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Differential Inhibition of *Arabidopsis* Methionine Adenosyltransferases by Protein *S*-Nitrosylation*

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In animals, protein *S*-nitrosylation, the covalent attachment of NO to the thiol group of cysteine residues, is an intensively investigated posttranslational modification, which regulates many different processes. A growing body of evidence suggests that this type of redox-based regulation mechanism plays a pivotal role in plants, too. Here we report the molecular mechanism for *S*-nitrosylation of methionine adenosyltransferase (MAT) of *Arabidopsis thaliana*, thereby presenting the first detailed characterization of *S*-nitrosylation in plants. We cloned three MAT isoforms of *Arabidopsis* and tested the effect of NO on the activity of the purified, recombinant proteins. Our data showed that incubation with GSNO resulted in blunt, reversible inhibition of MAT1, whereas MAT2 and MAT3 were not significantly affected. Cys-114 of MAT1 was identified as the most promising target of NO-induced inhibition of MAT1, because this residue is absent in MAT2 and MAT3. Structural analysis of MAT1 revealed that Cys-114 is located nearby the putative substrate binding site of this enzyme. Furthermore, Cys-114 is flanked by *S*-nitrosylation-promoting amino acids. The inhibitory effect of GSNO was drastically reduced when Cys-114 of MAT1 was replaced by arginine, and mass spectrometric analyses of Cys-114-containing peptides obtained after chymotryptic digestion demonstrated that Cys-114 of MAT1 is indeed *S*-nitrosylated. Because MAT catalyzes the synthesis of the ethylene precursor *S*-adenosylmethionine and NO is known to influence ethylene production in plants, this enzyme probably mediates the cross-talk between ethylene and NO signaling.

NO is a lipophilic, highly reactive gaseous molecule that plays important roles in regulation of stomatal closure (1), programmed cell death (2), abiotic stress (3), disease resistance (4, 5), and growth and development (6, 7). Because NO is involved in such many different processes it is not surprising that multiple NO sources are identified in plants providing NO for regulating the different physiological reactions. Recently, a hormone-activated NO-producing enzyme, which is involved in disease resistance, was found in *Arabidopsis* (8, 9). Other sources of endogenous NO are nitrate reductase (10, 11) and nitrite:NO reductase (12). Next to this enzymatic sources, non-enzymatic production of NO via reduction of apoplastic nitrite is also described (13).

Despite the multiplex importance of NO, less is known about the targets of this redox molecule in plant metabolism. Many of the biological functions of NO arise as a direct consequence of chemical reactions between proteins and NO or NO oxides generated as NO/O_2 or NO/superoxide reaction products. Different types of NO-dependent protein modification are described. NO is a precursor of the reactive nitrating species, peroxynitrite and nitrogen dioxide, which modify proteins to generate 3-nitrotyrosine as it is shown for the tyrosine residues 161 and 357 of α-tubulin (14). Protein nitration is an irreversible reaction, which is of importance for pathophysiological but probably not for signaling. Furthermore, NO can bind to metal ions of heme groups as it is reported for the activation of guanylate cyclase (15, 16) or it can form dinitrosyl complexes together with iron ions and low molecular weight thiols. Latter reactions seem to be important for iron uptake, trafficking, storage, and delivery in plant mesophyll cells (17, 18). Finally, NO can react with the thiol group of cysteine residues to form *S*-nitrosothiols (*S*-nitrosylation) as it is described *e.g.* for mammalian methionine adenosyltransferase (19) and ryanodine receptor/Ca^{2+} channel (20). Because of their reactivity with intracellular reducing agents like ascorbic acid or glutathione, and with reduced metal ions, especially Cu^{+}, the half-lives of *S*-nitrosothiols are very short (from seconds to a few minutes). This lability makes protein *S*-nitrosylation a very sensitive regulation mechanism, and there are many reports about the importance of this cysteine-dependent redox-based mechanism for controlling different cellular processes in animals (21–24).

Because most proteins possess cysteine residues, substrate specificity is a very important feature of endogenous protein *S*-nitrosylation. This includes structural factors that influence the susceptibility to *S*-nitrosylation like surrounding acidic or basic amino acids and the presence of a hydrophobic environment that enables the formation of *S*-nitrosylating species via the reaction between oxygen and NO (25). Additionally, trans-*S*-nitrosylation from the *in vivo* NO donor nitrosoglutathione (GSNO)[2] to proteins seemed to be promoted by acidic, basic, and hydrophobic side chains neighboring the target cysteine residue (23).

Until now more than 100 proteins were reported as targets for protein *S*-nitrosylation in animals. In plants there is experimental evidence for only three plant proteins to be regulated by *S*-nitrosylation. *Arabidopsis* hemoglobin 1 can scavenge NO through the formation of *S*-nitrosohemoglobin and *in vitro* experiments suggested that glyceraldehyde 3-phosphate dehydrogenase and the K^{+}-channel in guard cells are regulated by NO via *S*-nitrosylation (26–28). Recently, we identified 63 proteins from *Arabidopsis* cell cultures and 52 proteins from *Arabidopsis* leaves representing candidates for protein *S*-nitrosylation including stress-related, redox-related, signaling/regulating, cytoskeleton, and metabolic proteins (28). The latter group contains enzymes of the methylmethionine cycle: cobalamin-independent methionine synthase, *S*-adenosylhomocysteinase and *S*-methionine adenosyltransferase

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[2] The abbreviations used are: GSNO, nitrosoglutathione; GST, glutathione *S*-transferase; MS, mass spectrometry; MAT, methionine adenosyltransferase; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; AdoMet, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxlic acid.

