant leaves, diminishing by 74% after 3 d and by 91% after 4 d, whereas wild-type leaves lost 41 and 55% of their chlorophyll after 3 and 4 d, respectively (Figure 3B). Treatment of the *nos1* mutant leaves with the NO donor sodium nitroprusside (SNP) slowed dark-induced senescence and chlorophyll loss (leaves retained 43% of chlorophyll with SNP treatment compared with 9% without treatment after 4 d) (Figures 3A and 3B). Examination of cell viability showed that cell death was significantly higher in the *nos1* mutant as measured by Evans blue staining (Figure 3C). Evans blue measures cell death for an entire leaf. Trypan blue staining also showed higher rates of cell death in *nos1*, occurring as isolated dead cells in the mutant after 4 d of dark treatment (Figure 3A). These results indicate that NOS1 protects plants from dark-induced senescence and that the accelerated senescence of *nos1* leaves results from NO deficiency, because exogenous addition of NO can reverse the phenotype.

We next examined senescence of intact plants. Dark-induced leaf senescence occurs more slowly in intact *Arabidopsis* plants relative to detached leaves (Weaver and Amasino, 2001). Upon dark treatment for 4 d, no increase in yellowing was observed in both wild-type and mutant plants, although leaves began to lose pigment (Figure 4A). After dark treatment for 5 d, some older leaves of the mutant plants showed increased yellowing and collapse of leaf structure, whereas wild-type plants were still pale green with no visible yellowing (Figure 4A). At day 6, almost all of the older leaves were yellow and collapsed in the mutant, whereas only some of the older wild-type leaves showed yellowing and collapse. When chlorophyll levels were examined, mutant plants showed a more rapid loss of chlorophyll than wild-type plants, especially on days 4 and 5 (Figure 4B). In leaves from the mutant, chlorophyll levels decreased by 37% on day 4 and by 66% on day 5, whereas the levels in wild-type leaves were reduced by only 14% on day 4 and by 36% on day 5. After 6 d of dark treatment, wild-type and mutant plants were returned to light conditions (16 h of light, 8 h of dark) for 8 d. Approximately 40% of wild-type plants recovered, whereas only 5% of the mutants survived (Figures 4A and 4C). These results indicate that *NOS1* affects senescence and dark recovery rates of both detached and attached leaves. They also provide genetic evidence that NO is an endogenous regulator of plant leaf senescence.

nos1 Mutant Plants Have Enhanced Levels of ROS and Oxidized Proteins and Lipids

Senescence involves ROS and oxidative reactions that result in increases in lipid peroxidation (Buchanan-Wollaston, 1997; Jing et al., 2003; Laloi et al., 2004; Lam, 2004). NO is known to interact with ROS, especially during PCD, affecting the extent of ROS accumulation and cell death (Delledonne et al., 1998, 2001; Beligni et al., 2002; Beligni and Lamattina, 2002; Neill et al., 2002, 2003; Wendehenne et al., 2004; Zeier et al., 2004; Delledonne, 2005; He et al., 2005). In cell culture and during hypersensitive responses, NO works synergistically with H_2O_2 to induce cell death (Delledonne et al., 1998, 2001; Zhang et al., 2003; Romero-Puertas et al., 2004; Tada et al., 2004; Wendehenne et al., 2004; Delledonne, 2005). During senescence, however, NO appears to work as an antioxidant and slows degeneration. In rice, application of NO donors counteracts abscisic acid– and methyl jasmonate–induced leaf senescence and reduces H_2O_2 accumulation (Hung and Kao, 2003, 2004).

To determine what effect NOS1 has on ROS accumulation, young seedlings and detached leaves of wild-type and *nos1* plants were examined. Endogenous H_2O_2 and O_2^- levels were monitored with 3,3′-diaminobenzidine and nitroblue tetrazolium staining, respectively. We also examined H_2O_2 levels during dark-induced senescence in leaves of wild-type and mutant plants.

Basal H_2O_2 levels were higher in young *nos1* seedlings compared with wild-type seedlings when grown in peat soil under long-day conditions (Figure 5A). The H_2O_2 accumulated most intensely at the outer perimeter of older leaves. Likewise, higher levels of H_2O_2 were observed in detached leaves harvested from 3-week-old *nos1* plants (Figures 5B and 5C, day 0). When leaves were dark-treated to induce senescence, H_2O_2 accumulated more rapidly in *nos1* mutant leaves compared with wild-type leaves (Figures 5B and 5C). O_2^- levels were also higher in detached leaves from mutant plants compared with wild-type leaves at day 0, but, in contrast with H_2O_2, no distinct accumulation of O_2^- was found in wild-type and *nos1* leaves during dark-induced senescence (Figure 5D). These results indicate that NOS1 suppresses H_2O_2 and O_2^- accumulation in leaves of young plants and H_2O_2 accumulation during dark-induced senescence.

