

# Proteomic Identification of S-Nitrosylated Proteins in Arabidopsis<sup>1[w]</sup>

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Although nitric oxide (NO) has grown into a key signaling molecule in plants during the last few years, less is known about how NO regulates different events in plants. Analyses of NO-dependent processes in animal systems have demonstrated protein S-nitrosylation of cysteine (Cys) residues to be one of the dominant regulation mechanisms for many animal proteins. For plants, the principle of S-nitrosylation remained to be elucidated. We generated S-nitrosothiols by treating extracts from Arabidopsis (*Arabidopsis thaliana*) cell suspension cultures with the NO-donor S-nitrosoglutathione. Furthermore, Arabidopsis plants were treated with gaseous NO to analyze whether S-nitrosylation can occur in the specific redox environment of a plant cell in vivo. S-Nitrosylated proteins were detected by a biotin switch method, converting S-nitrosylated Cys to biotinylated Cys. Biotin-labeled proteins were purified and analyzed using nano liquid chromatography in combination with mass spectrometry. We identified 63 proteins from cell cultures and 52 proteins from leaves that represent candidates for S-nitrosylation, including stress-related, redox-related, signaling/regulating, cytoskeleton, and metabolic proteins. Strikingly, many of these proteins have been identified previously as targets of S-nitrosylation in animals. At the enzymatic level, a case study demonstrated NO-dependent reversible inhibition of plant glyceraldehyde-3-phosphate dehydrogenase, suggesting that this enzyme could be affected by S-nitrosylation. The results of this work are the starting point for further investigation to get insight into signaling pathways and other cellular processes regulated by protein S-nitrosylation in plants.

Overwhelming evidence suggests that nitric oxide (NO) is an integral part of normal physiological processes in animals (Nathan, 1995; Bogdan, 2001). By the late 1990s, NO was identified as an important messenger in plant defense signaling against microbial pathogens (Delledonne et al., 1998; Durner et al., 1998); it subsequently was shown to be a crucial player in the regulation of normal plant physiological processes including stomatal closure, growth, and development (Neill et al., 2002; Wendehenne et al., 2004), and recently, a hormone-activated NO producing enzyme was identified in Arabidopsis (*Arabidopsis thaliana*; Guo et al., 2003; Zeidler et al., 2004).

As a readily diffusible free radical, NO reacts with a variety of intracellular and extracellular targets and can act as activator or inhibitor of enzymes, ion channels, or transcription factors and in this way regulates specific processes during plant development and abiotic or biotic stress situations. The alteration of protein function/activity can be achieved by reaction of NO with sulfhydryl groups and transition metals (Stamler, 1994), and the resulting products, S-nitrosothiols and

metal nitrosyls, respectively, have intrinsic reactivities that enable local action.

The majority of all NO-affected proteins seem to be regulated by S-nitrosylation of a single critical Cys residue, which occurs by oxygen-dependent chemical reactions or by the transfer of NO from a nitrosothiol to a protein sulfhydryl group (transnitrosylation). Because of their reactivity with intracellular reducing agents, e.g. ascorbic acid or glutathione (GSH), and with reduced metal ions, especially Cu<sup>+</sup>, nitrosothiols are exceptionally labile. This lability results in tissue half-lives of seconds to a few minutes and therefore provides a very sensitive mechanism for regulating cellular processes. S-Nitrosylation is now regarded as posttranslational modification similar to phosphorylation.

In mammals, especially S-nitrosoalbumin, S-nitrosohaemoglobin, and S-nitrosoglutathione (GSNO) are discussed as in vivo NO reservoirs and NO donors (Stamler et al., 1992a; Jia et al., 1996; Kluge et al., 1997; Tsikas et al., 1999). In plants, a strong GSNO reductase activity was demonstrated recently for GSH-dependent formaldehyde dehydrogenase (Diaz et al., 2003), an enzyme previously identified as formaldehyde detoxifying protein in maize (*Zea mays*; Fliegmann and Sandermann, 1997; Wippermann et al., 1999) indicating that GSNO and the formation of other nitrosothiols might play an important role in NO signaling in plants as well (Durner et al., 1999; Diaz et al., 2003). On the other side, it is also speculated whether metal nitrosyls are the dominant compounds

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for NO storage and NO transport in plants (Garcia-Mata and Lamattina, 2003).

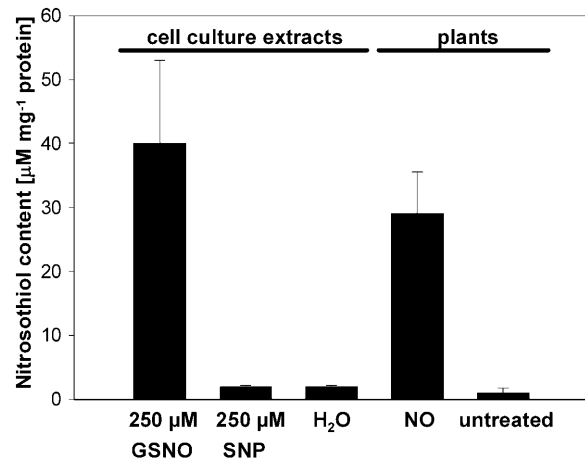
Until now, little has been known about the dimension let alone the physiological function of *S*-nitrosylation in plants, and no endogenous *S*-nitrosylated plant protein has been described. To identify possible targets of *S*-nitrosylation, SwissProt database was searched for the consensus motif of *S*-nitrosylation sensitive Cys residues (Stamler et al., 1997). This search revealed 103 matches in 99 sequences from the deduced Arabidopsis proteome (Huber and Hardin, 2004). Except for this bioinformatics strategy, no approaches were undertaken to identify *S*-nitrosylated plant proteins.