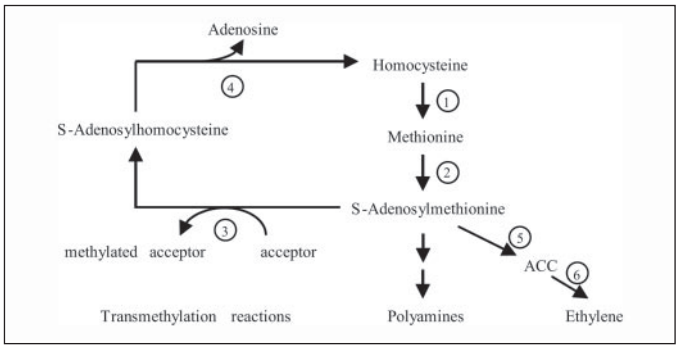

FIGURE 1. **Methylmethionine cycle in plants.** Enzymes: *1*, cobalamin-independent methionine synthase; *2*, methionine adenosyltransferase; *3*, AdoMet-dependent transmethylases; *4*, adenosylhomocysteinase; *5*, ACC synthase; *6*, ACC oxidase. The enzymes 1, 2, and 4 were previously identified as targets for protein *S*-nitrosylation (28).

(MAT) (see Fig. 1). MAT catalyzes the biosynthesis of *S*-adenosylmethionine (AdoMet), which is the most important methyl donor in transmethylation reactions and a substrate for the biosynthesis of polyamines and the plant hormone ethylene. Because of this important function of AdoMet it is not surprising that most species studied today have more than one MAT isoform (29). In mammals, two genes (MAT1A and MAT2A) encode different MAT isoenzymes that show an organ-specific expression pattern. MAT1A is expressed in the liver only, whereas MAT2A is expressed in all other tissues (30). However, the most interesting difference between these two isoenzymes is that MAT1A is reversibly inactivated by NO, whereas MAT2A is not. Responsible for the inactivation is the *S*-nitrosylation of the cysteine residue 121 of MAT1A, which is located within a flexible loop that can gate access to the active center (19, 31–37). Moreover, it has been shown that the inducible NO synthase controls the activity of liver MAT (31). Thus, NO regulates the synthesis of AdoMet and as consequence the synthesis of the metabolites AdoMet is necessary for.

In this paper, we report the differential inhibition of MAT isoenzymes of *Arabidopsis* by the NO donor GSNO. We isolated the coding sequences of three MAT isoforms and produced the corresponding enzymes as GST-fusion proteins in *Escherichia coli*. Activity assays with recombinant GST-MAT fusion proteins pretreated with GSNO demonstrated that the activity of isoenzyme MAT1 is reduced to 30%, whereas the other two isoforms are only weakly affected by this treatment. Site-directed mutagenesis and mass spectrometric analysis showed that *S*-nitrosylation of the cysteine residue 114 of MAT1 is responsible for the inhibition of the enzymatic activity. Thus, MAT1 represents the first metabolic plant enzyme that is regulated by *S*-nitrosylation of a redox-sensitive cysteine residue. Furthermore, our results led us to speculate about the involvement of MAT in the regulation of ethylene biosynthesis.

EXPERIMENTAL PROCEDURES

Structural Analysis—Amino acid sequences were aligned and modeled using SWISS-Model (www.expasy.ch). The crystal structure of *E. coli* methionine adenosyltransferase (Protein Data Bank code 1RG9, chains A and B) was used as template for the prediction of the putative conformations of MAT1, MAT2, and MAT3.

Isolation of Coding Sequences of MAT Isoforms—For cloning the cDNAs of the different MAT isoforms the λ phage-based site-specific recombination (Stratagene) was used (38). Briefly, RNA from *Arabidopsis* leaves and stems was used for reverse transcription-PCR using PfuTurbo DNA polymerase, gene-specific primers, and the following PCR conditions: 2 min at 94 °C, 35 cycles consisting of 30 s at 94 °C, 30 s at 57 °C for amplification of MAT1 and MAT3 cDNA, and 30 s at 54 °C for amplification of MAT2 cDNA, and 2.5 min at 72 °C, followed by a final extension step of 10 min at 72 °C. The introduction of the DNA recombination sequence (att) at the 5′- and 3′-end of the coding sequence of each isoform was achieved by PCR using the isoform-specific att-primers and the amplified cDNAs as template. The resulting PCR products were introduced into pDONR221 by recombination using BP Clonase enzyme mixture according to the instructions of the manufacturer. After verifying the sequences of the different MAT isoforms by sequencing they were transferred into the expression vector pDEST15 by recombination using LP Clonase enzyme mixture.

Expression in E. coli and Purification of Recombinant Proteins—*E. coli* strain BL21 DE3 pLysS harboring the plasmids pDEST15-MAT1, pDEST15-MAT2, pDEST15-MAT3, or pDEST15-MAT1/C114R were grown in Luria-Bertani medium until A_{600} ~0.5 was reached. Production of recombinant GST fusion proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. After incubation for 4 h at 37 °C bacterial cells were harvested by centrifugation. For protein isolation the cells were resuspended in an appropriate volume of buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 20% (v/v) glycerol, 20 mM β-mercaptoethanol, 1 mM dithiothreitol (DTT)) and disrupted by sonication. Cellular debris was removed by centrifugation (20,000 × *g*, 20 min, 4 °C). The recombinant GST fusion proteins were purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Biosciences). Adsorbed proteins were eluted from the matrix with 100 mM Tris-HCl, pH 8.0, containing 20 mM glutathione. After adding 20% (v/v) glycerol to the eluates they were frozen in liquid nitrogen and stored at −20 °C until analysis.