The higher levels of ROS in mutant plants led us to examine oxidative damage in the form of protein and lipid oxidation. Total soluble proteins from leaves were analyzed by SDS-PAGE. As a control, the protein gels were stained with Coomassie blue, and they showed that proteins appeared more degraded in the *nos1* mutant (Figure 6A, day 0). After 3 d of dark treatment, protein degradation in the mutant was even more apparent (Figure 6A). This loss of intact protein in the mutant corresponded to the

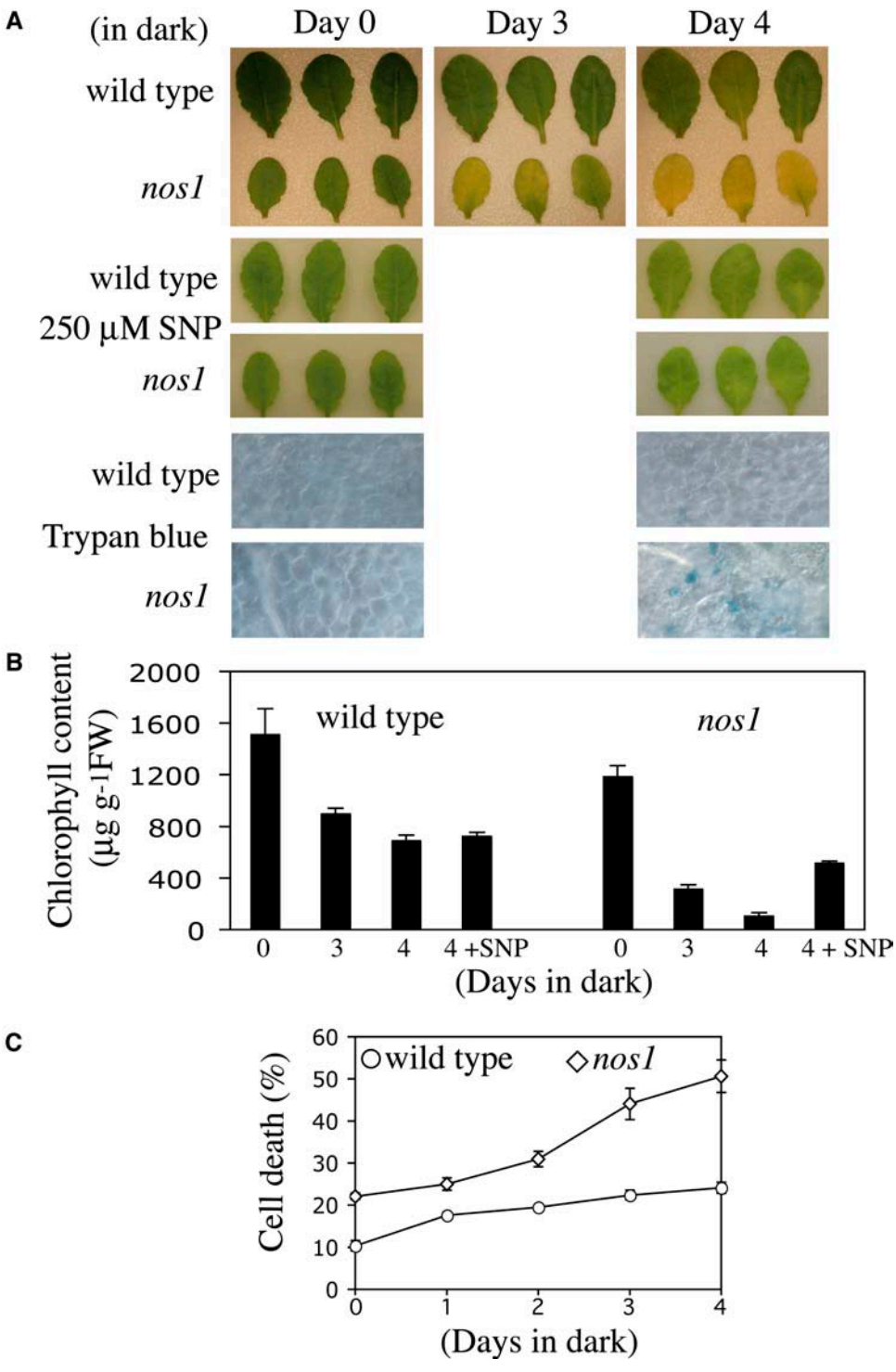

Figure 3. Leaf Senescence in Wild-Type and *nos1* Mutant Plants.

Fully expanded leaves detached from 3-week-old wild-type and *nos1* plants were placed in the dark.

(A) Images of untreated leaves or leaves treated with 250 μM SNP were taken at days 0, 3, and 4 of dark treatment. Images of leaves stained with trypan blue, an indicator of cell death, were taken at days 0 and 4. Six or more leaves were examined for each condition, and representative fields are shown.

(B) Chlorophyll content in detached leaves. Chlorophyll was extracted from wild-type and *nos1* leaves and measured spectrophotometrically as described in Methods. FW, fresh weight. Error bars indicate SD ($n = 6$).

(C) Cell death was measured spectrophotometrically as Evans blue staining in wild-type and *nos1* detached leaves as described in Methods. Error bars indicate SD ($n = 6$).

