Mitochondria play an essential role in nitric oxide (NO) signal transduction in plants. Using the biotin-switch method in conjunction with nano-liquid chromatography and mass spectrometry, we identified 11 candidate proteins that were S-nitrosylated and/or glutathionylated in mitochondria of Arabidopsis (Arabidopsis thaliana) leaves. These included glycine decarboxylase complex (GDC), a key enzyme of the photorespiratory C3 cycle in C3 plants. GDC activity was inhibited by S-nitrosogluthathione due to S-nitrosylation/S-glutathionylation of several cysteine residues. Gas-exchange measurements demonstrated that the bacterial elicitor harpin, a strong inducer of reactive oxygen species and NO, inhibits GDC activity. Furthermore, an inhibitor of GDC, aminoacetonitrile, was able to mimic mitochondrial depolarization, hydrogen peroxide production, and cell death in response to stress or harpin treatment of cultured Arabidopsis cells. These findings indicate that the mitochondrial photorespiratory system is involved in the regulation of NO signal transduction in Arabidopsis.

Nitric oxide (NO) has emerged as a new chemical messenger in plant biology. It can interact with a variety of intracellular and extracellular targets, acting as either a cytotoxic or a cytoprotective agent. NO stimulates seed germination in different species, and a decrease in NO levels has been associated with fruit maturation and senescence of flowers (Beligni and Lamattina, 2001). NO production has been observed in response to several biotic and abiotic stimuli, such as pathogen infection, bacterial elicitors, high temperature, osmotic stress, and UV-B light (Durner et al., 1998; Barroso et al., 1999; Krause and Durner, 2004; Zeidler et al., 2004; Shapiro, 2005; Corpas et al., 2008; Kolbert et al., 2008; Zhao et al., 2009).

Despite the proven importance of NO, little is known about signaling pathways downstream from it. During both programmed cell death and defense responses, NO requires cGMP and cADP rib as secondary messengers (Wendehenne et al., 2001). Furthermore, NO activates mitogen-activated protein kinases in different plant species during stress signaling (Nakagami et al., 2005). However, direct biological activity of NO arises from chemical reactions between proteins and NO itself (Foster and Stamler, 2004; Dahm et al., 2006). S-Nitrosylation is a labile post-translational modification with a half-life of seconds to a few minutes and represents a very sensitive mechanism for regulating cellular processes (Hess et al., 2005). More than 100 candidate S-nitrosylated proteins were identified from extracts of Arabidopsis (Arabidopsis thaliana) cultured cells treated with the NO donor S-nitrosoglutathione (GSNO) and from Arabidopsis leaves treated with gaseous NO (Lindermayr et al., 2005). Using the same proteomic approach, changes were characterized in S-nitrosylated proteins in Arabidopsis leaves undergoing a hypersensitive response (Romero-Puertas et al., 2008).

In animals, mitochondria play a crucial role in S-nitrosylation-dependent NO signaling (Foster and Stamler, 2004). The mitochondrion is an essential organelle for normal cellular function, being an important site of ATP synthesis and an integrator for apoptotic signaling (Skulachev, 1999). Mitochondria interact with NO at several levels. One particularly well-characterized example is the inhibition of complex IV (cytochrome c oxidase) via binding of NO to its binuclear CuB/heme a3 site (Cleeter et al., 1994). There are several reasons why S-nitrosylation may be an important mitochondrial regulatory mechanism. For example, mitochondria contain sizeable pools of thiols and transition metals, all of which are known to modulate nitrosothiol (SNO) biochemistry (Foster and Stamler, 2004). In addition, mitochondria are highly membranous and accumulate lipophilic molecules such as NO. Interesting in this respect is the fact that the formation of the S-nitrosylating intermediate...
$\text{N}_2\text{O}_3$ is enhanced within membranes (Burwell et al., 2006).

The role of mitochondria in stress-related responses has been investigated in both animals and plants. Endogenous nitrosylation of the catalytic Cys site of a subset of mitochondrial caspases serves as an on/off switch regulating caspase activity during apoptosis (Mannick et al., 2001). Moreover, cytochrome $c$, which is modified by NO at its heme iron during apoptosis, is released from mitochondria into the cytoplasm, which plays a critical role in many forms of apoptosis by stimulating apoptosome formation and subsequent caspase activation (Schonhoff et al., 2003). We previously showed that a prime target of NO in plants is the mitochondrial apparatus, causing an inhibition of KCN-sensitive respiration and an activation of alternative respiration via alternative oxidase (AOX; Huang et al., 2002; Krause and Durner, 2004; Livaja et al., 2008).

The aim of this study was to identify possible targets for S-nitrosylation in mitochondria of Arabidopsis leaves in order to gain more insight into the regulatory function of NO at the protein level. Using a proteomic approach involving the highly specific biotin-switch method for detection and purification of S-nitrosylated proteins (Jaffrey and Snyder, 2001) in conjunction with liquid chromatography and tandem mass spectrometry (nanoLC/MS/MS), we could identify 11 mitochondrial proteins as targets for S-nitrosylation. Among these identified proteins, we focused our attention on the P-subunit of the Gly decarboxylase complex (GDC), which is an integral part of the photosynthetic system. Since the release of apoptotic factors from mitochondria may be a result of inhibition of respiration, transition of mitochondrial permeability, and formation of reactive oxygen species (ROS; Saviani et al., 2002; Taylor et al., 2004; Chen and Gibson, 2008),

Figure 1. Harpin-dependent NO production in mitochondria. Arabidopsis cell cultures were treated either with extracts of nontransformed $E. \text{coli}$ (A) or with 35 $\mu$g mL$^{-1}$ recombinant partially purified harpin (B–D). Thirty minutes before microscopic analysis, cells were incubated with MitoTracker Red 580 as a mitochondria-specific dye (red fluorescence, center column) and DAF-2FM DA as an NO probe (green fluorescence, right column) and analyzed using a fluorescence confocal microscope (Zeiss LSM 510 NLO; 40× water lens). For NO scavenging, cells were incubated with 0.5 mM cPTIO 20 min before application of the NO probe. Colocalization of both fluorescent signals appears yellow (left column). A, Cells treated with extracts of nontransformed $E. \text{coli}$. The images were taken 6 h after treatment. B and C, Cells were treated with recombinant harpin. The images were taken 1 h (B) and 6 h (C) after treatment. D, Cells were treated with recombinant harpin followed by incubation with 0.5 mM cPTIO and DAF-2FM DA. The images were taken 6 h after treatment. NO fluorescence was completely scavenged by the cPTIO preincubation.
we investigated the molecular mechanism and the function of GDC-Cys modification in Arabidopsis.

