AtNOS1 was previously identified as a potential nitric-oxide synthase (NOS) in *Arabidopsis thaliana*, despite lack of sequence similarity to animal NOSs. Although the dwarf and yellowish leaf phenotype of *Atnos1* knock-out mutant plants can be rescued by treatment with exogenous NO, doubts have recently been raised as to whether AtNOS1 is a true NOS. Moreover, depending on the type of physiological responses studied, *Atnos1* is not always deficient in NO induction and/or detection, as previously reported. Here, we present experimental evidence showing that AtNOS1 is unable to bind and oxidize arginine to NO. These results support the argument that AtNOS1 is not a NOS. We also show that the renamed NO-associated protein 1 (AtNOA1) is a member of the circularly permuted GTPase family (cGTPase). AtNOA1 specifically binds GTP and hydrolyzes it. Complementation experiments of *Atnoa1* mutant plants with different constructs of AtNOA1 show that GTP hydrolysis is necessary but not sufficient for the physiological function of AtNOA1. Mutant AtNOA1 lacking the C-terminal domain, although retaining GTPase activity, failed to complement *Atnoa1*, suggesting that this domain plays a crucial role in planta. cGTPases appear to be RNA-binding proteins, and the closest homolog of AtNOA1, the *Bacillus subtilis* YqeH, has been shown to participate in ribosome assembly and stability. We propose a similar function for AtNOA1 and discuss it in the light of its potential role in NO accumulation and plant development.

Numerous studies have demonstrated that plants, like animals, generate nitric oxide (NO) to regulate a wide range of physiological processes. NO is involved in plant development; it represses flowering (1), reduces seed dormancy (2), and regulates germination (3). NO production has also been detected following different environmental stresses. For example, NO regulates stomata closure in response to abiotic stress (4), and in response to biotic stress, NO participates in induction of plant defenses (5–7).

Although NO plays a role as significant in plants as it does in animals, NO synthesis *in planta* is still a matter of debate (8). Two major routes have been proposed for NO formation in plants. The first one relies on the reduction of nitrite to NO. Several studies demonstrate that nitrate reductase, whose primary function is to catalyze the reduction of nitrate to nitrite, can convert nitrite to NO with low efficiency (9, 10). Nitric oxide can also be reduced to NO by a plasma membrane-bound nitrite:NO reductase (11), by a mitochondrial electron transport-dependent reductase (12), or nonenzymatically in acidic, reducing environments (13). The second probable NO biosynthetic pathway uses arginine as a substrate, following a reaction similar to that observed for the well-characterized animal NOSs. Indeed, several lines of evidence suggest the existence of a mammalian NOS-like enzyme in plants. Application of arginine analogs, inhibitors of animal NOSs, results in a reduction of NO detected in plants (5, 6, 14–17). Arginine-dependent citrulline formation, a co-product of the NOS reaction, has also been observed in plant extracts (5, 6, 15).

Two potential plant NOSs have been reported thus far, but in both cases, further investigation failed to confirm NO biosynthesis activity. Data demonstrating NOS activity of a variant form of the P protein of the glycine decarboxylase complex (18) were found to be nonreproducible and unreliable and thus were retracted (19). Crawford and co-workers (20) identified the second potential NOS in *A. thaliana*. AtNOS1, based on homology to a hypothetical snail NOS or NOS partner that cross-reacted with mammalian NOS antibody (23% identity, 39.5% similarity, 30.1% gap between AtNOS1 and the snail protein using a local alignment) (20, 21). Interestingly, AtNOS1 T-DNA knock-out plants (*Atnos1*) have a growth phenotype that can be rescued by the application of NO donor compounds. Moreover, chemical probes sensitive to NO indicated reduced NO levels in *Atnos1* compared with wild type plants (20, 22–24). However, several groups, including our laboratory and Crawford’s, cannot reproduce the originally reported NOS activity with recombinant AtNOS1, calling into question the true function of this protein (25–27).

AtNOS1 is a S61-amino acid protein that has no sequence homology to the animal NOSs. It belongs to the circularly permuted GTPase (cGTPase) family (28). The central domain of AtNOS1-(176–350) contains guanine-binding motifs (G...
motifs) characteristic of small GTPases like Ras, Rho, and Cdc42 (29), but in an unusual arrangement; G4-G5 are N-terminal of G1-G2-G3. Among the four subfamilies of cGTPase represented by YlqF (in Bacillus subtilis), YjeQ (in Escherichia coli), and YawG (in Saccharomyces pombe), Yqeh (in B. subtilis) is the closest homolog of AtNOS1 (30% identity, 43.6% similarity, 15.5% gap) (Fig. 2A). Both proteins contain four conserved cys teines in the N-terminal region (zinc-binding domain (ZBD)) that can form a zinc finger motif CXXC of the treble clef family (30, 31). They also possess a very similar C-ter minal domain (CTD) of unknown function (40.8% similarity, 22.3% gap). AtNOS1 contains an additional 101 residues at the N terminus comprising a predicted mitochondria targeting sequence and a short stretch of basic lysine residues (KKKKK).