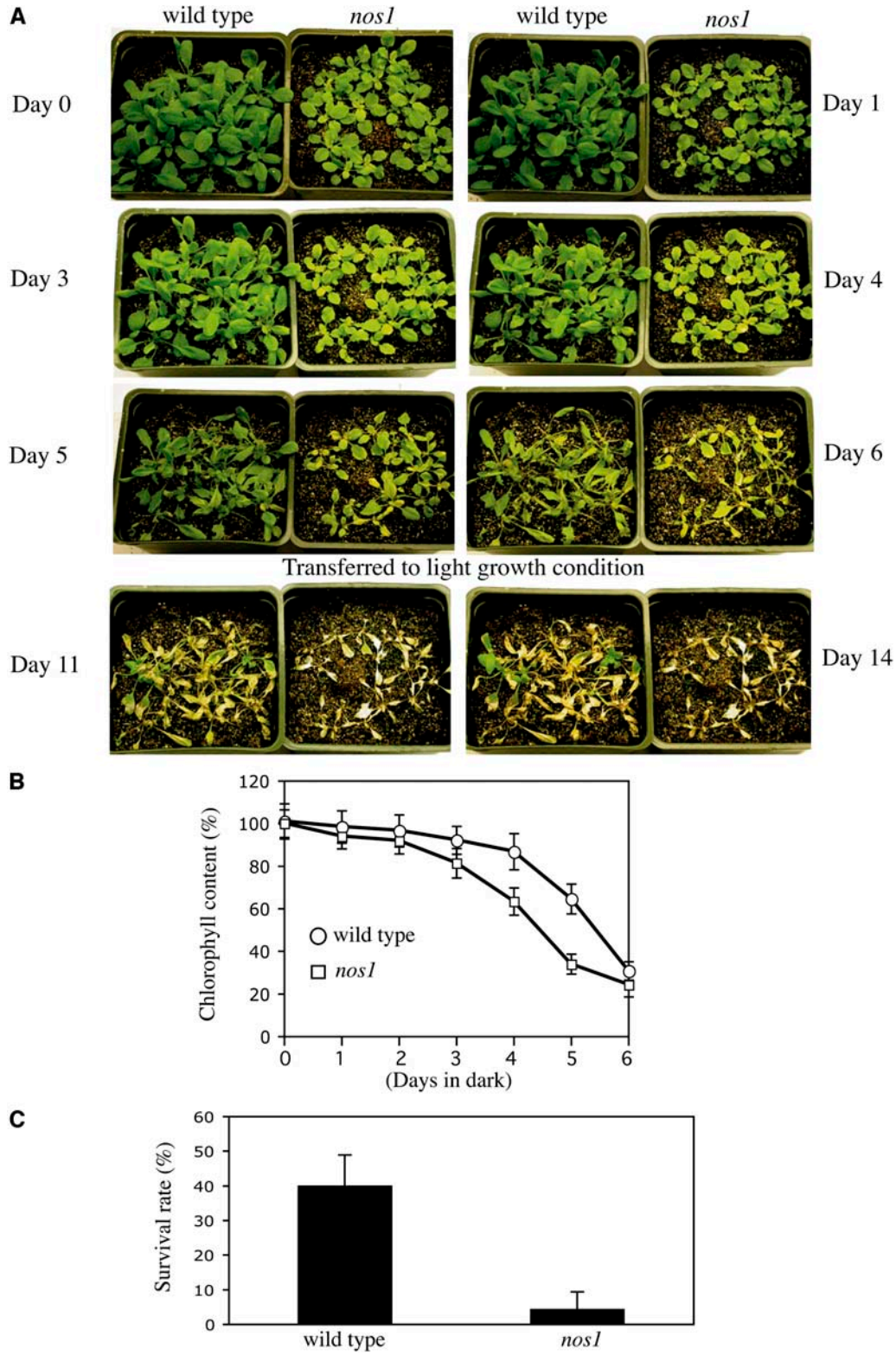

Figure 4. Comparison of Senescence Rates and Survival of Wild-Type and *nos1* Plants.

(A) Wild-type and *nos1* plants were grown for 3 weeks in long-day conditions (16 h of light, 8 h of dark), transferred to the dark for 6 d, and then transferred back to long-day conditions for 8 d.

(B) Chlorophyll content was determined in leaves of wild-type and *nos1* plants after 0 to 6 d of dark treatment as described for Figure 3. Error bars indicate SD ($n = 6$).

(C) Survival rates of wild-type and *nos1* mutant plants after transfer from dark (6 d) to light (8 d) growth conditions. Error bars indicate SD ($n = 6$).