RESULTS

Harpin-Induced Generation of NO in Mitochondria

While a strict relationship between harpin-induced generation of NO in mitochondria was proven in different organisms and in different signaling events (Millar and Day, 1996; Cooper and Davies, 2000; Huang et al., 2002; Krause and Durner, 2004), direct evidence for NO production in plant leaf mitochondria is still missing. To elucidate NO localization in mitochondria, we treated Arabidopsis cultured cells with the bacterial elicitor harpin because we previously showed that this is able to induce NO production within a few hours (Krause and Durner, 2004). To analyze NO production in mitochondria, Arabidopsis cultured cells were loaded with the NO-specific fluorescent dye diaminofluorescein (DAF-2FM DA) and with the mitochondria-specific dye MitoTracker Red 580. Fluorescence was visualized by confocal laser microscopy (Fig. 1) as described (Foissner et al., 2000; Pedroso et al., 2000; Beligni et al., 2002; Yao et al., 2002). As a control, we applied carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (cPTIO), an NO scavenger that does not react with any ROS (Barchowsky et al., 1999). After a 1-h treatment with 35 \( \mu \text{m} \) L \(^{-1} \) harpin, an NO burst became visible in Arabidopsis mitochondria, with increasing intensity during the next hours (Fig. 1). The NO scavenger cPTIO was able to inhibit the elicited burst.

Detection of S-Nitrosylated Proteins in Mitochondria

To identify S-nitrosylated proteins in mitochondria of Arabidopsis leaves, we used a modified biotin-switch method (Jaffrey and Snyder, 2001). The mitochondrial fraction from the leaves was prepared according to Keech et al. (2005). Since the mitochondrial proteome of Arabidopsis is well established (Kruft et al., 2001; Millar et al., 2001; Eubel et al., 2007), partially purified mitochondria were a useful experimental system to study posttranslational protein modification (Supplemental Fig. S1).

After treating mitochondrial proteins either with the trans-nitrosylating agent GSNO or with glutathione (GSH) as a negative control, S-nitrosylated proteins were biotinylated and separated by SDS-PAGE (Fig. 2; Supplemental Fig. S2). In order to identify S-nitrosylated proteins, GSNO- and GSH-derived eluates were subjected to nanoLC/MS/MS analysis. It was possible to identify 25 proteins with a significant score as judged by the Mascot search algorithm (score > 30). Five of these were chloroplast protein contaminations, while nine were not listed in the mitochondrial proteome (Millar et al., 2001) and did not harbor the mitochondrial targeting signal as identified by analysis using either TargetP 1.1 or iPSORT (Table I). Furthermore, mitochondrial fractions seemed to be contaminated by peroxisomes, as indicated by detection of catalase \( \beta \). Among 11 mitochondrial proteins in Table I, we could identify three subunits of the GDC, the key enzyme of the photorespiratory C\(_2\) cycle in plants, namely Gly dehydrogenase subunit P2 (15223217), Gly decarboxylase H1 (15226973), and Gly dehydrogenase subunit P1 (14596025).

Mass Spectrometric Analyses of GSNO-Treated P Protein

GSNO is able to S-nitrosylate or S-glutathiolate Cys residues, while both modifications can have different effects on protein conformation and activity. We investigated which kind of modification(s) resulted from the GSNO treatment of GDC, focusing our attention on the decarboxylating subunit, the P protein. In Arabidopsis, there are two genes encoding the P subunit, which share high homology. Since the production of recombinant P protein was not successful, we partially purified the P subunit from Arabidopsis mitochondria as described (Bourguignon et al., 1988) and tested its...
integrity by assaying it for the CO₂-exchange reaction (Supplemental Fig. S3). S-Nitrosylation of this partially purified P protein was confirmed using the biotin-switch method (data not shown). In addition, we used mass spectrometric analyses to detect GSNO-mediated modifications of the Cys residues, since this method allows the detection of both S-nitrosylation and S-glutathionylation. Protein S-glutathionylation is promoted by nitrosative stress and transient S-nitrosylation, but it also occurs in unstressed cells (Daile-Donne et al., 2009). After treating the P protein with GSNO and digesting it with trypsin, we analyzed the Cys-containing peptides for their modifications (Fig. 3; Supplemental Fig. S4). C₄₀₂ and C₄₆₃ were found to be S-glutathionylated after all treatments. In contrast, C₉₈, C₉₄₃, C₇₇₇, and C₁₀₂₂ were S-glutathionylated only after treatment with 1 mM GSNO (Table II). No S-nitrosylated Cys residues were detected by mass spectrometric analysis, probably because of the transient nature of this modification at the P protein, as reported for several other proteins (Martinez-Ruiz and Lamas, 2007).