Little is known concerning the function of cGTPases in eukaryotes that could shed light on the possible role of AtNOS1 in plants, particularly in regard to NO accumulation. Bacterial cGTPases are essential for cell growth (32–34). In bacteria and in some eukaryotes, this family of GTP-binding proteins is associated with RNA/ribosome binding function (28, 35–37). For example, Yqeh has been shown to be essential for the viability of B. subtilis (33) and to participate in ribosome biogene sis and assembly (38, 39). Eukaryotic homologs are found in other plants, such as tomato (64.7% identity) and rice (Q6YPG5, 60.5% identity) as well as in mice (NP_062810) and humans (NP_115689). Mammalian homologs have less homology to AtNOS1 (22.4 and 23.2% identity and 35 and 34.5% similarity for the mouse and the human homologs, respectively). These pro teins also contain a mitochondrial targeting peptide at their N termini and, like AtNOS1, they seem to localize in this organelle (22, 40).

The function of AtNOS1 as an authentic NOS has been recently questioned. This led to the renaming of AtNOS1 as NO-associated protein 1 (AtNOA1) (25). Nevertheless, publications still refer to AtNOS/A1 as a potential NOS (41, 42).

Here, we examined the ability of AtNOS1 protein to bind and oxidize arginine into NO, using several independent assays. We demonstrate that AtNOS1 is not a NOS but a functional GTPase. We show that its GTPase activity is necessary but not sufficient for its function in planta. This new activity is discussed in the context of the defective NO accumulation phenotype of Atnos1.

**EXPERIMENTAL PROCEDURES**

GTP and NADPH were purchased from Roche Applied Science, and (6R)-5,6,7,8-tetrahydro-t-bioperin (BH$_4$) came from Sigma, and (6R)-5,6,7,8-tetrahydro-t-bioperin (BH$_4$) came from Sigma, unless otherwise indicated.

Proteins Expression and Purification—Plasmids used for neuronal NOS (nNOS) expression, pCWori—Plasmids used for neuronal NOS (nNOS) expression, pCWori—Plasmids used for neuronal NOS (nNOS) expression, pCWori containing the rat nNOS cDNA and pGroESL for chaperone protein expression, were kindly provided by Dr. M. A. Sari (CNRS UMR 8601, Université René Descartes, Paris, France). The expression and purification of nNOS protein was conducted in the presence of arginine and BH$_4$, as previously reported (43). The protein was buffer-exchanged using a Sephadex G25 column prior to use.

The AtNOA1 cDNA clone was obtained from Arabidopsis Biological Resource Center. The cDNA encoding AtNOA1 full-length, the N-terminal deletion of 101 amino acids (Δ101) and the T327A mutant, the N-terminal truncation containing only the circularly permuted G-motif domains (CPG domains) and the CTD (residue 351–561) and the C-terminal truncated proteins (residues 102–350) were all generated by PCR and cloned into pET28 expression vector (Novagen) as N-terminal His$_6$ tag fusions. Plasmids were transformed in E. coli BL21 (DE3) cells. These cells were grown in LB media at 37 °C until A$_{600}$nm = 0.6, and expression was induced by 100 μM isopropyl 1-thio-β-D-galactopyranoside. After 20 h at 18 °C, cells were collected and lysed in buffer A supplemented with 5 mM imid azole (buffer A: 25 mM HEPES, pH 7.5, 300 mM NaCl, 2 mM MgCl$_2$, 10% glycerol, 2 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride). Soluble protein supernatant was applied to an Ni$^{2+}$-nitrilotriacetic acid-agarose column (Qiagen), washed with buffer A containing 20 mM imidazole, and eluted with 300 mM imidazole. Size exclusion chromatography (Superdex 200; Amersham Biosciences) in buffer B supplemented with 2 mM MgCl$_2$ was performed to further purify the proteins (buffer B: 50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 10% glycerol, and 2 mM dithiothreitol). Fractions of interest were pooled and concentrated before storage at −80 °C until further use. Protein concentrations were evaluated using the Bradford reagent (Bio Rad) and bovine serum albumin as a standard.

**NO Formation**—Rates of NO synthesis were determined at room temperature on a Cary 50 spectrophotometer using the oxyhemoglobin assay for NO (44). A 150-μl reaction containing 20 μM oxyhemoglobin, 100 units/ml superoxide dismutase and catalase, 10 μM BH$_4$, 10 mM CaCl$_2$, 10 μg/ml calmodulin (CaM), 1 mM NADPH, and 100 μM arginine in 50 mM Hepes buffer (pH 7.5) and 5 mM dithiothreitol was prepared. The addition of protein (nNOS or AtNOA1) initiated the reaction. The NO-dependent conversion of oxyhemoglobin to methemoglobin was monitored by scanning every 0.5 min between 380 and 450 nm. An extinction coefficient of 77 mm$^{-1}$ cm$^{-1}$ between the peak at 401 nm and the valley at 420 nm was used to quantify NO. All other NOS assays were conducted as previously described (45).