The aim of this study was to identify possible candidates for *S*-nitrosylation in Arabidopsis cell suspension cultures and leaves to get insight into the regulatory function of NO on protein level in plants. Recently, Jaffrey et al. (2001) developed a highly specific biotin switch method for detection and purification of *S*-nitrosylated proteins in animals (Jaffrey et al., 2001; Kuncewicz et al., 2003; Foster and Stamler, 2004; Martinez-Ruiz and Lamas, 2004). A proteomics approach using this method in combination with nano liquid chromatography and tandem mass spectrometry (nanoLC/MS/MS) allowed us to identify 63 proteins from GSNO-treated cell culture extracts and 52 proteins from NO-treated Arabidopsis leaves, which represent targets for *S*-nitrosylation in plants. These proteins include stress-related proteins, signaling/regulating proteins, redox-related proteins, and cytoskeleton proteins as well as metabolic enzymes. Strikingly, about 60% of the identified proteins were already described in the animal system in context with *S*-nitrosylation or *S*-glutathionylation, underlining the specificity of the method and indicating that NO-regulated processes in plants and animals have common features.

## RESULTS

### Generation of *S*-Nitrosothiols

To generate *S*-nitrosylated Arabidopsis proteins, we treated extracts from cell suspension cultures with the NO-donors GSNO and sodium nitroprusside (SNP). Furthermore, we exposed Arabidopsis plants to NO gas to investigate nitrosothiol formation in intact plants. Nitrosothiol contents were determined using the colorimetric assay developed by Saville (1958). Treatment of cell culture extracts with 250  $\mu\text{M}$  GSNO resulted in a nitrosothiol content of about 40  $\mu\text{M}/\text{mg}$  protein (Fig. 1). Although SNP is described to also have a strong *S*-nitrosylating potential (Stamler et al., 1992b), we could detect only very low amounts of nitrosothiols (2  $\mu\text{M}/\text{mg}$  protein) in the SNP-treated extracts (Fig. 1). The nitrosothiol content in NO-treated plants reached values up to 30  $\mu\text{M}/\text{mg}$  protein. In untreated cell culture extracts as well as in untreated plants, only traces of nitrosothiols (1–2  $\mu\text{M}/\text{mg}$  protein) could be measured (Fig. 1).



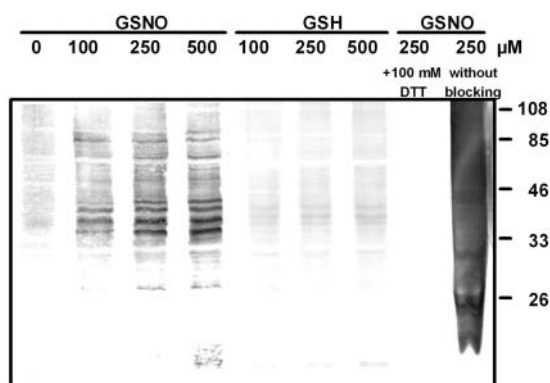
**Figure 1.** Nitrosothiol content in Arabidopsis cell suspension culture extracts after treatment with NO donors and in leaves of NO-treated Arabidopsis plants. After treatment of cell culture extracts with either 250  $\mu\text{M}$  GSNO, 250  $\mu\text{M}$  SNP, or water, remaining NO donors were removed by chromatography on a Sephadex G-25M column. Nitrosothiol contents were determined according to Saville (1958). Additionally, nitrosothiol content from leaves of Arabidopsis plants treated with NO gas and from leaves of untreated plants was measured. Values represent mean of at least two independent determinations.

### Detection of *S*-Nitrosylated Proteins

To detect *S*-nitrosylated proteins, we chose the biotin switch method that is based on the labeling of *S*-nitrosylated proteins with a biotin moiety specifically on *S*-nitrosylated Cys (Jaffrey et al., 2001). Biotinylated proteins can then be made visible by immunoblotting using anti-biotin antibody. After treatment of cell culture extracts with up to 500  $\mu\text{M}$  GSNO, a group of about 12 protein bands was detected representing *S*-nitrosylated proteins (Fig. 2). In the samples treated with increasing concentrations of GSH, however, only some weak protein bands could be seen, confirming that the detected band in the GSNO samples are attributed to NO (Fig. 2). Furthermore, in GSNO-treated samples that underwent the biotin switch procedure following treatment with 100 mM dithiothreitol (DTT), no protein bands could be detected (Fig. 2). This demonstrates that the anti-biotin antibody shows no unspecific cross reaction with unlabeled proteins and that no *in vivo* biotinylated proteins were detected. Analyses of GSNO-treated extracts that underwent the biotin switch method without a blocking step resulted in high grade of unspecific biotinylation (Fig. 2). In extracts treated with 250  $\mu\text{M}$  oxidized glutathione, 20 mM DTT, or water, no protein bands could be detected with the anti-biotin antibody (data not shown). Taken together, these results underline the specificity of the biotin switch method for detection of *S*-nitrosylated proteins.