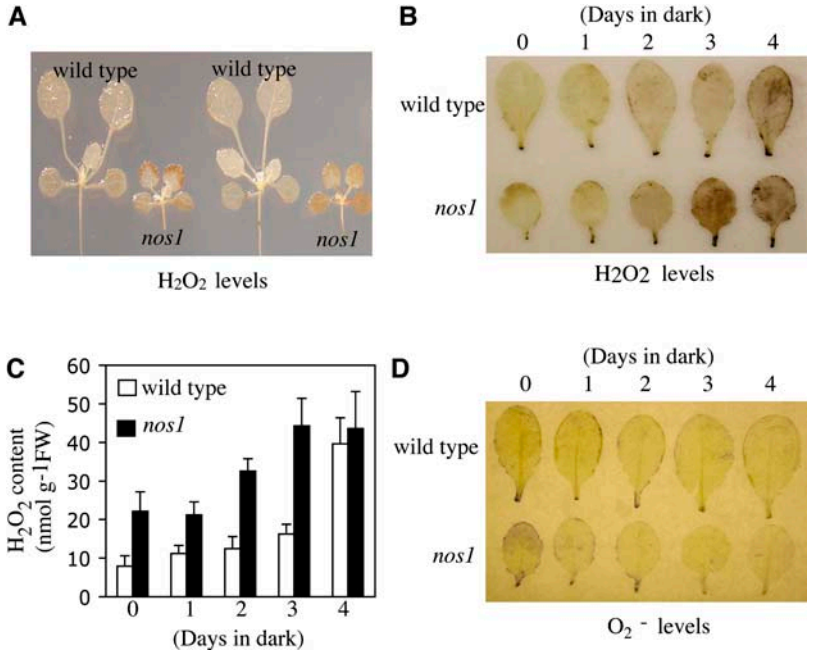

Figure 5. Comparison of ROS Levels in Wild-Type and *nos1* Plants.

(A) and **(B)** H_2O_2 levels were visualized by staining with 3,3′-diaminobenzidine in shoots of wild-type and *nos1* seedlings **(A)** and senescing leaves detached from wild-type and *nos1* plants dark-treated for the indicated times **(B)**.

(C) H_2O_2 concentrations were quantified in senescing leaves detached from wild-type and *nos1* plants dark-treated for the indicated times using an Amplex red H_2O_2/peroxidase assay kit (Molecular Probes). FW, fresh weight. Error bars indicate SD ($n = 6$).

(D) O_2^- levels in senescing detached leaves of wild-type and *nos1* plants were detected by staining with nitroblue tetrazolium.

maximal H_2O_2 accumulation at day 3 of dark treatment (Figure 5C). We then examined the extent of protein oxidation by measuring carbonylation levels, a marker of protein oxidation (Levine et al., 1990, 1994b; Johansson et al., 2004; Davletova et al., 2005). The *nos1* mutant leaves had much higher levels of oxidized proteins than did wild-type leaves before dark treatment (Figure 6B, day 0). After dark treatment, levels of oxidized protein in the wild type increased slightly after 1 d, then decreased to undetectable levels at day 3 (Figure 6B). In *nos1*, levels of oxidized protein decreased dramatically after 1 d of dark treatment, then disappeared by day 3 (Figure 6B).

Measurements of lipid peroxidation (malondialdehyde [MDA] levels) revealed that the *nos1* mutant had a higher level of MDA than the wild type before dark treatment (Figure 7, day 0). These levels did not increase significantly during the 3 d of dark treatment, whereas the levels of MDA in wild-type leaves were initially lower (56% of that in *nos1* at day 0) and increased somewhat during 3 d of dark treatment but remained lower relative to *nos1* (Figure 7).

DISCUSSION

NOS1 Is a mtNOS and Is Required for Arg-Stimulated NO Biosynthesis in Mitochondria

At present, the only known Arg-dependent NOS in plants is NOS1. In this study, NOS1 was localized to the mitochondria by demonstrating colocalization of NOS1-GFP and MitoTracker signals in roots. In addition, NOS1 was required for Arg-stimulated NO production in isolated mitochondria. NO production was visualized with the NO-sensitive dye DAF-FM DA, and the specificity of fluorescence signals was confirmed using the NO scavenger cPTIO. This test is important because the specificity of DAF-based dyes for NO in mitochondria has been questioned (Brookes, 2004). The Arg-stimulated activity was also inhibited by two mammalian NOS inhibitors (L-NAME and L-NMMA), indicating that a NOS activity is responsible for the NO production in mitochondria.

The localization of NOS1 to the mitochondria is surprising. The only reports of mitochondrial NO synthesis in plants document a nitrite-dependent activity (Tischner et al., 2004; Planchet et al., 2005). Other nitrite-dependent enzymes are located in the cytosol (nitrate reductase) or the plasma membrane (Ni–NO reductase) (Stohr et al., 2001; Stohr and Ullrich, 2002). By contrast, Arg-dependent NOS activities have been linked to peroxisomes (Barroso et al., 1999; Corpas et al., 2001, 2004; Prado et al., 2004) and chloroplasts (Foissner et al., 2000; Gould et al., 2003) but not to mitochondria. Our findings indicate that mitochondria are also an important source of Arg-derived NO in plants.

The localization of NOS1 to the mitochondria is also provocative in that it may provide a solution to a long-standing puzzle in mammalian systems. Much effort has been devoted to identifying and characterizing mtNOS in mammals. The reason for this intense interest is that NO regulates key functions of