Arginine Binding—Arginine-binding experiments were carried out with 100 nM [2,3,4-$^3$H]arginine (41 Ci/mmol; PerkinElmer Life Sciences) with or without unlabeled arginine (100,000-fold molar excess) in 120 μl of buffer B for 15 min on ice. Unbound ligand was removed with a 1-ml G-25 Superfine (Amersham Biosciences) column by centrifugation for 2 min at 1,000 × g. Bound [2,3,4-$^3$H]arginine was quantified by scintillation counting of 90 μl of filtrate.

Homology Modeling—Alignment of AtNOA1-(175–534) (containing CPG and CTD) and Yqeh-(59–369) was performed using the alignment program Tcoffee (46). A three-dimensional model for AtNOA1-(175–534) was generated by comparative protein modeling through satisfaction of spatial restraints with the program MODELLER (47) using the x-ray structure for gpYqeh-(59–369) as a template (48).

Complementation—Atnoa1 seeds were obtained from Dr. Nigel Crawford (University of California at San Diego). For constitutive expression of AtNOA1 or AtNOA1$_{H11001}$ in plants, the open

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AtNOS1/AtNOA1 Is a GTPase, Not a NO Synthase

AtNOS1/AtNOA1 is a GTPase, Not a NO Synthase—The AtNOS1 full-length (fl) and a deletion variant with the first 101 amino acids removed (Δ101) were expressed in E. coli and tested for their ability to generate NO from arginine. The N-terminal deletion increases the solubility and stability of the protein by removing a putative mitochondrial targeting sequence as well as additional residues not found in the bacterial homolog Yqeh (see Fig. 2A). Five different assays were used to assess the NO synthesis ability of AtNOS1, with the rat nNOS included in all assays as a positive control. First, the hemoglobin assay was used to follow the rapid conversion of oxyhemoglobin to methemoglobin by enzymatically generated NO. The reactions in the absence of protein were conducted to control for spontaneous hydrolysis. Reaction in the absence of protein were analyzed following the same procedure.

Rates of GTP hydrolysis were quantified by measuring \[^{32}\text{P}]\text{phosphate release (53). Reactions containing 1 mM [\gamma\text{-}^{32}\text{P}]\text{GTP (2 \mu Ci)} and varying amounts of cold GTP were prepared in 300 \mu L of buffer B supplemented with 5 mM MgCl}_2 and 200 mM KCl. The reaction was started by the addition of the protein. At various times, as indicated in the figures, 50-\mu L aliquots were mixed with 1 mL of activated charcoal (5% in 50 mM NaH}_2PO}_4). After a 1-min centrifugation, [\gamma-^{32}\text{P}]\text{phosphates in the supernatant were counted on a liquid scintillation counter. Counts/min were plotted as a function of the different GTP concentrations. Reactions in the absence of protein were conducted to control for spontaneous hydrolysis. Reaction in the absence of protein were analyzed following the same procedure.}

RESULTS

AtNOS1/AtNOA1 Is a GTPase, Not a NO Synthase—To demonstrate that Δ101 was indeed a functional GTPase, 20 or 40 \mu M protein was incubated with 500 \mu M GTP, 2 mM MgCl}_2, 200 mM KCl in buffer B at 37 °C overnight. Samples were boiled for 5 min to stop the reaction and precipitate the proteins and then were centrifuged for 5 min. The supernatant was analyzed by reverse phase HPLC on a Waters Sunfire C$_{18}$ 5 \mu M (4.5 \times 250-mm) column. Nucleotides were separated with an isocratic condition at 1 mL/min of 100 mM K$_2$PO$_4$, pH 6.5, 10 mM tetrabutylammonium bromide, 0.2 mM NaN$_3$, and 7.5% acetonitrile. Control reactions in the absence of protein were analyzed following the same procedure.

GTPase Activity—To demonstrate that Δ101 was indeed a functional GTPase, 20 or 40 \mu M protein was incubated with 500 \mu M GTP, 2 mM MgCl}_2, 200 mM KCl in buffer B at 37 °C overnight. Samples were boiled for 5 min to stop the reaction and precipitate the proteins and then were centrifuged for 5 min. The supernatant was analyzed by reverse phase HPLC on a Waters Sunfire C$_{18}$ 5 \mu M (4.5 \times 250-mm) column. Nucleotides were separated with an isocratic condition at 1 mL/min of 100 mM K$_2$PO$_4$, pH 6.5, 10 mM tetrabutylammonium bromide, 0.2 mM NaN$_3$, and 7.5% acetonitrile. Control reactions in the absence of protein were analyzed following the same procedure.