### Identification of Candidates for Protein *S*-Nitrosylation

Arabidopsis cell cultures were treated with 250  $\mu\text{M}$  GSNO or GSH and *S*-nitrosylated proteins were



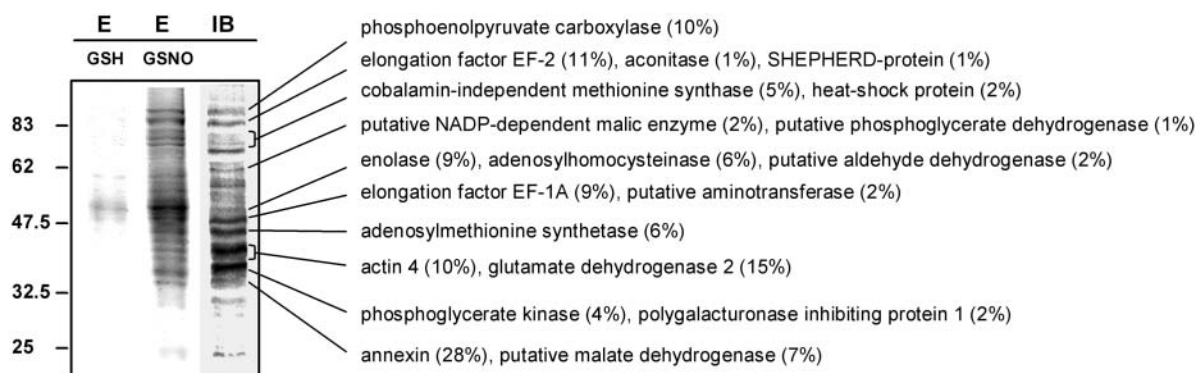
**Figure 2.** Detection of S-nitrosylated proteins of Arabidopsis cell culture extracts. Extracts containing 100  $\mu\text{g}$  protein were treated with different concentrations of GSNO or GSH and labeled with biotin using the biotin switch method. Additionally, proteins were S-nitrosylated with 250  $\mu\text{M}$  GSNO and reduced with 100 mM DTT after biotinylation. The sample on the right side was treated with 250  $\mu\text{M}$  GSNO and underwent biotin switch method without MMTS treatment (blocking step). Proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride-membrane. Detection of biotinylated proteins was achieved using anti-biotin antibody. The relative masses of protein standards are shown on the right.

subjected to the biotin switch method. Biotin-labeled proteins were purified by affinity chromatography on a neutravidin matrix and separated by SDS-PAGE (Fig. 3). In the eluate derived from GSNO treatment, 16 prominent protein bands could be detected, whereas only a few protein bands were visible in the GSH-derived eluate. Twelve protein bands that could be assigned to predominant bands of the immunoblot of GSNO-treated cell culture extracts were digested with trypsin and the resulting peptides were subjected to nanoLC/MS/MS (Fig. 3).

To also identify low abundant candidates for protein S-nitrosylation, proteins of GSNO- and GSH-treated extracts were labeled with biotin, affinity purified as

described above, and the eluates were subjected to nanoLC/MS/MS analysis. In the samples treated with the NO donor, 57 proteins could be identified comprising members of different functional families including stress-related proteins, signaling/regulating proteins, redox-related proteins, cytoskeleton proteins, and metabolic enzymes (Table I; supplemental material). More than 60% of the identified proteins are already described in the context of S-nitrosylation, S-glutathionylation, or redox-regulated processes (for references, see Table I and supplemental material), confirming that the identified proteins represent promising candidates for S-nitrosylation in plants. In the GSH-treated samples, 30 proteins could be identified (Table I; supplemental material). Twenty-seven of these are also identified in the GSNO-treated samples, and 18 of them are identified as targets for S-nitrosylation or S-glutathionylation in the animal system or have been reported to be involved in redox-regulated processes (for references, see Table I and supplemental material).

To get insight into NO-dependent protein regulation in plants, we also analyzed plants exposed to NO gas. Leaf extracts of NO-treated and untreated plants were subjected to the biotin switch method and affinity purified proteins were analyzed by nanoLC/MS/MS after tryptic digestion. The identified candidates for protein S-nitrosylation were divided into metabolic enzymes, proteins involved in photosynthetic processes, redox-related proteins, signaling/regulating proteins, stress-related proteins, and others (Table I; supplemental material). A total of 41 proteins have been identified in NO-treated extracts, and 19 of them have been described in context of redox-related processes, S-glutathionylation, or S-nitrosylation in plants or animals (for references, see Table I and supplemental material). In extracts of untreated plants, 25 proteins could be identified, and more than one-half of them (14) were found in the NO-treated samples. Western-blot analysis using antibodies raised against several of the identified proteins provide further



**Figure 3.** S-Nitrosylated proteins of Arabidopsis cell cultures. A total of 10 mg of cell culture proteins were treated with 250  $\mu\text{M}$  GSH or GSNO, subjected to the biotin switch method, and biotinylated proteins were purified by affinity-chromatography using neutravidin-agarose. Eluates (E) were separated by SDS-PAGE and visualized by Coomassie Blue staining. Protein bands corresponding to predominant bands of the immunoblot analysis (IB) were identified by nanoLC/MS/MS. The percentage of protein covered by the matched peptides is given in brackets. The relative masses of protein standards are shown on the left.