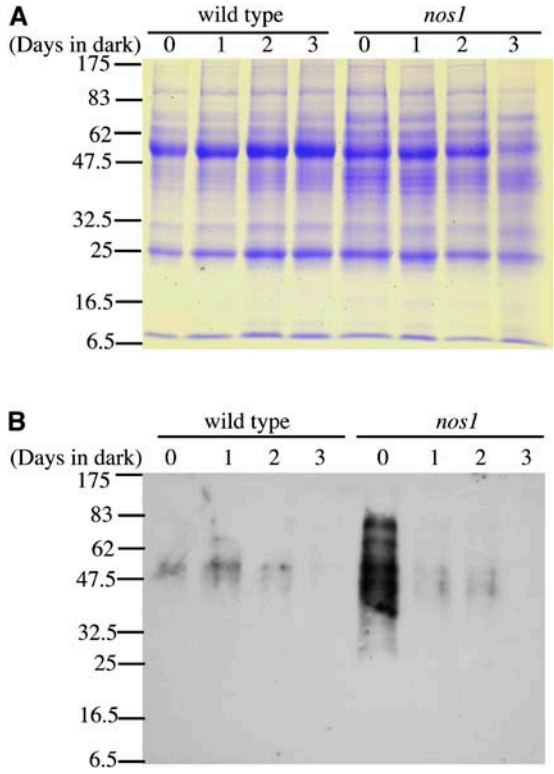

Figure 6. Analysis of Protein Degradation and Oxidation in Senescing Leaves of Wild-Type and *nos1* Plants.

(A) Total protein extracts from detached leaves of wild-type and *nos1* plants dark-treated for 0 to 3 d. Proteins were resolved by SDS-PAGE and stained with Coomassie blue.

(B) Extent of protein oxidation as revealed by treatment with dinitrophenylhydrazine and analysis by SDS-PAGE and protein gel blotting as described in Methods. Detached leaves of wild-type and *nos1* plants dark-treated for 0 to 3 d were examined as indicated.

mitochondria (reviewed in Brown and Borutaite, 2001; Moncada and Erusalimsky, 2002; Brown, 2003; Giulivi, 2003). First, NO reversibly inhibits oxygen consumption by competitively inhibiting cytochrome oxidase (Millar and Day, 1996; Caro and Puntarulo, 1999; Yamasaki et al., 2001; Cooper, 2002). (In plants, alternative oxidase is NO-resistant and thus can support respiration in the presence of NO [Millar and Day, 1997; Yamasaki et al., 2001].) Second, NO modulates ROS production by mitochondria (Sarkela et al., 2001). Third, NO regulates mitochondria-mediated apoptosis (Brown and Borutaite, 2001; Chung et al., 2001; Boyd and Cadenas, 2002; Blaise et al., 2005) by serving as an antiapoptotic agent (e.g., by blocking the activity of caspases [Torok et al., 2002]) or as a proapoptotic signal (e.g., by opening the permeability transition pore and inducing the release of cytochrome c [Brown and Borutaite, 2001; Saviani et al., 2002; Zottini et al., 2002]). Lastly, NO is a signal for mitochondrial biogenesis (Brown, 2003; Nisoli et al., 2003). Because NO can diffuse across membranes, it is possible that these effects can be accomplished by NO synthesized outside of the mitochondria; however, an internal source of NO could provide a direct internal signal (Boyd and Cadenas, 2002). There have been many reports of mitochondrial NO synthesis; unfortunately, a consensus about the identity of mtNOS has not been forthcoming.

The first reports of mtNOS described an Arg- and calcium-dependent activity in purified mitochondrial preparations (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Tatoyan and Giulivi, 1998). Efforts to identify mtNOS have implicated eNOS (Kobzik et al., 1995; Gao et al., 2004), iNOS (Tatoyan and Giulivi, 1998), and a subtype of nNOS (Kanai et al., 2001; Elfering et al., 2002; Riobo et al., 2002; Haynes et al., 2004). The preponderance of evidence currently supports nNOS (Haynes et al., 2004; Ghafourifar and Cadenas, 2005); however, no mitochondrial targeting sequence has been identified for any of these proteins. Several recent reports have raised questions about mtNOS and have concluded that the identification of mtNOS is still in doubt (Lacza et al., 2003; Brookes, 2004; Ghafourifar and Cadenas, 2005).

The discovery that NOS1 is targeted to the mitochondria raises the question, could NOS1 be a prototype for a mammalian mtNOS? Unlike the mammalian NOS enzymes, NOS1 is predicted to be in the mitochondria. The *nos1* knockout mutant provides genetic evidence that NOS1 is required for Arg-dependent NO synthesis in mitochondria. There are proteins with similar sequences to NOS1 in a variety of animals, including human, mouse, and rat (Zemojtel et al., 2004). These proteins may be the mammalian orthologs of NOS1 and, if located in the mitochondria, may be mammalian mtNOS enzymes.

NOS1 Attenuates ROS Levels and Oxidative Stress

It has been known for many years that ROS can be cytotoxic and contribute to disease and aging in animals (Ames et al., 1993; Hensley and Floyd, 2002) and defense responses in plants (Levine et al., 1994a; Lamb and Dixon, 1997). More recently, it has been shown that ROS also act as signals (Finkel, 2003; Laloi et al., 2004; Mittler et al., 2004). NO is intimately linked with ROS, and many processes that respond to one also respond to the other or both (Neill et al., 2002, 2003; Wendehenne et al., 2004). For example, NO reacts with O_2^-, producing peroxynitrite ($ONOO^-$), which itself is reactive and toxic. In animals, NO

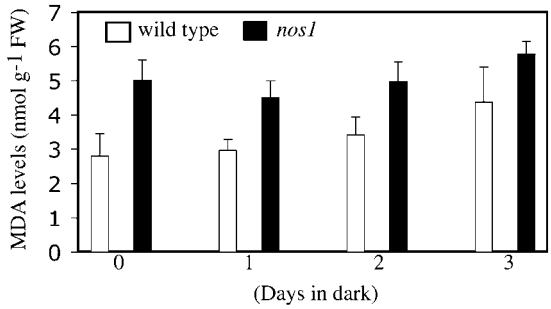

Figure 7. Analysis of Lipid Oxidation in Senescing Leaves of Wild-Type and *nos1* Plants.