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AtNOS1 Is Not a Nitric-oxide Synthase—AtNOS1 Is Not a Nitric-oxide Synthase—The AtNOS1 full-length (fl) and a deletion variant with the first 101 amino acids removed (Δ101) were expressed in E. coli and tested for their ability to generate NO from arginine. The N-terminal deletion increases the solubility and stability of the protein by removing a putative mitochondrial targeting sequence as well as additional residues not found in the bacterial homolog Yqeh (see Fig. 2A). Five different assays were used to assess the NO synthesis ability of AtNOS1, with the rat nNOS included in all assays as a positive control. First, the hemoglobin assay was used to follow the rapid conversion of oxyhemoglobin to methemoglobin by enzymatically generated NO. The reactions in the absence of protein were conducted to control for spontaneous hydrolysis. Reaction in the absence of protein were analyzed following the same procedure. AtNOS1 Is Not a Nitric-oxide Synthase—AtNOS1 Is Not a Nitric-oxide Synthase—The AtNOS1 full-length (fl) and a deletion variant with the first 101 amino acids removed (Δ101) were expressed in E. coli and tested for their ability to generate NO from arginine. The N-terminal deletion increases the solubility and stability of the protein by removing a putative mitochondrial targeting sequence as well as additional residues not found in the bacterial homolog Yqeh (see Fig. 2A). Five different assays were used to assess the NO synthesis ability of AtNOS1, with the rat nNOS included in all assays as a positive control. First, the hemoglobin assay was used to follow the rapid conversion of oxyhemoglobin to methemoglobin by enzymatically generated NO. The reactions in the absence of protein were conducted to control for spontaneous hydrolysis. Reaction in the absence of protein were analyzed following the same procedure.

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AtNOS1/AtNOA1 Is a GTPase, Not a NO Synthase

Structure-Function Analyses Reveal the Importance of the CPG and CTD Domains—Taking advantage of the close sequence similarity between Δ101 and YqeH (Fig. 2A), a model for the three-dimensional structure of Δ101 was built based on the x-ray crystal structure of the Geobacillus stearothermophilus YqeH (gsYqeH) (Fig. 2B). The model suggests that, like gsYqeH, the CPG domain of AtNOA1 displays a fold similar to that of canonical GTPases. In the GDP-bound form the nucleotide is probably exposed to the solvent. As observed with other small GTPases like Ras (29), the aspartate residue in the G4 motif (Asp-226; Fig. 2A) is positioned favorably to stabilize the guanine ring moiety. Introduction of fl wild type AtNOA1 into Attnoa1 mutant plants restored the wild type phenotype, including normal plant size and green coloration of leaves, as shown in Figs. 3, A and B (20, 48). In contrast, expression of a mutant AtNOA1D226N in Attnoa1 mutant plants failed to restore normal growth or leaf coloration to Attnoa1 (Figs. 3, A and B). This suggests that the disruption of GTP/GDP binding leads to loss of function of AtNOA1 in planta, thus highlighting the essential role of CPG domain in AtNOA1 physiological function.

Based on structural analysis of gsYqeH and other GTPases (48), GTP binding may trigger a conformational change in the connection between the CPG domain and the CTD. Repositioning of the CTD may modulate the potential GTPase activity of AtNOA1 or vice versa. In both cases, the spatial arrangement of the CTD in relation to the CPG domain suggests a possible important role of CTD in AtNOA1 function. The structure of AtNOA1 CTD is predicted to be very similar to that of gsYqeH. Insertions I2–I4 present in AtNOA1 are located in loops and β-turns (Fig. 2, A and B). Therefore, they are unlikely to change the overall fold of this domain from that found in gsYqeH. The arrangement of the CTD is interesting, since it displays a novel topology involving two pseudosymmetric β-sheet units. Structural similarity has been found between each of these subunits and the RNA-binding protein TRAP (Trp RNA-binding attenuating protein) (48). To assess the importance of this domain in planta, we conducted complementation experiments to evaluate if the CTD truncated AtNOA1 was able to function like fl AtNOA1 in plants. In contrast to what was observed with the introduction of the fl AtNOA1, introduction into Attnoa1 of AtNOA1-(1–386), which contains both the N-terminal zinc and the RNA-binding protein TRAP (Trp RNA-binding attenuating protein) (48). This suggests that CTD is necessary for AtNOA1 function in planta.

Attnoa1 and Δ101 Phenotypes—Attnoa1 and Δ101 plants failed to restore normal growth or leaf coloration to Attnoa1 (Figs. 3, A and B). This suggests that the disruption of GTP/GDP binding leads to loss of function of AtNOA1 in planta, thus highlighting the essential role of CPG domain in AtNOA1 physiological function.