**Table 1.** Selected candidates of identified *S*-nitrosylated proteins from *Arabidopsis* cell cultures and leaves

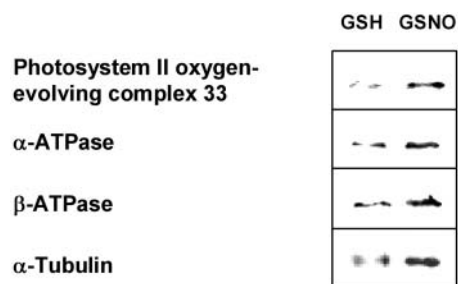
Cell culture extracts treated with GSNO or GSH and leaf extracts of NO-treated or -untreated *Arabidopsis* plants were subjected to the biotin switch method and analyzed by nanoLC/MS/MS after tryptic digestion. The MASCOT search engine was used to parse MS data to identify proteins from primary sequence databases. The best-matching peptide identifying the protein is given. If there were further peptides found, the number of the peptides is given. Hints confirming that the identified protein is a candidate for *S*-nitrosylation are given in the right column. The results of two separate experiments of each treatment were summarized in the table. Acc. No., Accession number.

Protein	Acc. No.	Molecular Mass	Identified Peptides (Score) GSNO/NO	Identified Peptides (Score) GSH/Untreated	Hints to <i>S</i> -Nitrosylation
<i>D</i>					
Stress-Related Proteins					
GST, putative	NP_565178	25,634	VTEFVSELR (79)	NPILPSDPYLR (38)	Sies et al. (1998); Ji et al. (2002)
Hsp 90, putative	AAL49788	80,036	ADLVNNLGTIAR (50) + 1		Jaffrey et al. (2001); Fratelli et al. (2002, 2003); Lind et al. (2002); Shenton and Grant (2003)
Cu/Zn-superoxide dismutase	NP_172360	15,088	AVVHADPDDLK (55) + 1		Lawler and Song (2002)
Redox-Related Proteins					
Glutathione peroxidase, putative	NP_192897	25,568	FAPTTSPSIEK (65) + 1	FAPTTSPSIEK (51)	Koh et al. (2001)
Peroxiredoxin-related	AAF66133	21,230	AVNVEEAPSDFK (37)		Motohashi et al. (2001); Fratelli et al. (2002, 2003); Lind et al. (2002)
Type 2 peroxiredoxin-related	NP_176773	17,417	APIAVGDVVPDGTISFF-DENDQLQTASVHSL-AAGK (51)		
Glutaredoxin, putative	NP_198853	11,749	LVPLLTEAGAIAGK (59)		Klatt et al. (2000); Song and Lee (2003)
Signaling/Regulating Proteins					
Elongation factor eEF-1 $\alpha$ -chain	S08534	49,457	VETGMIKPGMVVTF-APTGLTTEVK (61) + 4	IGGIGTVPVGR (58) + 2	Fratelli et al. (2002); Shenton and Grant (2003)
Elongation factor EF-2	A96602	94,185	AYLPVVESEFGFSSQLR (80) + 4	LWGENFFDPATR (43) + 2	
Elongation factor 1B $\alpha$ -subunit	NP_568375	24,186	TYISGDQLSVDDVK (67) + 1		
Elongation factor 1B $\gamma$ , putative	AAK59587	46,371	VPVLETPEGPIFESNAIAR (40) + 1		
Initiation factor eIF-4A1	CAC43286	41,823	GLDVIQQAQSGTGK (41)	GLDVIQQAQSGTGK (38)	Lind et al. (2002); Shenton and Grant (2003)
Initiation factor 5A-4-related	NP_173985	17,129	TYPQSAGNIR (58)		
Cytoskeleton Proteins					
Tubulin $\alpha$ 6 chain	JQ1597	49,506	AVFVDLEPTVIDEVR (84) + 1	DVNAAVGTIK (34) + 1	Jaffrey et al. (2001)
Tubulin $\beta$ 4 chain	S68122	49,876	AVLMDLEPGTMDSLR (82) + 2	GHYTEGAELIDSVLDVVR (71)	Jaffrey et al. (2001); Lind et al. (2002)
Actin 2/7	NP_196543	41,709	NYELPDGQVITIGAER (114) + 3	AGFAGDDAPR (53) + 1	Dalle-Donne et al. (2000, 2003); Jaffrey et al. (2001); Fratelli et al. (2002, 2003); Lind et al. (2002)
Actin depolymerizing factor 3-like	NP_851227	15,912	YAIFDFDFVSSEGVPR (54)		
Annexin	CAA67608	35,757	HYNDEDVIR (48) + 2	DALLANEATK (48) + 2	Liu et al. (2002); Kuncewicz et al. (2003)

(Table continues on following page.)