### Inhibition of Gly Decarboxylase Activity by GSNO

To elucidate the effects of S-nitrosylation/S-glutathionylation on GDC activity, we treated mitochondria with GSNO and monitored the GDC activity (Walker et al., 1982; Walker and Oliver, 1986). Following ¹⁴C-CO₂ release from [¹⁴C]Gly (i.e. the activity of P and H subunits), we could observe a significant reduction of decarboxylation, which was dependent on GSNO concentration (Fig. 4A). After 20 min, the GDC activity of mitochondria incubated with 0.25 mM GSNO showed an inhibition of 60% as compared with the GDC activity of untreated organelles. Treatment with 1 mM GSNO further decreased the decarboxylation to about 30%, similar to the inhibition obtained with the GDC inhibitor aminooacetonitrile (AAN; 10 mM). The incubation of mitochondria with the reducing agents dithiothreitol (DTT) and GSH increased decarboxylation activity by 76% and 15% in vivo, respectively, demonstrating the redox-dependent regulation of the P protein and suggesting a change in the mitochondrial redox status during purification. To further characterize the redox dependency of GDC activity, we tested the effects of two Cys-modifying agents on the enzymatic activity: N-ethylmaleimide, a highly reactive agent that covalently and irreversibly alkylates free Cys thiol groups, and 5,5'-dithiobis-(2-nitrobenzoic acid), an oxidizing reagent. Both treatments resulted in a complete inhibition of P/H protein activity within 20 min (Fig. 4A).

Modulation of GDC activity by GSNO was confirmed using Arabidopsis leaf slices as described by Navarre and Wolpert (1995). Incubation with 1 mM GSNO for 20 min resulted in an inhibition of GDC activity by 75%, which could be restored by 1 mM DTT (Fig. 4B). Moreover, to test if modulation of GDC activity was due to a direct effect of GSNO on the photosynthetic system, Arabidopsis leaf slices were incubated with inhibitors of complex I of the mitochondrial respiration system (rotenone) and of AOX (salicylhydroxamic acid [SHAM]). In animal systems, complex I has been shown to be regulated by S-nitrosylation and to be involved in reversible ROS production (Borutaite and Brown, 2006; Burwell et al., 2006). In plants, it was shown that the NO-induced AOX suppressed the production of ROS and cell death induced by KCN-sensitive respiration inhibition (Huang et al., 2002). Therefore, the inhibition of GDC activity after GSNO treatment could have been an indirect effect of ROS induced by inhibition of complex I. It is well known that the activity of complex I of the mitochondrial electron transport chain is inhibited by nitrosothiols and peroxinitrite (Carreras et al., 2004; Dahm et al., 2006). This inhibition results in increased production of ROS, which can cause oxidative damage of GDC (Taylor et al., 2002). However, blocking of complex I in mitochondria via rotenone did not affect

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**Table I. S-Nitrosylated proteins from Arabidopsis mitochondria**

Mitochondria extracts treated with GSNO or GSH were subjected to the biotin-switch method and analyzed by nanoLC/MS/MS after tryptic digestion. The Mascot search engine was used to parse MS data to identify proteins from primary sequence databases. The best-matching peptide identifying the protein is given. If there were additional peptides found, the number of the peptides is given. The asterisks indicate proteins not listed in the mitochondrial proteome (Millar et al., 2001) but with mitochondrial targeting signal (TargetP). Proteome analysis was carried out by TopLab.

| Protein Identifier | Protein Name | Molecular Mass | Identified Peptides (Score) |
|--------------------|--------------|----------------|----------------------------|
| 15223217           | Gly dehydrogenase subunit P2 | 18,000 | K.LTESPGLINSSPYEDGWMIK.V (87) +2 |
| 15221119           | Aminomethyltransferase | 44,759 | R.TGYTGEDGFESV/PDEH4AVDLAK.A (80) +10 |
| 15235745           | Ser hydroxymethyltransferase | 57,535 | K.LIVAGASAYAR.L (70) +9 |
| 15226973           | Gly deacylase H1 | 18,050 | K.LTESPGLINSSPYEDGWMIK.V (87) +3 |
| 14916970           | ATP synthase subunit α | 55,296 | K.AVDSLYPGR.G (44) +4 |
| 18394888           | Catalase 3 | 57,059 | R.LGPYNLQLPVNAPK.C (54) +4 |
| 30684419           | Lipoamide dehydrogenase 2 | 54,237 | K.HIVATGSVDK.S (47) +7 |
| 15221044           | Lipoamide dehydrogenase 1 | 54,239 | K.HIVATGSVDK.S (47) +4 |
| 79401911           | Unknown protein* | 78,638 | K.GFSFSSVDKS.S (42) +7 |
| 14596025           | Gly dehydrogenase subunit P1 | 113,852 | R.EY AAPAPWLR.S (35) +11 |
| 21537215           | Unknown protein* | 32,979 | K.SMN SNETSSEIQKPDYIHVR.A (28) +1 |
GDC activity within 30 min (Fig. 4B). Furthermore, neither increased ROS levels due to AOX inhibition nor reduced ROS levels due to catalase-dependent scavenging of ROS (data not shown) influenced the modulation of GDC activity.

Since it was not possible to demonstrate GSNO-dependent S-nitrosylation of the P protein via MS, we tested the effect of sodium nitroprusside (SNP) on the activity of partially purified P protein. SNP is an NO donor that is not able to S-glutathionylate Cys residues. As shown by Fig. 5, 250 μM SNP was able to inhibit the CO₂-exchange reaction, while the NO scavenger cPTIO (500 μM) almost completely prevented it. These data suggest that the activity of the P subunit of the GDC is inhibited by NO.

Inhibition of Gly Decarboxylase Activity by Harpin

Since protein S-nitrosylation is emerging as a specific and fundamental mechanism in NO signal transduction (e.g. during plant-pathogen interactions; Romero-Puertas et al., 2004), we tested the involvement of GDC inhibition in a stress-related response. Harpin is a proteinaceous bacterial elicitor of Erwinia, Pseudomonas, and Xanthomonas strains that can induce an oxidative burst and programmed cell death in various host plants. Leaf slices were incubated at room temperature with 35 μg mL⁻¹ recombinant harpin protein or with an extract of untransformed Escherichia coli DH5α as a control. GDC activity was recorded over a 4-h period. An inhibition of Gly decarboxylation in harpin-treated leaves was detected after 30 min and increased after 4 h to 61% in comparison with untreated and E. coli-treated samples (Fig. 6).