MAT Activity Assay—Purified enzymes were activated with 10 mM DTT (20 min, 4 °C), and residual DTT was removed using Micro Biospin 6 columns (Bio-Rad). The enzyme activity was assayed as described by (39) with minor modifications. Briefly, 50 μl of purified enzyme (~15 μg of protein) were added to 150 ml of a reaction mixture containing 100 mM Tris-HCl, pH 8.0, 30 mM $MgSO_4$, 10 mM KCl, 10 mM ATP, and 5 mM [^{35}S]Met (15 μCi). Control reactions contained all agents except for ATP. Reactions were incubated for 1–2 h at 35 °C and were terminated by placement on ice-water. Fifty microliters of the reaction mixture were then spotted onto chromatography paper P81 (2 cm × 2 cm) in duplicate or triplicate. The papers were air-dried and washed two times with ice-water for 5 min. The washed chromatography papers were transferred to scintillation vials containing 1 ml of 1.5 mM ammonium hydroxide. After 5 min, scintillation liquid was added, and the samples were counted by scintillation spectrometry.

Site-directed Mutagenesis—The modification of single nucleotide residues was performed as previously described (40). Briefly, for mutation, a pair of oligonucleotides was synthesized harboring the desired alterations. The size of the primers was adjusted to yield a melting temperature of 68 °C by using the following formula: $T_m = 81.5 + 0.41 \times$ GC (%) − 675/number of bases − sequence deviation (%). For amplification, 20 ng of plasmid DNA was used in a total volume of 15 μl, including 1 μM each primer, 200 μM dNTPs, and 1 unit of PfuTurbo DNA polymerase. After denaturation (2 min at 94 °C) 18 cycles were conducted, consisting of 45 s at 94 °C, 30 s at 55 °C, and 15 min at 72 °C, followed by a final extension step at 72 °C for 10 min. Subsequently, the parental and hemi-parental template DNA was digested with DpnI, and the amplified plasmids were transformed into *E. coli* DH5α. The mutation was verified by sequencing.

Mass Spectrometric Analyses—Purified recombinant MAT1 protein was dissolved in 0.1 M NH_4HCO_3 at 0.5 μg/μl. Aliquots of 10–20 μg of were treated with 500 ng of chymotrypsin at room temperature for 3 h. The digest was then diluted 1:8 into 50% (v/v) methanol/5% (v/v) formic

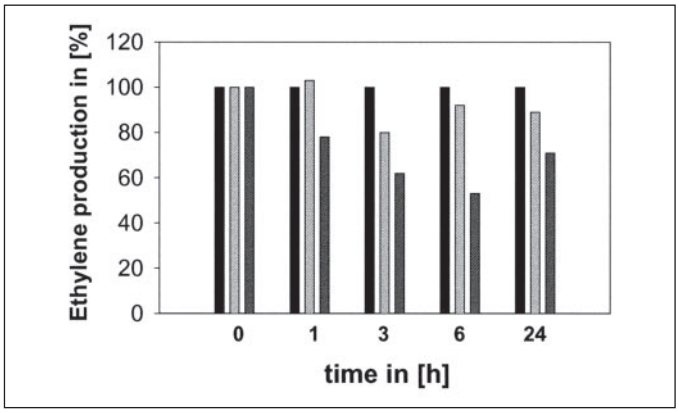

FIGURE 2. **Ethylene emission of *Arabidopsis* cell cultures after treatment with GSNO.** *Arabidopsis* cell cultures were treated with 0.5 mM (*light gray*) and 1 mM GSNO (*dark gray*). The cell cultures were air-tight and after the indicated times the produced ethylene was collected. After 30 min the amount of produced ethylene was determined as described previously (41).

acid. For analysis by mass spectrometry, this solution was applied to a Q-TOF Ultima™ Global (Micromass, Manchester, UK) by electrospray ionization using the nanospray kit coupled to a 100-μl Hamilton syringe. The mass spectrometer was calibrated using MS/MS fragments of GluFib (Sigma). Full mass scans of 1 s were recorded at a collision energy of 10 V, loss of the NO group was achieved at 20–25 V, and MS/MS fragmentation was achieved at 30 V. A number of scans recorded over time were combined and smoothed (Savitzky-Golay, 3/2).

Determination of Ethylene—Seven-day-old *Arabidopsis* cell cultures were portioned in 10-ml fractions in 25-ml Erlenmeyer flasks under sterile conditions. The following day the cell cultures were treated with water (control) or different concentrations of GSNO and incubated in dark at 26 °C (126 rpm). To measure the ethylene production of the cells the flasks were air-tight and after 30 min 1 ml of gas was collected from the gas phase of the cultures using a syringe. The produced ethylene was measured with a PerkinElmer Autosystem XL gas chromatograph equipped with a Porapak Q column (Supelco) and a flame ionization detector as described previously (41).

RESULTS

In a recent publication (28), we identified more than 100 proteins as *S*-nitrosylation targets, among them MAT, which is responsible for supplying AdoMet, *e.g.* for ethylene biosynthesis. Interestingly, NO inhibits ethylene production in plants (42, 43). We treated *Arabidopsis* cell cultures with different concentrations of the NO donor GSNO and determined their ethylene emission (Fig. 2). With 0.5 mM GSNO we detected a maximal inhibition of the ethylene production of 20% after a 3-h incubation. Longer incubation times reduced the inhibition of ethylene emission to 10% (6 and 24 h). Treatment with 1 mM GSNO decreased ethylene production to 55% after 6 h of treatment, and the production is restored with longer incubation times (24 h).