**Table 1.** (Continued from previous page.)

Protein	Acc. No.	Molecular Mass	Identified Peptides (Score) GSNO/NO	Identified Peptides (Score) GSH/Untreated	Hints to S-Nitrosylation
<i>D</i>					
Metabolic Enzymes					
Fru 1,6-biphosphate aldolase, putative	NP_190861	38,516	VSPEVIAEHTVR (85) + 4	LASINVENVETNR (64) +3	Fratelli et al. (2002); Lind et al. (2002); Ito et al. (2003); Shenton and Grant (2003)
Triosephosphate isomerase	T50646	27,138	VASPAQAQEVHDEL (109) + 3	VASPAQAQEVHDEL (68) +3	Fratelli et al. (2002); Ito et al. (2003); Shenton and Grant (2003)
GAPDH C-subunit	NP_187062	36,891	GILGYTEDDVVSTDFV-GDNR (92) + 4	AASFNIIPSSTGAAK (46) +3	Mohr et al. (1996, 1999); Klatt et al. (2000); Motohashi et al. (2001); Kuncewicz et al. (2003); Shenton and Grant (2003)
2-Phosphoglycerate hydrolase (enolase)	NP_181192	47,689	VTAAVPSGASTGIYEALELR (86) + 3		Fratelli et al. (2002); Lind et al. (2002); Shenton and Grant (2003)
Phosphoglycerate kinase	NP_178073	42,105	GVTTIIGGGDSVAAVEK (62) + 2		Fratelli et al. (2002)
Aconitase	Q8L784	108,133		INPLVPDLVIDHSVQV-DVAR (43)	Navarre et al. (2000)
SAM synthetase, putative	AAO11581	42,769	KPEEVGAGDQGHMF-GYATDEPELMLPLTH-VLTK (70) + 5	FVIGGPHGDAGLTGR (76) +4	Ruiz et al. (1998); Perez-Mato et al. (1999)
Adenosylhomo-cysteinase	CAB09795	51,447	LVGVSEETTGVK (48)		
Met synthase	NP_197294	84,304	IPSSEIADR (82) + 1	FALESFWDGK (53) +1	Yamazaki et al. (2004)
Cys synthase	CAA58893	33,842	IDGFVSGIGTGGTIT-GAGK (59)	LFVAIFPSFGER (35)	
ATP synthase CF1 $\alpha$ -chain	NP_051044	55,294	LIESPAPGIISR (49) + 2		Eaton et al. (2003)
ATP synthase CF1 $\beta$ -chain	NP_051066	53,900	YKELQDIIAILGLDELSEEDR (96) + 14	GIYPAVDPLDSTSTMLQPR (108) +6	
Proteins Involved in Photosynthetic Processes					
GAPDH precursor, chloroplast	JQ1285	42,481	VVDLADIVANNWK (74) + 1		Mohr et al. (1996, 1999); Klatt et al. (2000); Motohashi et al. (2001); Kuncewicz et al. (2003); Shenton and Grant (2003)
Rubisco large chain	NP_051067	52,922	ESTLGFVDLLR (90) + 6	LSGGDHIHAGTVVGK (56) +2	Marcus et al. (2003)
Rubisco small chain 1a precursor	RKMUA1	20,189	IIGFDNTR (45) + 1	IIGFDNTR (58) +1	Motohashi et al. (2001)
Rubisco activase, large subunit	AAA20202	51,948	VPLILGIWGGK (34)	GLAYDTSDDQQDITR (81) +3	Zhang and Portis (1999); Zhang et al. (2002)
PSII P680 47-kD protein	NP_051084	56,001	MPTFFETFPVVLVDGD-GIVR (85)		
PSII D2 protein, fragment	AAO13251	35,220	NILLNEGIR (31)		Carlberg et al. (1999)
PSII oxygen-evolving complex 33	NP_190651	34,998	NTAASVGEITLK (53) + 4		
Rieske Fe-S protein	CAC03598	24,334	VLFVPWVETDFR (63)		



**Figure 4.** Immunoblot analysis of in vitro *S*-nitrosylated proteins. Leaf extracts were treated with 250  $\mu\text{M}$  GSNO or GSH and analyzed with the biotin switch method. After biotinylation, proteins were purified with neutravidin-agarose, separated by SDS-PAGE, and immunoblotted with anti-PSII oxygen-evolving complex 33, anti- $\alpha$ -ATPase, and anti- $\beta$ -ATPase antibodies. Additionally, cell culture extracts were treated and prepared in the same way and analyzed with anti- $\alpha$ -tubulin antibody.

evidence that these proteins are indeed retained by the biotin switch method (Fig. 4).

In GSH-treated cell culture extracts and in untreated leaves, many proteins were identified that are also present in the NO-treated samples. Especially in the case of the untreated leaves of *Arabidopsis* plants, we think that these proteins represent in vivo *S*-nitrosylated proteins. Cell cultures as well as plants are known to show constitutive NO production due to the activity of NO producing enzymes and due to an enzyme-independent process in the apoplast of plant tissues (Guo et al., 2003; Bethke et al., 2004). However, despite constitutive basal *S*-nitrosylation, the detection of *S*-nitrosylated proteins in GSH-treated extract seemed to be contradictory because of the reducing activity of GSH. The reducing power of GSH is probably not strong enough for an effective reduction of all *S*-nitrosylated Cys residues. Furthermore, some *S*-nitrosylated proteins can only be reduced by the action of reducing enzymes like thioredoxin or glutaredoxin (Stamler et al., 2001). Taken together with the high specificity of the biotin switch method, we concluded that most of the identified proteins from the GSH-treated cell culture extracts and from the untreated leaves represent in vivo *S*-nitrosylated proteins, although we cannot exclude unspecific interactions of proteins with the biotinylation agent biotin-HPDP.

#### Inhibition of Glyceraldehyde-3-Phosphate Dehydrogenase Activity by NO

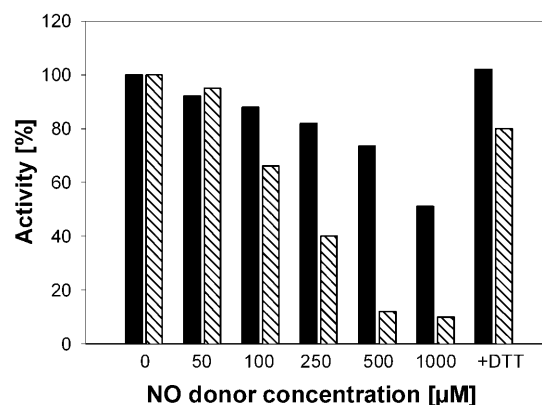
Although all of the proteins identified as candidates for *S*-nitrosylation contain at least one Cys residue, the effects of *S*-nitrosylation on enzyme activity or protein structure have to be elucidated. We have chosen glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the model enzyme since an easy and fast activity assay for this enzyme is already established (Mohr et al., 1996). Crude extracts of *Arabidopsis* cell cultures were incubated with different concentrations of GSH or the NO donors GSNO and SNP (Fig. 5). Whereas 1,000  $\mu\text{M}$  GSNO reduced the GAPDH activity up to