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Attnoa1 Binds GDP More Tightly than GTP—The Attnoa1 CPG domain displays a three-dimensional arrangement very similar to canonical GTPases, suggesting that Attnoa1 might be a functional GTPase. To assess this possibility, we first determined the ability of Attnoa1 to bind GDP and/or GTP using the fluorescent MantGDP (54). The addition of Δ101 to a solution of MantGDP led to a rapid increase in fluorescence, indi-
cating the binding of MantGDP to the protein (Fig. 4A, 1). The further addition of 50 μM ATP or CTP did not change the fluorescence, suggesting that neither ATP nor CTP were able to compete with MantGDP for binding (Fig. 4A, 2 and 3). Even higher concentrations of ATP or CTP (up to 500 μM) did not lead to any significant change in fluorescence (Fig. S1). This rules out the possibility of a low affinity binding of those nucleotide to Δ101. However, the addition of 50 μM GTP decreased MantGDP fluorescence by over 50%. This result argues that binding of MantGDP to Δ101 is reversible and specific. Both GTP and GDP were able to displace MantGDP; together these data indicate that AtNOA1 specifically binds the guanidine nucleotide. Magnesium salt regulates nucleotide release in small GTPases (55); however, its presence did not modify the observed GTP/GDP binding of AtNOA1. Moreover, it did not modify the high affinity of MantGDP for Δ101 (~560 nM; Table 2). A competition assay between MantGDP and either GDP or GTP was used to quantify GTP and GDP binding to AtNOA1 (Fig. 4B). Increasing amounts of GTP or GDP were added to the preformed MantGDP-Δ101 complex, and nucleotide exchange was monitored by following the decrease in fluorescence. The resulting IC₅₀ for GTP and GDP is a measure of their relative binding affinities for Δ101. GDP consistently bound more tightly than GTP to Δ101 with an approximately 3-fold difference in IC₅₀ that was little affected by the presence of MgCl₂ and KCl (Table 2).

AtNOA1 Is a Slow GTPase, Whose Activity Is Independent of Its ZBD and CTD—The ability of AtNOA1 to hydrolyze GTP was assessed by HPLC analysis of the reaction products of Δ101 with 500 μM GTP. After incubation at 37 °C overnight, in the absence of Δ101, very little GDP was produced with the majority of the guanine remaining as GTP (Fig. 5A, bottom). In the presence of Δ101, GTP was converted to GDP in a dose-dependent manner, with the majority of it hydrolyzed to GDP with the higher amount of Δ101 (Fig. 5A, top). The GTPase activity of Δ101 was quantified using an activated charcoal pull-down assay with radioactive [γ-³²P]GTP (Fig. 5B). In this assay, Δ101 was incubated at 37 °C with [γ-³²P]GTP and varying amounts of unlabeled GTP. The initial velocity of GTP hydrolysis was calculated from the counts/min data and used to determine the Kₘ and Vₘₐₓ values of the protein (64.5 ± 5.7 μM and 0.072 ± 0.01 min⁻¹, respectively). The GTPase activity required MgCl₂ (Fig. 6). The presence of either KCl or (NH₄)₂SO₄ was needed for the activity as well (data not shown). The conserved threonine residue of the G2 motif, within the Switch I region, is usually involved in coordination of Mg²⁺ via its side chain hydroxyl and is in contact with the γ phosphate of GTP via its main chain
NH. Mutation of this threonine residue to alanine has been shown to result in loss of GTPase activity of other GTPases (48, 56). Mutation of the corresponding threonine in H9004 (T327A) similarly abolished its GTP hydrolysis activity (Fig. 6). This result demonstrates that, although H9004 hydrolysis activity is low, this protein is an authentic GTPase. It also reveals the crucial role of the Switch I region for the GTPase activity of AtNOA1. Different constructs of AtNOA1 were also tested to determine whether the ZBD or CTD of AtNOA1 might alter its GTPase activity (Fig. 6). Removal of the ZBD or CTD did not significantly change its GTPase activity. AtNOA1-(102–350) and AtNOA1-(176–561) hydrolyzed GTP with activities corresponding to 80% and 130% of the GTPase activity of H9004, respectively.