A possible mechanism for NO-dependent decrease of ethylene production could be the *S*-nitrosylation of MAT. Searching the NCBI nucleotide data base we found four different MAT-encoding sequences in *Arabidopsis*. The deduced amino acid sequences of the different isoenzymes show high homology among each other (89.3–96.4% amino acid sequence identity) and possess seven highly conserved cysteines (Fig. 3). MAT1 and MAT3 have one additional cysteine at positions 114 and 302, respectively.

To investigate whether the activity of the *Arabidopsis* MAT isoenzymes is regulated by NO we first had to isolate the coding sequence of the *Arabidopsis* proteins. Because the localization of the cysteines of MAT2 and MAT4 are identical we concentrated our investigations on the isoforms 1, 2, and 3. The isolation of the cDNAs of these three MAT isoforms was achieved by reverse transcription-PCR using gene-specific oligonucleotides, and the amplified coding sequences were expressed in *E. coli* as inducible fusion proteins containing N-terminal glutathione *S*-transferase. After affinity chromatography on glutathione-Sepharose 4B, the three fusion proteins showed the expected relative molecular masses of 68 kDa in SDS-polyacrylamide gels.

The effect of NO on the activity of the different MAT isoforms was tested by incubating the purified enzymes with 1 mM GSNO and determining their activities afterward. The activity of MAT1 was reduced to ~30%, whereas neither the activity of MAT2 nor of MAT3 was significantly affected (Fig. 4*A*). The activity of the inhibited MAT1 could completely be restored by adding 10 mM DTT. Incubation of MAT1 with different doses of GSNO showed that a concentration of 10 μM already reduced the enzyme activity to 50% (Fig. 4*B*).

To further characterize the mechanism of GSNO-mediated MAT inhibition, we tested the influence of the cysteine residue-modifying agents *N*-ethylmaleimide (NEM) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) on the activity of the different MAT isoforms. NEM is a highly reactive agent that covalently and irreversibly alkylates free cysteine thiol groups. DTNB is an oxidizing reagent that acts by formation of a disulfide bond between itself and free cysteine thiol groups resulting in a dithiobenzoate complex with proteins. As shown in Fig. 4*C* exposure to 250 μM DTNB led to complete inactivation of MAT1, whereas MAT2 and MAT3 activity was reduced to 40 and 35%, respectively. We observed a similar result after treatment with 500 μM NEM. Although MAT1 was totally inhibited, MAT2 and MAT3 showed a residual activity of 40 and 20%, respectively (Fig. 4*C*).

E. coli MAT has been crystallized in a ternary complex with the *S*-adenosylmethionine and imidotriphosphate (44). Because this enzyme shares 54.4–55.4% identical amino acid residues with the *Arabidopsis* MAT isoforms, it was possible to use the structure of *E. coli* MAT as a template to model the hypothetical three-dimensional conformation of the *Arabidopsis* MAT isoenzymes. Actually, MAT is a tetramer of two asymmetric dimers. Each dimer has two-substrate binding site, which are located between the interface of the monomers and are gated by an flexible loop (34–37). The structural models gave us insight into the spatial disposition of the cysteine residues of the MAT isoforms to identify cysteine residues representing possible candidates for *S*-nitrosylation. All of the seven highly conserved cysteine residues are present as free amino acids, and therefore all are possible targets for NO. More interestingly, the additional cysteine residue of MAT1 (Cys-114, Fig. 3) is located directly next to the putative catalytic center as part of the active site loop (Fig. 5).

To elucidate whether this cysteine is a promising target for *S*-nitrosylation, we compared the amino acids around this cysteine to the putative acid-base motif for protein *S*-nitrosylation and to the potential motif for trans-*S*-nitrosylation (Table 1). The degenerated acid-base motif was constructed by comparing the flanking amino acids of NO-sensitive cysteine residues (45). The two amino acids positioned N-terminally of the cysteine (Thr and Lys) are part of the degenerated acid-base motif, whereas directly C-terminally of the cysteine a hydrophobic proline is located instead of an acidic amino acid in the motif (Table 1). However, the proline is followed by two acidic glutamate residues. The GSNO binding pocket was first defined for GSSG binding in the bacterial transcription factor OxyR (46). The potential motif for GSNO-mediated trans-*S*-nitrosylation was then identified by data base analysis and was shown to be generally present in some copies within ~650 substrates for

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MAT1  METFLFTSESVNEGHPDKLCDQISDAVLDACLEQDPDSKVACETCTKTNMVMVFGEITTKATVDYEKIVR 70
MAT2  ..............................................................I....... 70
MAT3  ..........................I.........E......................A.K........ 70
MAT4  ..S.......................I.........E........................N....Q... 70

MAT1  DTCRAIGFVSDDVGLDADKCKVLVNIEQQSPDIAQGVHGHFTKCPEEIGAGDQGHMFGYATDETPELMPL 140
MAT2  ....S...I..................................R..D....................... 140
MAT3  S...E...I.A.........N...................L..K..D....................... 140
MAT4  K...E.....A.......N.....................L..K...V...................... 140

MAT1  SHVLATKLGARLTEVRKNGTCAWLRPDGKTQVTVEYYNDKGAMVPIRVHTVLISTQHDETVTNDEIARDL 210
MAT2  .......I.............R.................N.....V........................ 210
MAT3  T.........K.......K..P..............K..G...I.......................A.. 210
MAT4  T.........K..........P...........I..I.ES.....V.....................A.. 210

MAT1  KEHVIKPVIPEKYLDEKTIFHLNPSGRFVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDR 280
MAT2  .......I.......D...................................................... 280
MAT3  ..........A....DN..................................................... 280
MAT4  ...................................................................... 280