90%, the same concentration of SNP reduced the GAPDH activity up to only 50%. Addition of 10 mM DTT to the inhibited enzymes completely restored the activity, confirming that the effect of the NO donors was due to *S*-nitrosylation of one or more critical Cys residues. The treatment of crude cell culture extracts with up to 1,000  $\mu\text{M}$  GSH did not affect GAPDH activity (data not shown).

## DISCUSSION

In mammals, protein *S*-nitrosylation is an accepted and intensively studied posttranslational modification and until now more than 50 *S*-nitrosylated proteins could be identified using the biotin switch method (Jaffrey et al., 2001; Kuncewicz et al., 2003; Foster and Stamler, 2004). In plants, however, the knowledge about the posttranslational modification of Cys residues by *S*-nitrosylation is rather limited and restricted to results obtained by protein database search for a putative *S*-nitrosylation motif (Huber and Hardin, 2004). To identify plant proteins sensitive to this posttranslational process, we treated extracts of *Arabidopsis* cell cultures with the NO donor GSNO, resulting in an effective formation of nitrosothiols (Fig. 1) and *S*-nitrosylated proteins (Fig. 2).

The proteomic analyses by nanoLC/MS/MS resulted in the identification of 67 proteins belonging to stress-related proteins, signaling/regulating proteins, redox-related proteins, cytoskeleton proteins, metabolic proteins, and others. Within the stress-related proteins, several proteins were identified that are already described to undergo *S*-nitrosylation in the animal systems. An interesting target in *Arabidopsis* is glutathione *S*-transferase (GST). In rats, microsomal and cytosolic GSTs showed differential activation and inhibition after treatment with GSNO, respectively (Ji



**Figure 5.** Effect of GSNO and SNP on GAPDH activity. Crude extracts of *Arabidopsis* cell cultures were treated with different concentrations of GSNO (diagonal lines) or SNP (black) and enzyme activity was determined according to Mohr et al. (1996). The GAPDH activity in untreated control extract was set at 100%. For restoring GAPDH-activity, 10 mM DTT was added to extracts with inhibited enzymes. For each concentration, measurements were done at least in triplicates.

et al., 2002). These results together with previous findings of increased microsomal GST activity after exposure to oxidants such as hydrogen peroxide and superoxide (Aniya and Anders, 1989) have led to the suggestion that this GST isoform may play a protective role under conditions of oxidative and nitrosative stress (Ji et al., 2002).

Another cluster of S-nitrosylated proteins in Arabidopsis includes several signaling and regulating factors. The identification of different elongation (eEF-1 and eEF-2) and initiation (4A-1 and 5A-4-related) factors as targets for S-nitrosylation suggest the assumption that NO encroaches in protein synthesis. Shenton and Grant (2003) identified elongation factors EF-1 $\alpha$  and EF-1 $\beta$  and initiation factor Nip 1 of yeast as targets for S-glutathionylation and demonstrated that protein synthesis is rapidly and reversibly inhibited by H<sub>2</sub>O<sub>2</sub> treatment.

Other candidates for S-nitrosylation in Arabidopsis are cytoskeleton proteins such as tubulin  $\alpha$ , tubulin  $\beta$ , actin-depolymerizing factor, and actin. Both tubulin variants and actin have previously been described to undergo S-nitrosylation in neuronal cells of mammals (Jaffrey et al., 2001). The dynamic nature of the cytoskeleton filaments allows cells a rapid response to intracellular and extracellular signals by changing shape and translocating intracellular organelles or vesicles. Conformational changes of cytoskeleton components due to S-nitrosylation might be involved in directing vesicles loaded with toxic metabolites to the infection site and deflating the contents into the extracellular space (Collins et al., 2003).

The proteomic analysis of Arabidopsis also revealed proteins related to enzymes of the antioxidant system. In plants, NO dramatically affects redox balance and genes involved in redox control (Wendehenne et al., 2004). Glutaredoxin, peroxiredoxin, and glutathione peroxidase are together with GSH and thioredoxin important components of the cellular redox-status controlling system. Thioredoxin and glutaredoxin function as redox-regulators of target enzymes and transcription factors and serve as hydrogen donor to peroxiredoxin and dehydroascorbate (Sha et al., 1997; Rouhier et al., 2001). Both proteins harbor two highly conserved Cys residues within their active site and represent possible targets for S-nitrosylation.

Additionally, several metabolic enzymes of Arabidopsis were identified as potential candidates for S-nitrosylation. Five enzymes of the glycolysis are sensitive to S-nitrosylation, however, only GAPDH with a Cys residue in the active center is shown to be inhibited by NO (Padgett and Whorton, 1995; Mohr et al., 1996; Fig. 5). Fru 1,6-biphosphate aldolase, triosephosphate isomerase, and 2-phosphoglycerate hydrolase undergo S-glutathionylation during oxidative stress (H<sub>2</sub>O<sub>2</sub> treatment) without dramatic effect on the enzyme activity (Shenton and Grant, 2003). However, recombinant Arabidopsis triosephosphate isomerase is reversibly inactivated by oxidized glutathione (Ito et al., 2003). The functional aspect of inhibition of

GAPDH activity could be the diversion of Glc equivalents into the pentose phosphate cycle and production of NADPH for controlling the redox status of the cell.