The Gly-Ser interconversion, catalyzed by GDC in conjunction with the Ser hydroxymethyltransferase, is an integral part of the photorespiratory metabolic pathway (Douce et al., 2001; Bauwe and Kolukisaoglu, 2003). Therefore, we investigated the role of GDC...
during plant defense responses in vivo by monitoring the amino acid content in Arabidopsis leaves (Blackwell et al., 1990; Abe et al., 1997; Heineke et al., 2001). The concentration of harpin used was able to cause cell death after 24 h when infiltrated into Arabidopsis leaves, but not after 30 min (in vitro experiments; data not shown). The inhibition of GDC activity observed after harpin treatment led to a distinctly elevated leaf Gly-Ser ratio after 4 h relative to the control treatment with *E. coli* extract (Fig. 7). As a second control, leaves were infiltrated with the GDC inhibitor AAN, which also resulted in a fast increase in Gly-Ser within 30 min (Fig. 7).

### Mitochondrial and Cellular Responses to GDC Inhibition

To confirm the role of GDC in the harpin-induced plant defense response, Arabidopsis plants and cultured cells were treated with the GDC inhibitor AAN, which has no side effects on dark respiration and CO₂ fixation (Usuda and Edwards, 1980; Creach and Stewart, 1982) and is able to mimic the toxin victorin-induced mitochondrial oxidative burst in oat (*Avena sativa*) cells (Yao et al., 2002).

Stress-induced ROS generation has been reported as an essential signal for activation of resistance in sev-
eral plants (Desikan et al., 1998; Delledonne et al., 2001; Krause and Durner, 2004). Energy dissipation mechanisms such as photorespiration or the water-water cycle (Asada, 2006) prevent excessive photo-reduction of oxygen, which could potentially generate an excess of ROS, leading to photooxidation (Kozaki and Takeba, 1996; Osmond et al., 1997). Thus, inhibition of GDC should result in enhanced ROS. Photometric determination of hydrogen peroxide (H$_2$O$_2$) was performed using the ROS-specific fluorescent dye dichlorofluorescein (H$_2$DCF-DA; Allan and Fluhr, 1997; Yao et al., 2002). ROS production in Arabidopsis cultured cells was monitored over time with a fluorescence plate reader (Fig. 8A), whereas the localization of ROS in the cells was analyzed with a confocal microscope (Fig. 8, B and C). The fluorescent signal of ROS increased already 10 min after addition of AAN to 1-week-old cultured cells, whereas untreated cells showed almost no fluorescence even after 60 min. In the presence of Gly, ROS accumulation increased dramatically (Supplemental Fig. S5), which could possibly result from glyoxylate-induced ROS production in peroxisomes. Catalase completely scavenged the H$_2$O$_2$ produced in the assay, demonstrating the specificity of the fluorescent dye. In contrast, pretreatment with the AOX inhibitor SHAM increased H$_2$O$_2$ accumulation after 60 min in Arabidopsis cells treated with AAN. H$_2$O$_2$ production after inhibition of the Gly decarboxylase was further confirmed in Arabidopsis leaves using H$_2$DCF-DA. The site of ROS production was identified with double staining using MitoTracker Red 580 (Yao et al., 2002; Krause and Durner, 2004). The intracellular localization of H$_2$O$_2$ production in the AAN-treated samples matched that of the mitochondria-staining dye (Fig. 8C).

Plant mitochondrial responses to stress signals are similar to those of animal mitochondria and include a collapse of the transmembrane potential ($\Delta$$\Psi_m$), release of cytochrome c, and decrease in ATP production (Saviani et al., 2002; Tiwari et al., 2002; Krause and Durner, 2004). Normally, the mitochondrial H$_2$O$_2$ burst appears to precede the breakdown in the $\Delta$$\Psi_m$ probably as a result of perturbation of the mitochondrial electron transport chain (Pellinen et al., 1999; Yao et al.,

Figure 5. CO$_2$-exchange reaction modulated by NO. The activity of P protein was determined by measuring the amounts of $[^{14}$C] bicarbonate fixed to the carboxyl group carbon atom of Gly in the presence of a saturating amount of H protein. Proteins were treated with 1 m$m$ DTT, 250 $\mu$g SNP, and/or 500 $\mu$g cPTIO for 10 min, and the activity was measured 30 min after the addition of bicarbonate. As a source of H protein, 100 $\mu$g of proteins of the light fraction from size-exclusion chromatography was used. P protein derived by chromatography, 30 $\mu$g; black bars, H + P proteins; striped bar, P protein alone. Each value represents the mean of at least three technical replicates. Different letters indicate values statistically different based on a one-way ANOVA followed by Tukey’s honestly significant difference post hoc test ($F = 12.8$, df = 5, $P < 0.01$).

Figure 6. Modulation of Gly decarboxylase activity by harpin. Leaf slices were treated with 35 $\mu$g mL$^{-1}$ recombinant harpin or with an extract of nontransformed E. coli at room temperature after equilibration in MOPS buffer for 1 h at 30°C. Treatment with the GDC inhibitor AAN was used as a control. The Gly decarboxylase activity in untreated control was set to 100%. Data shown are means ± SD of three independent experiments. Different letters indicate values statistically different based on a one-way ANOVA followed by Tukey’s honestly significant difference post hoc test ($F = 7.8$, df = 5, $P < 0.01$).
Inhibition of GDC by Harpin and NO