MAT1  SGAYIVRQAAKSVVANGMARRALVQVSYAIGVPEPLSVFVDTYETGLIPDKEILKIVKESFDFRPGMMTI 350
MAT2  ...........................................G...............T.......... 350
MAT3  ...............A.L...CI....................K..T....D..VLI..A........A. 350
MAT4  ............I..S.L...VI..................S.G..K.......E............IS. 350

MAT1  NLDLKRGGNGRFLKTAAYGHFGRDDPDFTWEVVKPLKWDKPQA 393
MAT2  ............Q.............................. 393
MAT3  .........F..Q........................PKA--- 390
MAT4  .........................A...........SN.V.. 393
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FIGURE 3. **Alignment of the amino acid sequences of *Arabidopsis* MAT isoforms.** GenBank™ accession numbers are as follows: MAT1 (NP_849577), MAT2 (AAA32869), MAT3 (AAD31573), MAT4 (AAO11581). Highly conserved cysteine residues are bordered, and Cys-114 of MAT1 is marked with an *arrow*. Amino acids forming the active site loop acids are highlighted with *gray*. *Dots* mark amino acids identical to the sequence of MAT1.

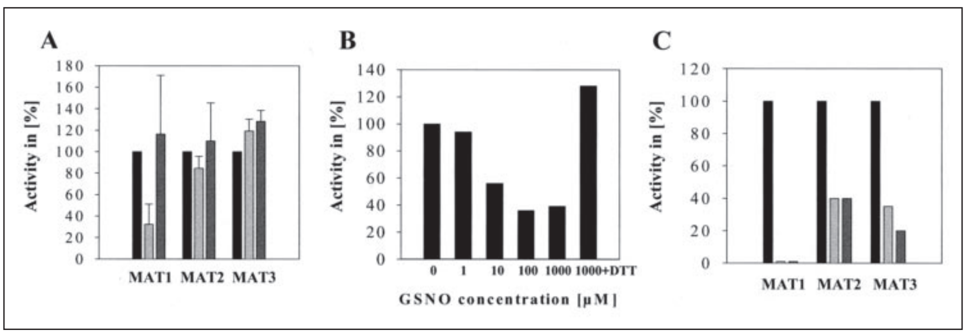

FIGURE 4. **Differential inhibition of *Arabidopsis* MAT isoforms by GSNO.** *A*, recombinant MAT isoenzymes were produced in *E. coli* and purified as described under "Experimental Procedures." Purified enzymes were incubated in the absence (*black*) or presence (*light gray*) of 1 mM GSNO for 20 min at room temperature before enzyme activity was measured. For restoring MAT activity, 10 mM DTT was added to extracts with inhibited enzymes (*dark gray*). The activity of untreated enzymes was set to 100%. Values are the mean ± S.D. of at least four different measurements. *B*, dose-dependent inhibition of *Arabidopsis* MAT1 was analyzed by incubating purified, recombinant MAT1 with different concentrations of GSNO for 20 min at room temperature before measuring the enzyme activity. Values are the mean of at least three different experiments. *C*, SH-modifying agents DTNB and NEM reduce activity of *Arabidopsis* MAT isoforms. Recombinant enzymes purified from *E. coli* were incubated with 250 mM DTNB (*light gray*) or 500 mM NEM (*dark gray*) for 20 min at room temperature. Afterward, the activity of the enzymes were measured. Control treatment was done with water (*black*). The activity of the controls was set to 100%. Values represent means of two independent experiments.

protein *S*-nitrosylation, including bacterial transcription factor OxyR, annexin-6, glutathione *S*-transferase-μ, and semaphoring-4D (23). In MAT1 the GSNO binding motif for trans-*S*-nitrosylation is also present including the Cys-114 (Table 1). The three-dimensional structure of *Arabidopsis* MAT1 revealed that the basic (Lys-113) and acidic (Glu-116, Glu-117) amino acids side chains of the *S*-nitrosylation promoting motifs are placed close to the SH group of Cys-114 (Fig. 6). As a result of the model, the distance of the thiol group of Cys-114 to the ϵ-amino group of Lys-113 is 7.7 Å and to the γ-carboxyl group of Glu-116 and Glu-117 is 4.5 and 5.3 Å, respectively. Other amino acids that could be important for optimal GSNO binding are Asp-37 and Glu-314, whose γ-carboxyl groups are modeled in a distance of 13.9 and 12.0 Å, respectively, to the thiol group of Cys-114 (data not shown).

To determine whether the Cys-114 is *S*-nitrosylated, we analyzed GSNO-treated and untreated MAT1 by mass spectrometry after digestion with chymotrypsin. This protease generated an analyzable peptide including Cys-114. We observed a peptide of m/z = 860.3728 in good agreement with the expected m/z value of a doubly charged peptide of

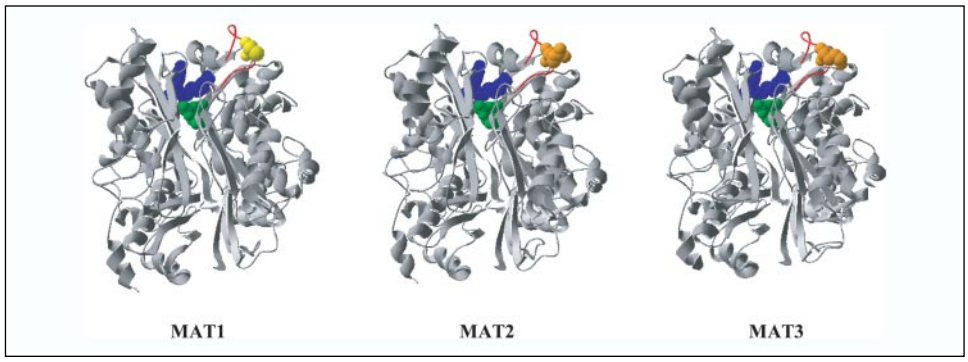

FIGURE 5. **Structural models of *Arabidopsis* MAT isoforms.** The three-dimensional structures of the MAT isoforms were modeled using SWISS-MODEL (56) with the crystal structure of *E. coli* MAT as template (PDB code: 1RG9, chains A and B). Dimers of the actually tetrameric MAT are shown. The two active centers of the dimer are located between the interface of two monomers, whereas only one is indicated by bound *S*-adenosylmethionine (*blue*) and imidotriphosphate (*green*), respectively. Cys-114 of MAT1 (*yellow*) is positioned within the active site loop (*red*), and the corresponding amino acids at this position of MAT2 (Arg) and MAT3 (Lys) are shown in *orange*.