Detached leaves were dark-treated for 0 to 3 d, and total lipid was extracted and assayed for MDA levels as described in Methods. FW, fresh weight. Error bars indicate SD ($n = 6$).

inhibits oxygen reduction by cytochrome oxidase, resulting in higher production of H_2O_2 (Sarkela et al., 2001). In these capacities, NO acts as a prooxidant. However, NO can also act as an antioxidant by scavenging free radicals and inhibiting oxidant formation (Wink et al., 2001; Mohanakumar et al., 2002; Sharpe et al., 2003). In fact, NO is a more potent inhibitor of lipid peroxidation than vitamin E (Rubbo et al., 2000).

Both antioxidant and prooxidant effects of NO have been described in plants. Reports of prooxidant effects show that NO inhibits or represses ROS-scavenging enzymes, including ascorbate peroxidase and catalase (Clark et al., 2000; Murgia et al., 2004). Reducing NO levels by NO dioxygenase in transgenic *Arabidopsis* plants results in a reduction of H_2O_2 levels during bacterial infection, indicating that NO normally increases ROS levels during an infection (Zeier et al., 2004). By contrast, other reports indicate that NO acts as an antioxidant. In transgenic tobacco (*Nicotiana tabacum*) plants expressing alfalfa (*Medicago sativa*) hemoglobin (a NO-scavenging enzyme), ROS levels are higher than in control plants during bacterial infection, indicating that NO normally suppresses ROS accumulation (Seregelyes et al., 2003). NO donors counteract photooxidative damage during treatment with methyl viologen herbicides by reducing H_2O_2, O_2^-, and ·OH radical levels and slowing ion leakage, protein and lipid oxidation, loss of chlorophyll, and protein degradation (Beligni and Lamattina, 1999, 2002). The authors concluded that NO acts as an antioxidant by reacting directly with ROS. In barley (*Hordeum vulgare*) aleurone layers, NO treatments delay gibberellic acid–induced PCD, which is mediated by ROS (Beligni et al., 2002). In this case, NO appeared to act both as an antioxidant and as a signal that delays the loss of catalase and superoxide dismutase. In rice, NO donor treatments reverse abscisic acid– and methyl jasmonate–induced increases in H_2O_2 and oxidized protein levels (Hung and Kao, 2003, 2004).

The studies described above indicate that NO can affect ROS levels and oxidative damage. The phenotypes of the *nos1* mutant provide critical genetic data supporting the linkage between NO and ROS. In addition, the *nos1* data indicate that the role of NOS1 is to reduce the level of ROS (H_2O_2 and O_2^-) and oxidized proteins and lipids in plants grown under nonstress conditions. NOS1 also reduces the level of H_2O_2 production and protein degradation during dark-induced senescence. We cannot say whether NO made by NOS1 acts directly as an antioxidant or as a signal that indirectly modulates ROS accumulation, but we can conclude that the resulting NO reduces levels of oxidized proteins and lipids.

Mitochondria, Oxidative Damage, Leaf Senescence, and Cell Death: NOS1 Provides a Common Link

Numerous reports have shown that senescence and cell death involve ROS and oxidative damage (Jing et al., 2003; Overmyer et al., 2003; Wendehenne et al., 2004). They also involve mitochondria, because mitochondrial metabolism is altered during senescence and mitochondria can contribute to cell death (Robson and Vanlerberghe, 2002; Saviani et al., 2002; Fridovich, 2004; Laloi et al., 2004; Vacca et al., 2004; Yao et al., 2004). Mitochondria also contribute to ROS production (Moller, 2001; Tiwari et al., 2002; Overmyer et al., 2003; Mittler et al., 2004). NO can affect mitochondrial metabolism and modulate ROS accumulation, senescence, and cell death (Tiwari et al., 2002; Jing et al., 2003; Overmyer et al., 2003; Vacca et al., 2004). All of these linkages share a common element: NOS1. The *nos1* mutant shows more rapid senescence and cell death in dark-treated plants and detached leaves. NOS1 also reduces ROS levels and oxidative damage. NOS1 is located in mitochondria and is needed for Arg-dependent NO synthesis in mitochondria. These results indicate that NOS1, located within the mitochondria, plays a protective role in the cell by inhibiting oxidative processes that lead to cell death and senescence.

An important point to note is that the above discussion highlights the protective effect of NO and NOS1 during normal growth and dark-induced senescence. This should not be confused with the cytotoxic effect of NO during hypersensitive responses, in which NO works with H_2O_2 to induce PCD. Such dual effects of NO have long been noted in animals, in which protective effects occur at low concentrations of NO normally found in physiological conditions in cells that are not stressed or infected, whereas high levels induce inflammation and pathological effects (Wink et al., 2001). Antagonistic effects dependent on NO concentration have also been reported in plants (Leshem and Haramaty, 1996; He et al., 2004).