GDC, together with Ser hydroxymethyltransferase, is responsible for the conversion of Gly to Ser during photosynthesis. During this metabolic process, CO₂ and NH₃ are produced while ATP and reducing equivalents are consumed, which makes photosynthesis an energetically wasteful process (Wingler et al., 2000). It has been described that oat GDC is involved in the response to the toxin victorin produced by the fungus *Cochliobolus victoriae* (Navarre and Wolpert, 1995; Yao et al., 2002). The P subunit of the GDC was found to bind victorin in vivo, but only in susceptible genotypes. The H subunit bound victorin in vivo in both susceptible and resistant genotypes (Navarre and Wolpert, 1995). In addition, application of the GDC inhibitor AAN resulted in apoptosis-like cell death and disease symptoms (Yao et al., 2002). In this study, we observed a clear modulation of the GDC activity by redox agents in both intact mitochondria and plant leaves following ¹⁴CO₂ release from [¹⁴C]Gly (i.e. the H-dependent P protein reaction). The decarboxylation activity of GDC was significantly inhibited by GSNO as well as after blocking of the Cys residues with Cysteine-modifying chemicals. By contrast, strongly reducing agents such as DTT promoted GDC activity (Fig. 4A). The requirement of a reduced environment for both

DISCUSSION

Programmed cell death as observed in the form of a hypersensitive response in several plant-pathogen interactions exhibits similarities to programmed cell death as seen in animal apoptosis, including chromatin condensation and fragmentation (Mur et al., 2008) and caspase-like proteolytic activity (Lam and del Pozo, 2000). Furthermore, it has been shown that NO and ROS play major regulatory and antimicrobial roles in plant defense responses (Delledonne et al., 2001). In animal cells, mitochondria are major sites of the formation of redox compounds and major targets of NO/ROS-induced damage (Skulachev, 1996). By contrast, large quantities of ROS and NO are produced in plastids and peroxisomes of plants, especially in photosynthetic cells (del Rio et al., 2003). Nonetheless, plant mitochondria also are responsible for the production of ROS and are a site of oxidative damage (Taylor et al., 2002). In this paper, we not only showed that NO and ROS accumulate in Arabidopsis mitochondria during stress responses but also that NO is capable of modulating an essential mitochondrial function, photosynthetic metabolism, as an integral part of this response.
CO₂ exchange and decarboxylase activity of GDC in vitro has been described previously (Hiraga and Kikuchi, 1980; Walker and Oliver, 1986). Inactivation of GDC after treatment of mitochondria with N-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid) showed that Cys residues are important for the enzymatic activity.

In animal systems, the toxicity of NO has been associated with inhibition of cellular respiration (Clementi et al., 1998). Inactivation of GDC after treatment of mitochondria with N-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid) showed that Cys residues are important for the enzymatic activity.

In general, the role of S-nitrosothiols as primers for S-glutathionylation should be considered, with special emphasis given to GSNO as a reagent that can induce not only S-nitrosylation but also S-glutathionylation of GDC (rotenone) and AOX (SHAM), we showed that a direct action of NO on the GDC is responsible for its decreased activity (Fig. 4B).

**Posttranslational Modification of GDC by NO**

MS analysis of partially purified P protein (Table II) suggested the possibility that the modulation of the GDC was due to an S-glutathionylation and not to an S-nitrosylation of specific Cys residues. Although the biotin-switch method is specific for detection of SNO-modified Cys, we tested the activity of the purified P protein after treatment with the nonglutathionylating agent SNP and analyzed if NO is able to modify GDC activity (Fig. 5). The inhibition of GDC activity observed after SNP treatment and the ability of cPTIO to scavenge this effect proved that NO is able to modulate GDC activity per se, confirming the strict connection between the two posttranslational modifications. In general, the role of S-nitrosothiols as primers for S-glutathionylation should be considered, with special emphasis given to GSNO as a reagent that can induce not only S-nitrosylation but also S-glutathionylation of...
proteins (Martinez-Ruiz and Lamas, 2007). In several proteins, “competition” between S-glutathionylation and S-nitrosylation has been compared, which showed that there is considerable variation among different proteins regarding their tendencies to undergo either modification.

The modulation of a key enzyme of the photorespiratory system via S-nitrosylation suggests a direct role of this metabolic pathway in the regulation of the redox state of the cell. In addition, GDC was found in root and etiolated tissue of different plants, in C4 plants, and in a wide range of organisms from bacteria to humans. Taken together, the data suggest a non-photorespiratory role of the GDC complex (Navarre and Wolpert, 1995). For this reason, we investigated the state of GDC during a plant stress response. Using the Arabidopsis-harpin interaction as a model system (Xie and Chen, 2000; Krause and Durner, 2004; Livaja et al., 2008), we demonstrated that GDC activity is inhibited in Arabidopsis leaves after harpin treatment (Figs. 6 and 7). Thus, harpin inhibits not only the cytochrome c-dependent respiration (Xie and Chen, 2000; Livaja et al., 2008) but also the photorespiratory system in Arabidopsis. This reduces the cellular capacity of ATP synthesis and increases the production of ROS, which leads to an overreduction and overenergization of the entire cell. Recent studies revealed the importance of energy dissipation in mitochondria.

Oxidative stress by excess light is involved in the regulation of respiratory gene expression and the modulation of respiratory properties, especially up-regulation of AOX (Yoshida et al., 2006, 2007, 2008).