TABLE 1

Comparison of the Cys-114-flanking amino acid sequences of MAT1 with the acid-base and the potential GSNO binding motif for protein *S*-nitrosylation

The acid-base motif for protein *S*-nitrosylation was defined by analyzing the primary sequence around NO-sensitive cysteine residues (bold) (45). The GSNO binding motif was identified by database analysis using the eMOTIF scan (23,57)

Motif	Sequence
Acid-base motif	[GSTCYNQ][KRHDE]**C**[DE]
GSNO binding motif	[H, K, R]C[hydrophobic]X[D, E]
MAT1	^{112}T K **C** P E E^{117}

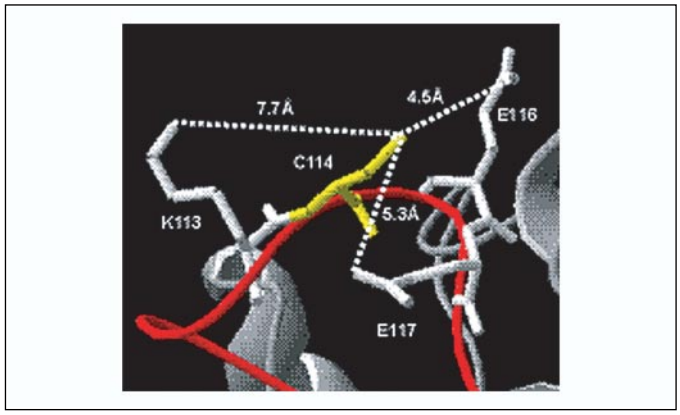

FIGURE 6. **Part of the structural model of *Arabidopsis* MAT1 showing the active site loop.** The structural model of *Arabidopsis* MAT1 was generated using SWISS-MODEL (56) with the crystal structure of *E. coli* MAT as template (PDB code: 1RG9, chains A and B). The active site loop is marked in *red*. The distances between the thiol group of Cys-114 and the side chains of the surrounding basic (Lys-113) and acidic (Glu-116, Glu-117) amino acids are illustrated.

TABLE 2

Determination of *S*-nitrosylation of Cys-114 by mass spectrometry.

Purified, reduced, recombinant MAT1 was incubated with 1 mM GSNO or water for 20 min at room temperature and precipitated with acetone. After chymotryptic digestion of the GSNO-treated and untreated MAT1, peptides containing Cys-114 were analyzed by mass spectrometry to determine *S*-nitrosylation of Cys-114. Expected *m*/*z* values for the different digests are shown to the left and the measured values to the right.

Sequence	Treatment	*m*/*z*	
		Expected	Observed
^{112}TKCPEEIGAGDQGHMF^{127}	−GSNO	860.3696	860.3728
	+GSNO	874.8696	874.8655

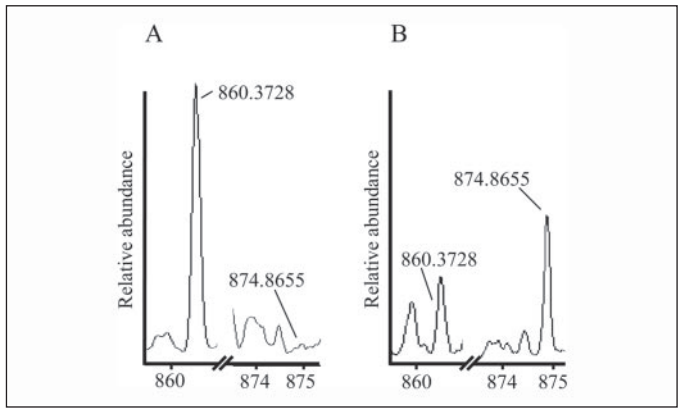

FIGURE 7. **Analysis of *S*-nitrosylation of Cys-114 by mass spectrometry.** Purified, reduced, recombinant MAT1 was treated with 1 mM GSNO or water for 20 min at room temperature and precipitated with acetone. After chymotryptic digestion, Cys-114 containing peptides of untreated (*A*) and GSNO-treated (*B*) MAT1 were analyzed by mass spectrometry to determine *S*-nitrosylation of Cys-114.

m/*z* = 860.3696 of the untreated MAT1 (Table 2 and Fig. 7*A*). The peptide of *m*/*z* = 860.3728 was identified as the expected Cys-114-harboring peptide by MS/MS *de novo* sequence analysis (data not shown). The signal was drastically reduced when the GSNO-treated MAT1 was analyzed, and the corresponding peptide with the *S*-nitrosylated cysteine residue (increased in mass by 29) could instead be detected (Table 2 and Fig. 7*B*). Furthermore, increasing the collision energy for the observed *S*-nitrosylated peptide of *m*/*z* = 874.8655 resulted in the partial loss of the NO group (loss of mass 30) and appearance of the expected corresponding product ion of *m*/*z* = 859.8708 (expected 859.8696).