The second important group of metabolic enzymes undergoing S-nitrosylation includes enzymes involved in sulfur metabolism, such as Cys synthase, S-adenosylhomocysteinase, vitamin B12-independent Met synthase, and S-adenosylmethione (SAM) synthetase. In plants, the latter three proteins are part of the methylMet cycle that provides activated methyl groups in the form of SAM for methylation of many different cell components and ingredients such as DNA, lignin, and flavonoids. Inhibition of SAM synthetase by NO is already described for the rat enzyme (Ruiz et al., 1998; Perez-Mato et al., 1999). In plants, SAM is a precursor for ethylene biosynthesis. NO regulates ethylene production in plants (Leshem and Haramaty, 1996), and S-nitrosylation of SAM synthetase or other enzymes of the methylMet cycle might mediate the crosstalk between ethylene and NO signaling.

To analyze S-nitrosylation processes in photosynthetic active tissue, we exposed Arabidopsis leaves to NO gas. Many chloroplast proteins identified as targets for S-nitrosylation are regulated in a redox-dependent manner including Gln synthase, NADPH-dependent GAPDH, Rubisco, and Rubisco activase (Ruelland and Miginiac-Maslow, 1999; Motohashi et al., 2001; Zhang et al., 2002; Marcus et al., 2003). The latter one plays a pivotal role in regulating the activity of the Calvin-Benson cycle and is regulated by thioredoxin-f (Zhang et al., 2002), a disulfide oxidoreductase, which controls the activity of many enzymes by reducing disulfide bridges and protecting single Cys residues from oxidation (Ruelland and Miginiac-Maslow, 1999; Motohashi et al., 2001; Sparla et al., 2002). In the large subunit of Rubisco, a Cys residue is adjacent to the active site and has been suggested to play a role in Rubisco activity and degradation (Marcus et al., 2003). Oxidizing conditions, both in vitro and in vivo, inhibited Rubisco activity and stimulated its degradation that in several instances was prevented by thiol reducing agents (Mehta et al., 1992; Desimone et al., 1996). In addition to these key enzymes of the Calvin-Benson cycle, proteins of PSII seemed especially to be targets for NO. Phosphorylation of PSII reaction center proteins D1 and D2, as well as other subunits of PSII, was found to be stimulated by moderately thiol-reducing conditions and kept at a high level also under highly reducing conditions (Carlberg et al., 1999). Furthermore, reversible inhibition of photophosphorylation in chloroplasts by NO was demonstrated (Takahashi and Yamasaki, 2002), suggesting that proteins of the energy transduction system in chloroplast thylakoids could be affected by S-nitrosylation.

In sum, the identification of plant proteins being potential targets for S-nitrosylation in vivo is a promising starting point to get insight in physiological as well as regulatory functions of NO in plants. The effect

of *S*-nitrosylation on the identified plant proteins, if enzyme activities are inhibited or enhanced due to *S*-nitrosylation or if a structural alteration followed by change of the protein function is the result of the modification, has to be analyzed. Additionally, these results will probably give hints to the regulation of crosstalk between more plant specific NO-, salicylic acid-, and jasmonic acid/ethylene-dependent signaling pathways.

## MATERIALS AND METHODS

### Chemicals

The NO donors GSNO and SNP were purchased from Alexis (Grünberg, Germany) and Fluka (Neu-Ulm, Germany), respectively. GSH, methyl methanethiosulfonate (MMTS), neocuproine, and anti-biotin mouse monoclonal antibody were from Sigma (Taufkirchen, Germany). *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridylthio) propionamide (biotin-HPDP) and neutravidin-agarose were purchased from Perbio (Bonn).

Standard chemicals of analytical grade were from Sigma (Taufkirchen, Germany), Roth (Munich), and Boehringer (Mannheim, Germany).

### Plant Material

*Arabidopsis* (*Arabidopsis thaliana*) plants (ecotype Columbia) were cultivated in a growth chamber for 6 weeks at 23°C during the day and 18°C at night (14 h). The experimental setups to study the effect of NO on whole plants consisted of controlled-environment cabinets as well as complete instrumentation to adjust and control gaseous NO through an electrochemical sensor. *Arabidopsis* plants were treated with NO concentrations of 1,250  $\mu\text{L/L}$  for 10 min under light (Huang et al., 2004). At this NO concentration, the plants did not show any symptoms. Twenty minutes after the treatment, leaves were harvested and stored at -20°C. Cell suspension cultures of *Arabidopsis* (ecotype Columbia) were grown in dark at 26°C (126 rpm) in PS medium as described (Huang et al., 2002).

### Determination of Nitrosothiol Content

Nitrosothiol content was determined according to the procedure of Saville (1958). Briefly, 180  $\mu\text{L}$  of each sample were incubated with 30  $\mu\text{L}$  of 0.5% ammonium sulfamate in water for 2 min. A total of 300  $\mu\text{L}$  of 2.7% sulfanilamide and 0.25%  $\text{HgCl}_2$  in 0.4 N HCl were added, followed by 240  $\mu\text{L}$  of 0.1% *N*-(1-naphthyl) ethylenediamine in water. The reference was incubated without  $\text{HgCl}_2$ . The concentration of formed azo compound was determined after 20 min by measuring the absorption at 540 nm. The nitrosothiol content was quantified according to a standard curve created with GSNO.