Potential Involvement of GDC in Plant Defense Responses

The possible role of GDC in pathogen responses has been investigated upon victorin treatment of oat. In this system, application of the GDC-specific inhibitor AAN resulted in a highly localized accumulation of H2O2 within mitochondria followed by disease symptoms similar to those induced by victorin and during apoptosis-like cell death (Yao et al., 2002). Here, we demonstrated that AAN is able to induce an oxidative burst in cultured cells and in leaves of Arabidopsis (Fig. 8). Whereas catalase was able to scavenge AAN-induced ROS, inhibition of AOX (by SHAM) further increased the ROS signal 1 h after incubation (Fig. 8). The use of a mitochondria-specific dye allowed us to localize the ROS burst in Arabidopsis mitochondria, although peroxisomal ROS production cannot be excluded, since peroxisomes are localized next to mitochondria. The direct relationship between inhibition of GDC activity and ROS levels is difficult to explain. The oxidation of Gly by GDC provides NADH for the mitochondrial electron transport chain. Inhibition of

![Figure 9. AAN-induced depolarization of mitochondrial membranes. AAN was used to study the effect of GDC inhibition in Arabidopsis. A, One-week-old cultured Arabidopsis cells were treated for 30 min with different inhibitors on the 96-well plate in the grow chamber. Afterward, JC-1 dye (5 μg mL⁻¹), to probe the mitochondrial membrane potential, and 10 mM AAN were added and the measurement was started. The mitochondrial depolarization was indicated by a decrease in the red-green fluorescence intensity ratio. The data represent the status of the mitochondrial membrane 30 min after AAN treatment. Black bars indicate the percentage of depolarization. SHAM, 2 mM; catalase, 100 units mL⁻¹; cyclosporine A, 50 μM. Cyclosporine A was used as a control for complete polarization (100%). The experiment was repeated three times with similar results. Different letters indicate values statistically different based on a one-way ANOVA followed by Tukey’s honestly significant difference posthoc test (F = 532.6, df = 7, P < 0.01). B, Model proposed to explain the relation between GDC and AOX in modulating depolarization mitochondrial membranes.](https://plantphysiol.org)
GDC would lead to undersupply of NADH to the respiratory chain. This shortage of the electron donor pool might cause the production of partially reduced oxygen species. However, there might be other, still unknown connections between the inhibition of GDC on the one hand and ROS production as a result of perturbation of the energy metabolism in mitochondria on the other hand.

In animal cells, the increase of mitochondrial ROS, together with an elevation of cytosolic Ca\(^{2+}\), contributes to the opening of the mitochondrial permeability transition pore (PTP). The PTP depolarizes mitochondria, leading to mitochondrial swelling and subsequent release of cytochrome \(c\) from the intermembrane space (Goldstein et al., 2000). It has been demonstrated that the mitochondrial PTP participates in NO-induced cell death in plants (Saviani et al., 2002). In concurrence, our data show a 50% loss of membrane potential in Arabidopsis cells 30 min after treatment with AAN, whereas this loss is limited by the action of the alternative respiratory pathway (Fig. 9). Moreover, the scavenging effect of catalase proves that ROS are directly involved in activating depolarization of mitochondrial membranes in plants. Among the proteins released from mitochondria after depolarization of the mitochondrial membrane are cytochrome \(c\) and apoptosis-inducing factor. The latter protein moves directly to the nucleus, where it causes chromatin condensation and nuclear fragmentation. Cytochrome \(c\) activates a caspase signaling cascade that selectively cleaves vital substrates in the cell, including the nuclease responsible for DNA fragmentation (Green and Reed, 1998). In agreement with this scheme of actions, inhibition of Arabidopsis GDC by AAN resulted in cell death (Fig. 10).

In conclusion, we showed that GSNO is able to modulate the photorespiratory pathway by \(S\)-nitrosylation/\(S\)-gluthationylation of critical Cys residues of the \(P\) subunit of the GDC in Arabidopsis leaves. Moreover, we observed that this inhibition is part of the stress-related response of Arabidopsis to the bacterial elicitor harpin and that GDC inhibition alone is able to activate a redox response that triggers mitochondria perturbation and cell death. Taken together, these data reinforce the model of cross talk between NO/ROS and mitochondria in the activation of stress-related responses in plants.

**MATERIALS AND METHODS**

**Harpin Purification**

The bacterial elicitor harpin was purified by *Escherichia coli* DHSa transformed with a pBluescript SK+ vector carrying the full-length *Pseudomonas syringae* 61 hrpZ open reading frame as described by Krause and Durner (2004). Proteins of nontransformed *Escherichia coli* were purified as the one carrying the harpin-encoding vector and used as a control.
Partial Purification of Mitochondria

Crude, well-coupled mitochondria were isolated and purified by differential centrifugation as described by Keech et al. (2005), with a nonreducing medium replacing the extraction buffer to avoid interference with the biotinylation process. All procedures were carried out at 4°C in detergent-free vessels. Arabidopsis leaves (15 g, from 5- to 6-week-old plants) were ground with 20 mL of grinding medium (0.5 mM Suc, 60 mM Tris, 10 mM EDTA, 25 mM tetrasodium pyrophosphate, 1% [w/v] polyvinylpyrrolidone-40, 0.5% [w/v] defatted bovine serum albumin, 100 mM potassium phosphate [pH 7.5], 1 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, and 1 µM pepstatin) using a pestle and a small amount of quartz (fine granular, washed and calcined, guaranteed reagent grade; Merck). The extract was filtered through a 20-µm nylon mesh and centrifuged at 2,500g for 5 min to remove most of the intact chloroplasts and thylakoid membranes. The supernatant was transferred to a new tube and centrifuged at 15,000g for 15 min. The pellet obtained was suspended in the same medium (0.5 mM Suc, 10 mM Tris, 10 mM potassium phosphate [pH 7.5], and Complete Protease Inhibitor Cocktail [Roche Applied Science]) and centrifuged at 15,000g for 15 min. The resulting supernatant was discarded, and the pellet containing the crude mitochondria was resuspended in 0.5 mL of wash medium or a specific assay medium. Cytochrome c oxidase activity, succinate-dependent respiration rate, and total protein concentration were estimated as described (Braxton, 1976; Neuburger et al., 1985; Livaja et al., 2008). Mitochondria were used immediately after preparation.