Generation of a mutant of *Arabidopsis* MAT1 where the Cys-114 was changed to arginine by site-directed mutagenesis revealed that Cys-114 is indeed responsible for the GSNO-mediated inhibition of the enzyme activity. The recombinant enzyme mutant was incubated with 1 mM GSNO, and the activity was measured. As shown in Fig. 8 GSNO treatment inhibited the mutated enzyme to only ~15%, and activity could be completely restored with 10 mM DTT. Surprisingly, incubation of the MAT1 mutant with 250 μM DTNB or 500 μM NEM reduced the enzyme activity up to 95% similar as for the wild-type form.

DISCUSSION

Nitric oxide plays a key role in plant signaling, development, metabolism, and defense. However, in most cases neither the target molecules nor the way of action of NO are known. An important mechanism how NO can affect protein activity/function is the modification of sulfhydryl groups known as protein *S*-nitrosylation. Here we describe the differential inhibition of *Arabidopsis* MAT isoforms by NO and the possible physiological function of inhibition of MAT1.

MAT1 Is Inhibited by S-Nitrosylation in Vitro—Recently, we identified *Arabidopsis* MAT as target for protein *S*-nitrosylation using the biotin switch method (28). In *Arabidopsis* four MAT isoforms exist with very similar amino acid sequences. The importance of sulfhydryl protection reagents for mammalian and plant MAT during protein extraction and for determination of enzyme activity suggests the presence of crucial cysteine residues in the primary structure of MAT (31, 39, 47).

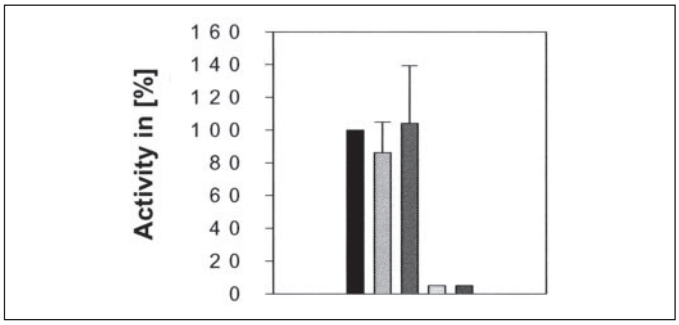

FIGURE 8. **Effect of GSNO and SH-modifying agents on MAT1/C114R.** Purified, recombinant MAT1/C114R was treated with GSNO and SH-modifying agents for 20 min at room temperature, and enzyme activity was determined afterward. The activity of the control treatment (water) was set to 100%. The activities for the different treatments are depicted from left to right in the order: water, 1 mM GSNO, 1 mM GSNO followed by 10 mM DTT, 250 μM DTNB, and 500 μM NEM. Values represent means of at least two independent experiments.

Actually, *Arabidopsis* MAT isoforms possess up to eight in part highly conserved cysteines. These observations led us to evaluate the effect of NO on *Arabidopsis* MAT isoenzymes. Our data showed that incubation of the purified, recombinant isoenzymes with 1 mM GSNO resulted in blunt inhibition of MAT1, whereas MAT2 and MAT3 were affected only weakly. The inhibition was reversed when the reducing agent DTT was added to the GSNO-treated samples (Fig. 4*A*). The reversible effect of NO is characteristic for protein *S*-nitrosylation and has been documented for the activity of other NO-sensitive proteins like glyceraldehyde-3-phosphate dehydrogenase (28) and argininosuccinate synthetase (48).

Comparing the cysteine composition of the three MAT isoforms we identified the Cys-114 of MAT1 as the most promising target of NO-induced inhibition of MAT1, because this residue is located nearby the putative substrate binding site of this enzyme and is absent in MAT2 and MAT3 (Figs. 3 and 5). Experimental evidence for the importance of this amino acid residue for GSNO-induced MAT1 inhibition was given by the MAT1 mutant MAT1/C114R. The inhibitory effect of GSNO was drastically reduced when Cys-114 of MAT1 was replaced by arginine demonstrating that this cysteine is the main target for NO-dependent inhibition of MAT1. Furthermore, mass spectrometric analyses of Cys-114-containing peptides obtained after chymotryptic digestion revealed that Cys-114 of MAT1 is indeed *S*-nitrosylated after GSNO treatment (Table 2 and Fig. 7).

Quite low GSNO concentrations are necessary to inhibit MAT1 activity (Fig. 4*B*). The reason for the high sensitivity of MAT1 to NO might be the presence of *S*-nitrosylation-promoting amino acids around Cys-114. Actually we identified the acid-base motif for protein *S*-nitrosylation (25) as well as to the GSNO binding motif (23) (Table 1). The acid-base motif contains acidic (Asp, Glu) and basic (Arg, Lys, His) residues, which promote *S*-nitrosylation of the NO-sensitive Cys residues. The basic amino acids support the H^+ release of the thiol group of the Cys, whereas acidic amino acid side chains promote NO^+ donation of the NO donor (19). In MAT1 the NO-sensitive Cys-114 is surrounded by basic (Lys-113) as well as acidic (Glu-116, Glu-117) amino acids, whose side chains are positioned close to the thiol group of Cys-114 (Fig. 6). This facilitates on one side the H^+ release of the SH group of Cys-114 on the other side the NO^+ release of GSNO.