FIGURE 3. Complementation of Atnoa1 with mutants of AtNOA1. A (from left to right), 2-week-old seedlings of wild type Col-0, Atnoa1, and Atnoa1 transformed with either AtNOA1D226N or AtNOA1fl. Seeds were planted on Murashige-Skoog medium, incubated at 4 °C for 3 days, and then transferred to 22 °C for germination and further growth. The size bars correspond to 3 mm. The arrowheads point to the emerging rosette leaves. B, Western blot of leaf extracts using anti-FLAG antibody to assess the expression of FLAG-tagged AtNOA1D226N and AtNOA1 in Atnoa1 plants. C shows that the C-terminal truncated AtNOA1 fails to complement Atnoa1 mutant plants and shows (from left to right) wild type, Atnoa1, and Atnoa1 transformed with Atnoa1-(1–386) (white bars, correspond to 2 cm). Seed germination was synchronized by cold treatment, and photographs were taken 4 weeks after germination. D, Western blot of leaf extracts of wild type, Atnoa1, and Atnoa1 plants expressing HA-tagged AtNOA1-(1–386) using anti-HA antibody. In both B and D, the Rubisco protein band stained with Coomassie Blue shows comparable loading of protein extracts.
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pmol of NO/min/mg of protein at room temperature as measured with the oxyhemoglobin assay and as previously published (43). The NOS activity reported for the recombinant AtNOS1 was ~30 pmol of NO/min/mg of protein (20), which is in the same range and should have been detected by this assay. Indeed, this level of activity should have resulted in a detectable optical difference of 0.01 between 401 and 420 nm in our experiments (with 155 μg of protein in a 150-μl reaction for 5 min). This was not observed even at later time points and higher protein concentrations. A third assay, the very sensitive detection of radiolabeled citrulline from [3H]arginine, also failed to detect NOS activity of AtNOS1. In addition, AtNOS1 did not have electron transfer and arginine binding (Fig. 1B) activities, two properties of NOS-like enzymes. Moreover, the structure of the bacterial homolog gsYqeH and the modeling of AtNOS1 do not reveal any fold that might account for a NOS function or the binding of necessary cofactors. In summary, our data show that AtNOS1 does not possess any of the expected characteristics of a NOS or NOS-like enzyme (Table 1). These findings are consistent with the recent communications questioning the NOS activity of AtNOS1 (25, 27) and the renaming of AtNOS1 as AtNOA1.

Characterization of AtNOA1, a Plant cGTPase—The amino acid sequence of AtNOA1 reveals the presence of a circularly permuted GTP-binding domain (57). Unlike the classical small GTPases like Rho, Ras, and Ran that have been extensively studied, little is known about the cGTPase family and its GTPase activity, especially in eukaryotes. Moreover, the catalytic glutamine or histamine found in the G3 motif of small GTPases, which maintains the water molecule in an orientation necessary for hydrolysis, is replaced with a hydrophobic residue in AtNOA1 and its homologs (Val-349 in AtNOA1; see Fig. 2A). Although such a mutation in Ras disrupts GTP hydrolysis, many HAS GTPases (hydrophobic amino acid substituted for catalytic glutamine residue GTPases) retain their GTPase activity (58).

We demonstrated by HPLC analysis that AtNOA1 is able to hydrolyze GTP to GDP and showed the requirement for both MgCl2 and a monovalent salt, such as KCl or (NH4)2SO4 (Fig. 4A; data not shown for (NH4)2SO4 effect). The GTPase activity of AtNOA1 was higher and more reproducible in the presence of KCl than in the presence of (NH4)2SO4. This stimulating effect of potassium ions on GTP hydrolysis has been observed in another HAS GTPase, MnnE (59). In that case, the positive charge of the potassium ion stabilizes the transition state in MnnE. Further study of the AtNOA1 GTPase mechanism of catalysis is required to determine if this is also the case for AtNOA1. Examination of GTP binding by AtNOA1 revealed additional interesting characteristic of this cGTPase. Although magnesium plays an inhibitory role in guanine nucleotide exchange in small GTPases (55, 60), the presence of MgCl2 did not alter AtNOA1 affinities for GTP, GDP, or a fluorescent GDP analog (Table 2), as has been reported for the Rho GTPases (61). This characteristic might explain why magnesium ions were not detected in the crystal structure of the bacterial homolog gsYqeH and the other cGTPase YjeQ (48, 62). Nonetheless, magnesium ions are essential for GTPase activity and thus may be bound only transiently to assist catalysis. Sec-

| Buffer B with 200 mM KCl, 5 mM MgCl2 | Kd (MantGDP) | μα | μα |
|---|---|---|---|
| MantGDP fluorescence change (in arbitrary units (μM)) | 546 ± 98 | 5.2 ± 0.9 | 18.9 ± 2.5 |
| MantGDP fluorescence change (in arbitrary units (μM)) | 577 ± 237 | 6.0 ± 1.8 | 15.4 ± 1.8 |