Another question that arises from our results is, how significant is ROS production by mitochondria in producing oxidative stress? High levels of ROS are produced by several sources in plant cells, including chloroplasts, peroxisomes, and NADPH oxidases in the apoplast. In mammalian systems, the role of mitochondria in producing ROS is well established (Balaban et al., 2005), but there is some disagreement about the importance of plant mitochondria in producing ROS. Some authors hold that mitochondria are a significant source (Moller, 2001; Tiwari et al., 2002; Overmyer et al., 2003; Mittler et al., 2004), but others do not (Laloi et al., 2004). There are numerous reports of linkages between each of the following: senescence (and cell death), ROS production, oxidative damage, and altered mitochondrial metabolism (Sweetlove et al., 2002; Tiwari et al., 2002; Jing et al., 2003; Overmyer et al., 2003; Vacca et al., 2004). For example, ROS production in mitochondria increases significantly in dark-induced senescent pea leaves (Jimenez et al., 1998) and in aged potato (*Solanum tuberosum*) tubers (Boveris et al., 1978). Our data do not resolve this issue, but they do indicate that NOS1, which produces NO in mitochondria, reduces ROS accumulation and the resulting oxidative damage in entire leaves. Such protection is important for mitochondria because mitochondrial proteins are especially vulnerable to oxidative stress (Sweetlove et al., 2002; Bartoli et al., 2004).

Our data also suggest that because *nos1* mutant plants suffer from a heavier burden of ROS and oxidative damage, they are more vulnerable to dark-induced senescence. This view is consistent with the free radical theory of aging in animals, which states that ROS and oxidative damage promote aging, cellular senescence, and PCD (Finkel and Holbrook, 2000; Hensley and Floyd, 2002; Balaban et al., 2005). For example, oxidation of proteins increases with age (Oliver et al., 1987; Stadtman, 2001) and is associated with increased protein degradation (Levine et al., 1981), cellular deterioration, and disease (Stadtman, 2001;

Nystrom, 2005). In plants, however, these linkages are not so straightforward. For example, protein oxidation increases with age only during the vegetative phase and then declines dramatically during flowering (Johansson et al., 2004). In our studies, levels of protein oxidation were much higher in growing *nos1* mutant plants but decreased dramatically when leaves were induced to senesce by dark treatment. It is unlikely that the dark treatment alone induced this decline, because protein oxidation levels are not much affected by light conditions, as shown in a previous study (Johansson et al., 2004). Thus, in both the findings from Johansson et al. (2004) and our results, levels of protein oxidation in *Arabidopsis* do not always correlate with aging or senescence.

METHODS

Bioinformatic Analysis of Intracellular Location

Sites for computational analysis of the NOS1 protein sequence were as follows: MitoProtII (http://ihg.gsf.de/ihg/mitoprot.html), PSORT (http://psort.ims.u-tokyo.ac.jp/), and TargetP (http://www.cbs.dtu.dk/services/TargetP/).

Plant Materials and Growth Conditions

Wild-type and *nos1* mutant plants of *Arabidopsis thaliana* ecotype Columbia were grown in peat soil (Sun Gro Horticulture) with fertilizer (Pete's 20-10-20; McConkey). All plants were grown under a light cycle of 16 h of light and 8 h of dark at 24°C. For plants grown on agarose plates, seeds were surface-sterilized, first in 70% ethanol for 5 min and then in 5% bleach for 15 min, washed with water, and plated on germination medium as described (Guo et al., 2001). Plates were kept at 4°C for 2 d and then germinated vertically at 24°C under continuous light.

Constructs and Plant Transformation

NOS1 cDNA (1.7 kb) was amplified by RT-PCR and confirmed by sequencing. The following primers were used in the PCR to create *Xho*I and *Spe*I sites: 5′-GAGCTCGAGATGGCGCTACGAACACTC-3′ and 5′-TCGACTAGTCGAAAGTACCATTTGGGTCT-3′. The resulting *NOS1* cDNA was cloned into the pGEM-T Easy vector (Promega). *p35S-NOS1-GFP* reporter constructs were generated by making translational fusions of the 1.7-kb *Xho*I-*Spe*I fragment into the *Xho*I-*Spe*I sites of p35S-GFP-JFH1 vector. This GFP vector was kindly provided by J.F. Harper (University of Nevada, Reno) (Hong et al., 1999). In the fusion construct, the stop codon TGA of NOS1 was replaced with CGA followed by CTAGTGATGGFP. Transgenic *Arabidopsis* plants were produced by vacuum-infiltrating 4-week-old plants in *Agrobacterium tumefaciens* culture containing the appropriate construct (Bechtold et al., 1993). Seeds from treated plants were collected and screened for kanamycin resistance. Transgenic plants identified in this generation were classified as T1 plants.