### Gel Electrophoresis and Western-Blot Analysis

Proteins were separated by SDS-PAGE on 12% polyacrylamide gels (Laemmli, 1970), were transferred onto polyvinylidene difluoride membranes and blocked with 1% nonfat milk powder and 1% bovine serum albumin. The blots were incubated with anti-biotin mouse monoclonal antibody conjugated with alkaline phosphatase at a dilution of 1:10,000 for 1 h. Antibodies raised against PSII oxygen-evolving complex 33,  $\alpha$ -ATPase,  $\beta$ -ATPase, and  $\alpha$ -tubulin were incubated for 1 h, followed by incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase. Cross-reacting protein bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates.

### Biotin Labeling of *S*-Nitrosylated Proteins

Frozen *Arabidopsis* cells were ground under liquid nitrogen to a fine powder. Proteins were extracted by adding 12 mL of HEN buffer (25 mM HEPES, pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine) puffer to 10 g of cell powder. Cell debris was removed by centrifugation (20,000g, 10 min, 4°C) and

protein concentration was determined according to Bradford (1976) with bovine serum albumin as standard.

The *in vitro* *S*-nitrosylation and subsequent biotinylation of *S*-nitrosylated proteins were done as described by Jaffrey (2001) with minor modifications. After treating the supernatant with GSNO for 20 min at room temperature (RT), the proteins were incubated with 20 mM MMTS and 2.5% SDS at 50°C for 20 min with frequent vortexing for blocking nonnitrosylated free Cys residues. Residual MMTS was removed by precipitation with 2 volumes of -20°C acetone and the proteins were resuspended in 0.1 mL of HENS buffer (HEN buffer containing 1% SDS)/mg protein. Biotinylation was achieved by adding 2 mM biotin-HPDP and 1 mM ascorbate and incubation at RT for 1 h.

### Purification of Biotinylated Proteins

After removing biotin-HPDP, the precipitated proteins were resuspended in 0.1 mL of HENS buffer/mg of protein and 2 volumes of neutralization buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). A total of 15  $\mu\text{L}$  of neutravidin-agarose/mg of protein were added and incubated for 1 h at RT. The matrix was washed extensively with 20 volumes of washing buffer (600 mM NaCl in neutralization buffer) and bound proteins were eluted with 100 mM  $\beta$ -mercaptoethanol in neutralization buffer.

### nanoLC/MS/MS Analyses

Proteins were dissolved in 50  $\mu\text{L}$  of 0.1 M  $\text{NH}_4\text{HCO}_3$ /10% acetonitrile and digested with 3  $\mu\text{g}$  of trypsin at 37°C overnight. Bands from stained SDS gels were cut out, washed, and treated with trypsin according to Shevchenko et al. (1996). Digested peptides were extracted by vortexing for 3 h with 100  $\mu\text{L}$  of 5% formic acid. All tryptic peptide samples were dried and redissolved in 50  $\mu\text{L}$  0.1% trifluoroacetic acid and 5% acetonitrile. Peptides were separated by reversed-phase chromatography using an UltiMate Capillary/Nano liquid chromatography system (LC Packings, Amsterdam). Portions of 20- $\mu\text{L}$  sample were loaded to a precolumn (300  $\mu\text{m}$   $\times$  5 mm, 5  $\mu\text{m}$  C18, 100 Å, PepMap, LC Packings) and eluted and fractionated on a self-packed analytical column (75  $\mu\text{m}$   $\times$  120 mm packed with YMC-Gel ODS-A (3  $\mu\text{m}$  C18; YMC, Kyoto) with a gradient of 5% to 55% acetonitrile at a flow rate of 150 nL/min in 40 min. Eluted peptides were continuously delivered to a Q-ToF Ultima mass spectrometer (Waters/Micromass, Manchester, UK) by electrospray and analyzed by MS/MS employing data-dependent analysis (3 most abundant ions in each cycle; 0.3 s MS *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 pkl-format using MassLynx 4.0 ProteinLynx. The resulting pkl-files were used for searching the NCBInr (Viridiplantae) protein database by Mascot search program (Matrixscience, London) with 0.2 D mass tolerance. Only matches calculated as significant by the Mascot search algorithm were considered in the protein identification.

### GAPDH Activity Assay

The activity of GAPDH was determined according to Mohr et al. (1996) with some modifications. Crude extracts of *Arabidopsis* cell suspension cultures (in 50 mM Tris-Cl, pH 7.5) were reduced with 10 mM DTT for 20 min at room temperature. Residual DTT was removed with Sephadex G-25M columns. A total of 150 to 300  $\mu\text{g}$  protein were incubated with 50  $\mu\text{M}$  arsenate and 100  $\mu\text{g/mL}$  3-phosphoglycerinaldehyde and were adjusted to 950  $\mu\text{L}$  with 50 mM Tris-Cl, pH 7.5. The reaction was initiated by adding 50  $\mu\text{L}$  of 10 mM  $\text{NAD}^+$  and the reduction of  $\text{NAD}^+$  to NADH was monitored at 340 nm.

Inhibition assays were done as described in (Mohr et al., 1999) with minor modifications. In brief, after DTT treatment and desalting, reduced cell culture extracts were incubated with different concentrations of GSNO, SNP, or GSH at room temperature. After 2 min, the treated cell culture extracts were added to the assay mixture and GAPDH activity was determined subsequently as described above.

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