**DISCUSSION**

From AtNOS1 to AtNOA1—AtNOS1 was reported in 2003 to have NOS activity (20). However, using a range of assays with varying sensitivities to monitor different properties of NOSs, our results indicate that this protein does not have NOS-like activities (Table 1). The NO formation activity of AtNOS1 was reported to be regulated by Ca2+-CaM binding (20); thus, the rat nNOS isoform was chosen as a positive control in our experiments, since its activity is also regulated by Ca2+-CaM binding (43). Reactions containing AtNOS1 or Δ101, arginine, and NADPH, as an electron donor, failed to produce NO (oxyhemoglobin assay; Fig. 1A) or NO-derived nitrite (Griess assay) regardless of the presence of CaM, CaCl2, BH4, and flavines like FMN or FAD. Under the same conditions, nNOS produced 147
ond, neither ATP nor CTP could compete with the fluorescent GDP analog, even when present at high concentrations (2500-fold molar excess, Figs. 4A and S1). Thus, AtNOA1 appears to specifically bind the guanosine nucleotide, a characteristic shared with the bacterial cGTPases YqeH, YlqF, and YloQ (33, 63). Third, the higher affinity of AtNOA1 for GDP than for GTP (Fig. 4B and Table 2) might explain its slow steady-state GTP hydrolysis rate. Indeed, its $V_{\text{max}}$ of 0.07 $\mu$mol/min/100 μg protein falls within the range of nonactivated Ras (0.028 min$^{-1}$) or EF-Tu (0.036 min$^{-1}$) GTPase activity (64, 65). It is also comparable with nonactivated cGTPase YjeQ (0.15 min$^{-1}$) of E. coli (66, 67) and YloQ of B. subtilis (0.22 min$^{-1}$) (63) but unexpectedly lower than YqeH high intrinsic GTPase activity (0.93 min$^{-1}$) (39).

Small GTPases like Ras or Rho interact with a guanine nucleotide exchange factor or GTPase-activating protein, which leads to a 102- to 103-fold increase of the rate of GTP hydrolysis (68). Interestingly, for several bacterial cGTPases, interaction with ribosome subunits modulates their GTPase activity; YjeQ GTPase activity in vitro was enhanced 160-fold in the presence of the 30S subunit (67, 69), whereas the 50S subunit stimulates the activity of YlqF (35). The slow steady-state GTP hydrolysis rate of AtNOA1 suggests that it may require a guanine nucleotide exchange factor or a GTPase-activating protein to reach a physiologically relevant GTPase activity. It remains to be determined whether ribosome and/or RNA binding enhances AtNOA1 GTPase activity.

Despite the circular permutation of the G domain, the GTPase domain folding in YloQ and YjeQ, as well as in YqeH and the model of AtNOA1, is similar to the one observed with classical small GTPases (48, 62, 70). However, the rearrangement of the G subdomains leads to repositioning at the C-terminal end of the G3 region (Switch II; Fig. 2A). This region contains the traditional catalytic glutamine residue and is therefore essential for catalysis. In all members of the cGTPase family, a C-terminal domain connects directly to G3 due to the sequence permutation of the G protein homology regions (28). This suggests that the CTD may influence the GTPase activity or vice versa (i.e. GTP hydrolysis may modulate CTD function). In our study, removal of either the ZBD or the CTD did not significantly change the GTPase activity of AtNOA1. This is particularly interesting considering that not only the GTPase activity...
domain but also the CTD is important for the function of AtNOA1 in planta (Fig. 3). As G proteins cycle between an inactive GDP-bound and an active GTP-bound state, they undergo conformational changes that allow for interaction with effectors. We suspect that the CTD of AtNOA1 has a critical function in planta that is modulated by GTP hydrolysis.

What Function of AtNOA1 Could Account for the Impaired NO Accumulation in Atnoa1 Mutant Plants?—We suspect that AtNOA1 binds ribosomes and consequently plays a role in their proper assembly and/or stability, which leads to appropriate levels of protein synthesis. Although Zeomjojel et al. (27) previously speculated that AtNOA1 is involved in mitochondrial ribosome biogenesis and/or translation, our suspicion is based on several observations. First, AtNOA1 belongs to the Era/Obg subfamily of small GTPases that have been predicted and/or shown to be associated with ribosomes (36, 69). Studies show that bacterial YlfF participates in the final steps of 50 S ribosomal subunit assembly (35, 37), evidence points toward a role of YjeQ in 30 S ribosome biogenesis and subunit association (69, 71), and preliminary results indicate that YloQ activity is enhanced by purified E. coli ribosomes (63). Second, in eukaryotes, the yeast cGTPase Nug1 associates with 60 S preribosomal particles (72). Third and of particular relevance is the finding that YqeH, the closest bacterial homolog of AtNOA1, is involved in 30 S subunit biogenesis in B. subtilis (38, 39). Since YqeH complements the Atnoa1 mutant (48, 73), it is very likely that AtNOA1 plays a similar role in plants as YqeH does in bacteria. The ZBD, CTD, or both may play such a ribosome/RNA-binding role. Indeed, zinc finger motifs have nucleic acid-binding properties. The treble clef motif found in AtNOA1 is associated with many types of activities, from binding nucleic acids, proteins, or small molecules to phophodiestere bond hydrolysis. Interestingly, the ribosomal proteins L24E and S14 contain such a domain (30). Also, the unique structure of the C-terminal domain of gsYqeH is found in the predicted structure of the modeled AtNOA1 and is similar to the TRAP protein, which has the ability to bind RNA (48). In particular, two triads of residues involved in RNA binding in the TRAP protein (74) are well conserved in AtNOA1 and its homologs (e.g. Atp83, Trp-491, and Arg-530 and Phe-401, Arg-407, and Asp-409 in AtNOA1). The x-ray structure of gsYqeH and the predicted structure of the modeled AtNOA1 suggest that these residues are exposed and match the spatial arrangement observed in the RNA-binding site of TRAP (48). Whether the CTD binds RNA alone or in association with the N-terminal domain remains to be demonstrated, but the essential role of the CTD in AtNOA1 function (Fig. 3, C and D) suggests that it may fulfill the important role of RNA/ribosome binding.