Isolation and Staining of Mitochondria

Leaves (50 g) were harvested from 3-week-old seedlings. Mitochondria were isolated according to Michalecka et al. (2004). The purified mitochondria were resuspended in wash medium supplemented with 5% (v/v) DMSO, frozen in liquid nitrogen, and stored at −80°C until used. Mitochondria were stained with 500 nM MitoTracker Red 580 (Molecular Probes).

NO Measurements in Isolated Mitochondria

Isolated mitochondria (20 μl) were incubated on ice for 30 min with Arg (2.5 mM) alone or with cPTIO (500 nM) or the mammalian NOS inhibitors L-NMMA and L-NAME (5 mM). After incubation, mitochondria were loaded with 15 μM DAF-FM DA in the dark for 20 min at ambient temperature. After this loading period, the mitochondria were pipetted gently onto a glass microscope slide using a 200-μL pipette tip with the last 5 mm removed and then covered with a glass cover slip. Fluorescent signals were detected using a confocal microscope. Emission light was collected at 515 ± 10 nm, and excitation was at 488 ± 10 nm. Signal intensities were quantified using Photoshop (Adobe Systems).

Dark-Induced Senescence

Fully extended leaves detached from 3-week-old plants were placed onto 9-mm-diameter Petri dishes with double-layer Whatman filter papers at bottom containing 15 mL of distilled water without (untreated) or with 250 μM SNP. Petri dishes were sealed with Parafilm tape to avoid NO escaping, wrapped with double-layer aluminum foil, and kept at ambient temperature. For intact plant senescence experiments, 3-week-old plants grown in peat soil with 16 h of light and 8 h of dark were transferred from the growth room to a dark room. After 6 d of dark treatment, plants were moved back to the growth room with 16 h of light and 8 h of dark, and surviving plants were counted 8 d later.

Measurement of Chlorophyll Content

Chlorophyll was extracted from individual leaves with 80% acetone. Chlorophyll content was determined spectrophotometrically at 663 and 646 nm according to Lichtenthaler (1987).

Measurement of Cell Death

Cell death, indicated as loss of plasma membrane integrity, was measured spectrophotometrically by Evans blue staining of detached leaves as described (Wright et al., 2000; Rea et al., 2004) with minor modifications. Briefly, detached leaves, completely submerged in a 0.1% (w/v) aqueous solution of Evans blue dye (Sigma-Aldrich), were subjected to two 5-min cycles of vacuum followed by 30 min under vacuum. The leaves were then washed three times with distilled water (15 min each). Dye bound to dead cells was solubilized in 50% (v/v) methanol and 1% (w/v) SDS at 60°C for 30 min and then quantified by absorbance at 600 nm. For 100% cell death, the detached leaves were heated at 100°C for 5 min before staining. Two to three leaves were pooled for each sample. Six samples were analyzed for each data point. This experiment was repeated three times with equivalent results.

Cell death was visualized in senescing detached leaves by lactophenol–trypan blue staining followed by destaining in saturated chloral hydrate as described (Koch and Slusarenko, 1990).

Histochemical Staining for H_2O_2 and O_2^- Detection

Production of H_2O_2 in young seedlings and detached leaves was measured by staining plants with 3,3′-diaminobenzidine as described (Rea et al., 2004) and then boiling in 96% ethanol for 10 min. Detection of O_2^- with nitroblue tetrazolium was performed essentially according to Jabs et al. (1996) and Overmyer et al. (2000).

H_2O_2 Measurement

Frozen leaves (0.2 g) were ground to a powder under liquid nitrogen, and H_2O_2 was extracted from leaves according to the method described previously (Rao et al., 2000). H_2O_2 concentration was measured with an

Amplex red H_2O_2/peroxidase assay kit (Molecular Probes). Six samples were analyzed and averaged for each data point.

Protein Extraction and Detection of Oxidized Proteins

Protein was extracted from detached leaves of *Arabidopsis* according to Martinez-Garcia et al. (1999). The protein concentration was determined with the Bio-Rad protein assay kit.

Oxidized proteins were detected by measuring carbonyl content in extracted proteins from detached leaves according to Levine et al. (1990). 2,4-D–treated protein samples (10 μg of protein per lane) were resolved by SDS-PAGE (10% acrylamide gels). After electrotransfer of the proteins to polyvinylidene difluoride membranes, dinitrophenylhydrazine moieties were detected with mouse anti-dinitrophenyl primary antibodies (dilution, 1:2500 [v/v]; Sigma-Aldrich) and peroxidase-labeled anti-mouse antibody (dilution, 1:3000 [v/v]; Amersham Biosciences). Immunosignal was developed using enhanced chemiluminescence detection reagents (Amersham Biosciences).

Detection of Lipid Peroxidation

The extent of lipid peroxidation in leaves was estimated by measuring the amount of MDA, a decomposition product of the oxidation of polyunsaturated fatty acids, as described (Havaux et al., 2003). Briefly, one adult leaf was ground in 1 mL of chilled reagent (0.25% [w/v] thiobarbituric acid in 10% [w/v] trichloroacetic acid). After incubation at 90°C for 20 min, the extracts were cooled at room temperature and centrifuged at 12,000*g* for 15 min. The absorbance of the supernatant was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm.

Accession Number

The Arabidopsis Genome Initiative locus identifier for *Arabidopsis* NOS1 is At3g47450.

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