Detection of S-Nitrosylated Proteins

To detect S-nitrosylated proteins, we adopted the biotin-switch method, a three-step procedure that converts S-nitrosylated Cys residues into biotinylated Cys residues (Jaffrey and Snyder, 2001). Mitochondria (around 15 mg total wet weight) were incubated with the S-nitrosylating agent GSNO (1 mM; Alexis) or with GSH (1 mM; Sigma-Aldrich) in the dark at room temperature for 20 min with frequent vortexing. Mitochondrial proteins were precipitated by adding 1.0 mL of 100% acetone for 20 min at −20°C to remove the excess of GSNO/GSH. The samples were centrifuged at 10,000g for 10 min, and pellets were resuspended at a final concentration of 1 mg mL−1 and assayed with the biotin-switch method. The remaining proteins were analyzed by nanoLC/MS/MS essentially as described (Lindemayr et al., 2005).

Purification of P Protein from Mitochondria Extract

P protein purification from mitochondria extract was essentially as described by Bourguignon et al. (1988). Arabidopsis mitochondria (about 70 mg of protein) were diluted in dilution buffer (5 mM MOPS, 5 mM Tris, 1 mM β-mercaptoethanol, 1 mM EGTA, 20 mM pyridoxal phosphate, 1 mM Ser, 4 µM leupeptin, and 0.1% Triton X-100, pH 7.0) to 40 mg mL−1. Total release of the matrix protein was achieved by three cycles of freeze-thaw (liquid N2 for 2 min followed by 30°C until thawed). The suspension of broken mitochondria was centrifuged at 100,000g for 2 h to remove all the mitochondrial membranes. The supernatant-containing soluble protein was concentrated by a Centriprep-30 (Amicon) and centrifuged at 3,600×g for 30 min in a swinging rotor (Rotanta 460R; Hettich Zentrifugen). P protein was purified as described (Bourguignon et al., 1988). After an initial size-exclusion chromatography step with a HiPrep 16/60 Sephacryl S-300 HR (GE Healthcare) column connected to an AKTAexplorer PPLC device, an anion-exchange step was applied (HTRAP DEAE FF, GE Healthcare). Final fractions were combined, concentrated with Amicon Ultra-4 10 kD (Millipore), and stored at −80°C (on addition of 20% glycerol).

Identification of S-Nitrosylated Proteins: Matrix-Assisted Laser-Desorption Ionization Time of Flight MS and NanoLC/MS/MS

Peptides were visualized by colloidal Coomassie Brilliant Blue staining, excised from SDS gels, and completely digested by washing in 25 mM NH4HCO3. Digested peptides were extracted by vortexing for 3 h with 100 mL of 5% formic acid. All tryptic peptide samples were dried and redissolved in 50 mL of 0.1% trifluoroacetic acid and 5% acetonitrile. Peptides were separated by reverse-phase chromatography using an UltiMate Capillary/Nano liquid chromatography system (LC Packings) and eluted and fractionated on a self-packed analytical column (75 µm × 120 mm) packed with YMC-Pack ODS-A (3 µm C18; YMC) with a gradient of 5% to 55% acetonitrile at a flow rate of 150 mL over 40 min. Eluted peptides were continuously delivered to a Q-Tof Ultima mass spectrometer (Waters/Micromass) by electrospray and analyzed by MS/MS employing data-dependent acquisition (three most abundant ions in each cycle; 0.3 s MS, mass-to-charge ratio [m/z] 400–2,000, and maximum 4.8 s MS/MS, m/z 50–3,000; continuum mode, 60 s dynamic exclusion). The MS/MS raw data were processed and converted into Micromass pkf format using MassLynx 4.0 ProteinLynx. Resulting pkf files were compared with those of theoretical trypsin digestions and searched against predicted masses derived from the National Center for Biotechnology Information genomic database using ProFound software (Genomic Solutions). The Mascot search engine was used to parse MS data to identify proteins from primary sequence databases. Proteome analysis was carried out by TopLab.

Posttranslational Modifications of GDC: Nano-HPLC-MS23 and Data Analysis

For mass spectrometric analyses, partially purified P protein was digested by trypsin at 37°C for 1 h in 100 mM Tris-HCl, pH 6.5. The reaction was done in the dark to avoid light-dependent decomposition of the modifications. The trypsin/neutral protein ratio was 1:20. Protein digests were analyzed by online nanoLC/MS/MS. The samples were separated on an in-house-made 10-cm reverse-phase capillary emitter column (d. 75 µm, 5 µm ProteoSil 120-5-C18 ac-ECPS) using 120-min linear gradients from 0% to 40% acetonitrile/0.1% formic acid with a Flow Rheos 2200 nanoflow system (Flux Instruments) at a flow rate of 270 nL min−1. The LC setup was connected to an LTQ-Orbitrap classic (Thermo Fisher) equipped with a nanoelectrospray ion source (Proxeon Biosystems). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS, MS2, and MS3 acquisition. Survey full-scan MS spectra (m/z 350–1,800) were acquired in the Orbitrap with resolution R = 7,500 at m/z 400. The six most intense ions were then sequentially fragmented in the linear ion trap using collisionally induced dissociation at normalized collision energy of 35. In the case of a resulting neutral loss of 9.7 m/z or 14.5 m/z in the MS2 spectra in the three most abundant peaks, indicating the loss of NO, these fragments were selected for further MS3 fragmentation. Former target ions selected for MS3 were dynamically excluded for 30 s. Total cycle time was approximately 3 s. The general mass spectrometric conditions were as follows: spray voltage, 1.4 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 200°C. Ion selection thresholds were as follows: 500 counts for MS2 and 500 counts for MS3. An activation q = 0.25 and activation time of 30 ms were applied in both MS2 and MS3 acquisitions.

Peptides and proteins were identified via automated database searching (Bioworks 3.3.1; SP1) of all MS2 and MS3 against an in-house-curated database (36,361 protein sequences). Spectra were normally searched with a mass tolerance of 1.5 atomic mass units for the parent mass and 0.5 atomic mass units for fragment masses, with semitryptic specificity allowing two missed cleavages. All modifications were set to be variable: oxidation of Met, nitrosylation and glutathionylation of Cys. Proteome analysis was carried out in cooperation with the Helmholtz Zentrum Munich core facility.