Based on its sequence, AtNOA1 is predicted to be targeted to either mitochondria (score of 77.9% on TargetP, 6.5 on Psort) or chloroplasts (6.5 on Psort). According to the same localization programs, both the tomato (64.7% identity) and rice (60.5%) homologs are predicted to be imported into chloroplasts. Moreover, in contrast to evidence for mitochondrial localization of AtNOA1 in Arabidopsis roots (22), a recent publication has shown that AtNOA1 co-localized with chloroplasts in leaves and is imported into isolated leaf chloroplasts (73). Regardless of whether AtNOA1 is in mitochondria and/or chloroplasts, these are both sites of electron transfer that can lead to reactive oxygen species (ROS) production (75). We propose that the defective ribosome/RNA assembly in Atnoa1 leads to increased production of ROS, such as superoxide ion (O$_2^-$), hydroxyl radical (OH$^-$), and hydrogen peroxide (H$_2$O$_2$) in either or both of these organelles. Consistent with this proposition, Atnoa1 mutant plants exhibit a constitutively elevated level of ROS and oxidized lipids and proteins (22, 76).

We propose that the elevated amount of ROS observed in the Atnoa1 mutant is responsible for the reduced NO accumulation, since NO can react very quickly with O$_2^-$ and lipid radicals (77) and thus reduce the amount of detectable NO. Indeed, peroxynitrite generated by the rapid reaction between NO and O$_2^-$ is unable to activate the extensively used fluorescent probe diaminofluorescein (78). Moreover, although there is an abundant literature documenting the lower accumulation and/or detection of NO in Atnoa1 (20, 22, 24, 79, 80), there are also reports showing that Atnoa1 is not always impaired in NO accumulation. For example, in response to H$_2$O$_2$, to iron, to indole 3-butyric acid, to Verticillium dahliae toxins, or to zeatin, NO production is as high in Atnoa1 as in wild type plants (23, 41, 42, 81, 82). The presence of a nitrite-dependent NO production pathway in Atnoa1 might account for those conflicting observations. Consideration of subcellular localization of ROS and NO may be another way to reconcile these differences. If both reactive species are produced in the same subcellular location, NO detection may be inefficient due to its rapid interaction with the elevated levels of ROS in Atnoa1; in contrast, elevated ROS levels in Atnoa1 in a subcellular localization different from that of NO production may not affect the detected NO levels. Thus, it is likely that the association of AtNOA1 with NO is the result of the pleiotropic effects of malfunctioning organelles that overproduce ROS, which can rapidly react with NO, thereby reducing the amount of NO free to react in the various NO detection assays. A very recent study shows that a mutant of AtNOA1 (also called rif1 for resistant to inhibition by ESM) has reduced chloroplastic protein synthesis and elevated expression of methylerythritol phosphate (MEP) pathway enzymes (73). Although exogenous application of NO partially rescued the pale yellowish leaf phenotype of Atnoa1/ rif1 mutant plants, the other physiological traits associated with Atnoa1/rif1 were not rescued (e.g. chloroplastic protein synthesis, MEP pathway regulation). The rescue of the morphological phenotype of Atnoa1 by application of SNP was the central argument in favor of a direct relation between AtNOA1 and NO production (20). The recent result with rif1 reinforces the possibility that the connection between NO and AtNOA1 is indirect. The addition of exogenous NO might rescue the pale phenotype via its antioxidant property that counteracts the high ROS environment of Atnoa1. However, this antioxidant effect is not sufficient to rescue the loss of function of AtNOA1 associated with deregulation of the MEP pathway enzymes and protein synthesis in chloroplasts (73).

In conclusion, our study provides strong evidence that AtNOA1 is not an NOS, but a cGTPase, whose enzyme activity is necessary but not sufficient for its function in planta. Characterization of this new class of plant GTPases demonstrates the importance of a previously uncharacterized protein
domain, the CTD. Additional studies are needed to elucidate the role of AtNOA1 in plants, in particular its possible function in mitochondrial and/or chloroplastic ribosome biogenesis and maintenance.

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