The second important *S*-nitrosylation motif promotes binding of GSNO in a way that the NO group of GSNO is positioned close to the sulfhydryl group of the reactive cysteines. The putative GSNO-binding motif was identified by molecular modeling and data base screening and seems to be present in some proteins, which are already reported to be sensitive to *S*-nitrosylation (23). The optimal positioning of GSNO is achieved through hydrogen bonding between the γ-glutamyl amine of GSNO and the γ-carboxylate of the acidic amino acid C-terminal of the target Cys (23). In MAT1 the γ-carboxylate of Glu-116 or Glu-117 could be responsible for optimal binding of GSNO. Additionally, Asp-37 and Glu-314, whose side chains are located 13.9 and 12.0 Å away from the thiol group of Cys-114, respectively, could also be involved in optimal positioning of GSNO for an effective NO transfer. As a result of the binding of NO to Cys-114 the flexible loop could take a conformational change resulting in a restricted access of the substrate binding site.

The NO sensitivity of MAT1, the reduced NO sensitivity of MAT1/C114R, and the presence of *S*-nitrosylation-promoting amino acids around Cys-114 demonstrate that Cys-114 is the critical site for redox regulation of MAT1. However, treatment of the mutant MAT1/C114R with the sulfhydryl group-blocking agents NEM and DTNB resulted in almost total loss of activity like it was found for MAT1. Apparently, alkylation of other cysteine residues of MAT1 and the mutated enzyme lead to alterations in their protein structures, which might have a negative effect on enzyme activity. This could also be the reason for the reduced activity of MAT2 and MAT3 after treatment with the thiol-modifying agents, although the effect is not as drastic as it is for MAT1 and its mutant. The inhibition of MAT2 to 80%, and the activation of MAT3 up to 120% after GSNO treatment is probably also a result of *S*-nitrosylation of one or more of the highly conserved cysteines suggesting that modification of cysteine residues that are not located near the substrate binding site also can influence enzyme activity. For thioredoxin, for example, it is described that Cys-69 is *S*-nitrosylated under basal conditions, whereas the redox-active cysteines, Cys-32 and Cys-35, are not (49). *S*-Nitrosylation of Cys-69 is required for scavenging reactive oxygen species and for preserving the redox-regulatory activity of thioredoxin. Although there is no doubt about the importance of Cys-114 for the regulation of MAT1 activity, *S*-nitrosylation or probably also *S*-glutathionylation of other cysteines of MAT1 might have a weak effect on the activity of MAT1. Furthermore, it should be mentioned that redox-sensitive cysteine residues are also subject to multiple other redox-based modifications, resulting in the formation of sulfenic acid (S-OH), sulfinic acid ($S\text{-}O_2H$), sulfonic acid ($S\text{-}O_3H$), or intramolecular disulfides (S-S), which all may alter protein function/activity (23, 46).

Possible Physiological Function of S-Nitrosylation of MAT1—In animals, protein *S*-nitrosylation regulates many different processes like protein-protein interactions (50), apoptosis (49), signal transduction through Src (51), and protein ubiquitylation (23). In contrast, investigation of the regulatory functions of protein *S*-nitrosylation in plants is just at the beginning. Until now a physiological function of *S*-nitrosylation is only described for *Arabidopsis* nonsymbiotic hemoglobin AHb1. This protein is able to scavenge NO through formation of *S*-nitrosohemoglobin and reduce NO emission during hypoxic stress, suggesting a role in NO detoxification (26). Furthermore, there is some evidence that outward-rectifying K^+ channels of *Vicia faba* guard cells are regulated by *S*-nitrosylation of cysteine sulfhydryl groups influencing stomatal closure (27). The importance of *S*-nitrosothiols in plant defense was recently uncovered by Feechan *et al.* (52). They observed that GSNO reductase knock-out mutants of *Arabidopsis* are more susceptible to pathogens than wild-type plants and that, like in mice and yeast, GSNO reductase controls the levels of *S*-nitrosothiols in plants (52, 53). The protective function of GSNO reductase seemed to be based on the stimulation of salicylic acid-dependent defense pathways.

As observed in a variety of plants, endogenously produced NO and ethylene appear to be natural regulators for plant growth, plant development, and plant defense reactions (54). Ethylene and NO monitoring

of both vegetative and generative plant organs revealed that temporal progress of maturation and senescence go hand in hand with a significant decrease of endogenous NO emission and increase of ethylene emission indicating that NO has opposite effects to ethylene (55). A linkage of NO and ethylene emission in senescing foliage and in fruit, flower, and vegetable ripening was also reported by Leshem and colleagues (42, 43), who demonstrated the effect of NO in extending the post-harvest life of fresh horticultural products by reducing ethylene synthesis. These observations together with our results that NO donors markedly reduce ethylene emission of *Arabidopsis* cell cultures (Fig. 2) suggest that NO directly acts by down-regulating ethylene synthesis. Because inhibition of MAT1 activity by *S*-nitrosylation will result in reduction of the SAM pool and therefore will affect ethylene biosynthesis, we assume that this protein could be a switch point for regulating cross-talk between NO and ethylene signaling. Interestingly, MAT is probably not the only target where NO can influence ethylene biosynthesis. Leshem *et al.* (43) showed that carnations (*Dianthus caryophyllus*) immersed in solutions containing low concentrations of different NO donors in the presence of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) significantly reduced endogenous ethylene output, suggesting that NO inhibits the ACC/ethylene step. Additionally, *S*-nitrosylation of *S*-adenosylhomocysteinase and cobalamin-independent methionine synthase could affect ethylene biosynthesis (28), because both enzymes are part of the methylmethionine cycle and activation/inactivation of these enzymes might also influence the AdoMet pool (Fig. 1). In sum, we have provided the first detailed molecular characterization of *S*-nitrosylation in plants. *S*-Nitrosylation of MAT1 but also of other enzymes of the methylmethionine cycle may have a regulatory function on ethylene biosynthesis.

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Differential Inhibition of *Arabidopsis* Methionine Adenosyltransferases by Protein *S*-Nitrosylation

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