Gly Decarboxylase Activity

GDC activity in Arabidopsis leaf slices and intact mitochondria was determined following the Gly decarboxylase activity in vitro, by means of the P/H subunit activity (Somerville and Ogren, 1981). Leaf slices were prepared as described (Navarre and Wolpert, 1995; 2-×-7-mm slices). GDC activity was assayed with 8 mM [14C]Gly (8.75 µCi, labeled at the carboxyl group) to 500 μL of assay buffer (4 mM sodium phosphate, pH 7.2, 0.3 mM sorbitol, 20 mM MOPS, pH 7.2, 1 mM KCl, 4 mM MgCl2, and 0.1% bovine serum albumin) containing 100 μg of mitochondrial protein or 10 leaf slices. Reaction mixtures were placed in 2-mL Eppendorf tubes suspended over 1.4 mL of Oxsolve C400 scintillator fluid (Zinsser Analytic) in 20-mL scintillation vials. Reactions were initiated by adding [14C]Gly and terminated by injecting 100 μL of 6 M acetic acid.
acid into the reaction mixture. The vials were left overnight to permit trapping of 14C02, the reaction tubes were removed, and the trapped 14C radioactivity was determined by scintillation counting (Beckman LS6000SC).

**Gas-Exchange Measurements**

The photosynthesis rate in vivo was monitored following the response of the photosynthesis (A) to the intracellular CO2 mole fraction (Ci). Rates of photosynthesis and dark respiration in leaves were determined under the growth conditions using a portable infrared gas analyzer system (LI-6400; LI-COR Biosciences). One leaf was placed into the leaf chamber of the LI-6400 and exposed to 75, 250, or 400 μmol m⁻² s⁻¹ light at a flow rate of 300 μmol s⁻¹. Leaf temperature was maintained at 25°C during measurement. To calculate the CO2 compensation point, a cycle with six CO2 reference concentrations was used: 400, 300, 200, 100, 50, and 400 μmol.

**Gly-Ser Content**

For amino acid determination, 100 mg of leaf material was ground in liquid nitrogen and extracted in 1 mL of 80% ethanol for 30 min. After centrifugation for 10 min at 24,000g, the supernatant was vacuum dried and the dried extract was dissolved in 1 mL of 0.3 M sodium phosphate (pH 6.8) and 0.4% tetrahydrofuran. Samples were incubated for 30 min and then diluted 1:50 with the same phosphate buffer. Individual amino acids were separated by HPLC and quantified as described (Hagemann et al., 2005).

**Cytofluorometric Analysis of NO and ROS Formation**

For the detection of NO or ROS in mitochondria, untreated or harpin-treated (35 μg mL⁻¹) cultured cells of Arabidopsis were stained in the dark with 0.5 μM MitoTracker Red 580 (Invitrogen) as mitochondria-specific marker (Yao et al., 2002; Krause and Dunner, 2004). After incubation for 20 to 30 min in the presence or absence of the NO scavenger cPTIO (0.5 mM; Alexis), 2 μM NO-specific dye DAF-2FM DA (Molecular Probe) or 10 μM ROS-sensitive dye H2DCF-DA (Invitrogen) was added to the cells and incubated for 10 min under continuous shaking. Fluorescence development was observed and photographed with a Zeiss confocal microscope (LSM 510 NLO). The mitochondrial-dependent red fluorescence was excited using a 543-nm laser and detected through a long-pass filter LP 565 to 615 nm. The NO- or ROS-dependent green fluorescence was excited with a 488-nm argon laser and visualized through a band-pass filter BP 500 to 550 nm. Images were analyzed with LSM Image Browser (Zeiss).

**Determination of Mitochondrial ΔΨm**

Changes in mitochondrial ΔΨm in response to different elicitors were monitored using the mitochondrial potential sensor JC-1 (Invitrogen; Yao et al., 2002). Cultured cells of Arabidopsis were stained with the 5 μg mL⁻¹ JC-1 dye and incubated for 30 min in the dark with continuous shaking. Stained cells were diluted with PS medium, placed on a black 96-well microplate (Nunc), and treated with the elicitors. Changes in fluorescence intensities were measured immediately after treatment, every 5 min over 100 min, using a Tecan GENios reader (excitation, 488 nm; emission 1, 535 nm; emission 2, 595 nm).

**Cell Death Assay**

Cell death, measured as loss of plasma membrane integrity, was detected by double staining of harvested cultured cells of Arabidopsis with Evans blue (Sigma-Aldrich) to stain dead cells and fluorescein diacetate to stain live cells. The excitation and emission wavelengths of the two dyes were different and not interfering (Evans blue: excitation, 543 nm and LP filter 650 nm; fluorescein diacetate: excitation, 488 nm and BP filter 505-530 nm). Harvested suspension cells were stained with 0.01% Evans blue for 5 min. Stained cells were then incubated for a further 5 min with 0.01% fluorescein diacetate and treated with different inhibitors or water. Confocal microscopy images were obtained using a Zeiss LSM 510 NLO, and images were analyzed with LSM Image Browser (Zeiss).

**Statistical Analysis**

All experiments had more than two levels of the independent variable. For this reason, we carried out a one-way ANOVA, followed by Tukey’s honestly significant difference posthoc test, in which sets of two means at a time were compared. Letters indicate statistically different values.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT4G33010 and AT2G26080.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Mitochondria fraction purification degree.

**Supplemental Figure S2.** Controls for the biotin-switch method.

**Supplemental Figure S3.** Activity assay for purified P protein.

**Supplemental Figure S4.** Mass spectrometric analyses of partially purified P protein.

**Supplemental Figure S5.** AAN-induced ROS production in Arabidopsis cell cultures.

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