THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 8, Issue of February 21, pp. 6490–6494, 2003
Printed in U.S.A.

Identification of a Novel Protein with Guanylyl Cyclase Activity in *Arabidopsis thaliana**

Received for publication, October 28, 2002, and in revised form, December 1, 2002
Published, JBC Papers in Press, December 12, 2002, DOI 10.1074/jbc.M210983200

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Guanylyl cyclases (GCs) catalyze the formation of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) from guanosine 5′-triphosphate (GTP). While many cGMP-mediated processes in plants have been reported, no plant molecule with GC activity has been identified. When the *Arabidopsis thaliana* genome is queried with GC sequences from cyanobacteria, lower and higher eukaryotes no unassigned proteins with significant similarity are found. However, a motif search of the *A. thaliana* genome based on conserved and functionally assigned amino acids in the catalytic center of annotated GCs returns one candidate that also contains the adjacent glycine-rich domain typical for GCs. In this molecule, termed AtGC1, the catalytic domain is in the N-terminal part. AtGC1 contains the arginine or lysine that participates in hydrogen bonding with guanine and the cysteine that confers substrate specificity for GTP. When AtGC1 is expressed in *Escherichia coli*, cell extracts yield >2.5 times more cGMP than control extracts and this increase is not nitric oxide dependent. Furthermore, purified recombinant AtGC1 has Mg^{2+}-dependent GC activity *in vitro* and >3 times less adenylyl cyclase activity when assayed with ATP as substrate in the absence of GTP. Catalytic activity *in vitro* proves that AtGC1 can function either as a monomer or homo-oligomer. AtGC1 is thus not only the first functional plant GC but also, due to its unusual domain organization, a member of a new class of GCs.

Guanylyl cyclases (GCs)[1] (EC 4.6.1.2) catalyze the formation of guanosine 3′,5′-cyclic monophosphate (cGMP) from guanosine 5′-triphosphate (GTP). cGMP acts as second messenger in many prokaryotes and all eukaryotes (1) including plants. In higher plants cGMP-mediated processes control phytochrome-dependent gene expression required for chloroplast development and anthocyanin biosynthesis (2–4). The light down-regulated gene asparagine synthetase has been reported to be controlled by a Ca^{2+}/cGMP-dependent pathway that activates other light responses and complementary loss- and gain-of-function experiments have identified a 17 base pair *cis*-element within the asparagine synthetase promoter that is both necessary and sufficient for this regulation (5). This *cis*-element may well be the target for a conserved phytochrome-generated repressor whose activity is regulated by calcium and cGMP (5).

In plants as well as in animals, nitric oxide (NO) is operating as a redox-active signaling molecule, and NO donors have been shown to induce expression of some defense-related genes, and tobacco mosaic virus-dependent increases of NO synthase activity occur in resistant plants only (6). NO does elevate cGMP levels in plants, and NO-induced expression of some defense-related genes was found to be mediated by the second messengers cGMP and cyclic ADP-ribose, both of which also operate in animal responses to NO (6).

Cellular cGMP levels are also increased transiently after application of the plant hormone gibberellic acid (GA) in barley aleurone layers and GC inhibition prevents the GA-induced increase in cGMP and inhibits GA-induced α-amylase synthesis and secretion (7). Both processes can be restored by exogenous application of membrane-permeant analogs of cGMP thus establishing cGMP as second messenger critical for α-amylase synthesis and/or secretion.

The regulation of ion transport is also in parts dependent on cyclic nucleotides (8, 9). Such regulation can occur in plant voltage-gated K^+ channels where binding of cGMP modulates the voltage/current relationship (8). Plants also contain cyclic nucleotide-gated low affinity cation channels where binding of cAMP and cGMP to the intracellular portion leads to direct gating (10). Recently, voltage-independent channels without selectivity for particular monovalent cations have been characterized in *Arabidopsis thaliana*. Voltage-independent channels showed no selectivity among monovalent cations, and their gating was found to be voltage-independent, while micromolar concentrations of cAMP or cGMP at the cytoplasmic side of the plasma membrane caused rapid decreases in channel open probability (9). It was shown that short term unidirectional Na^+ influx is reduced in the presence of cyclic nucleotides and that membrane-permeable cyclic nucleotide can improve salinity tolerance presumably by reducing net Na^+ uptake.

Cell-permeable cGMP and cAMP analogs elicit elevation of cytosolic Ca^{2+} in tobacco protoplasts and cause a physiological swelling response in plant protoplasts (11). Opening of the stomatal pore, which results from a swelling of the two neighboring guard cells, has been observed in response to cell-permeable cGMP analogs and is suppressed by guanylyl cyclase inhibitors (12). Like protoplast swelling, stomatal aperture regulation is likely to be tuned by Ca^{2+} and cGMP cross-talk (13).

Since significant and transient increases in intracellular cGMP levels, *e.g.* in response to the plant hormones GA (7) and cytokinins (13) as well as vertebrate atrial natriuretic peptides and immunoreactant plant natriuretic peptides (13–15), have been reported, it is reasonable to presuppose GC activity in

* This work was supported by the National Research Foundation of South Africa. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AAM51559.

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[1] The abbreviations used are: GC, guanylyl cyclase; NO, nitric oxide; GA, gibberellic acid; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside.

plants. However, no plant molecule with GC activity has been identified to date, and this failure could be explained by an unusually high level of divergence in plant GCs that has put them outside the detection limit of "Blast" searches or biochemical tools such as specific antibodies against GCs from *e.g.* bacteria or animals. Alternatively, plant GCs may not be homologous to currently annotated GCs and thus not easily identified. In both cases, however, we hypothesized that plant GCs may contain a significant degree of similarity to the catalytic center from previously identified nucleotide cyclases and GCs in particular. Consequently, we aligned designated catalytic domains (16–22) from vertebrates, lower eukaryotes, and prokaryotes with a view to deduce a GC catalytic domain search motif. Such a motif would then be used to do pattern searches of the complete *A. thaliana* genomic sequence to identify candidate proteins for functional testing.

EXPERIMENTAL PROCEDURES

Sequence Analyses—GCs were retrieved from NCBI, and their catalytic domains were used for Blast (23) queries of "The Arabidopsis Information Resource" database and GenBank™. The catalytic domains were aligned using Clustal X (24), and the alignment at the catalytic center of the catalytic domain was used to derive the search motif. The derived search motif was tested for accurate and specific detection of nucleotide cyclases by querying the Protein Information Resource (www-nbrf.georgetown.edu) using the Pattern Match option on the PIR-NREF link. The search motif was used to query The Arabidopsis Information Resource database using the Patmatch link in The Arabidopsis Information Resource.

Cloning and Protein Expression—Total RNA was isolated from 3-week-old seedlings using the RNeasy plant mini kit (Qiagen GmbH, Hilden, Germany) in combination with DNase treatment using RNase-free DNase Set (Qiagen GmbH) according to the manufacturer's instructions. First strand AtGC1 cDNA was synthesized from total RNA with 1 μM Primer GC1fwd (5′-CAC TGT GGA TCC ATG TGG CCT CTT TGT TTT CTG-3′) incorporating a 5′ *Bam*HI restriction site (underlined), 1 μM Primer GC1rev (5′-CTG ACT CTC GAG CTA ATA TCC GTT CTG GTT CC-3′) incorporating a 5′ *Xho*I restriction site (underlined) using reverse transcriptase (Promega Corp.). Double-stranded AtGC1 cDNA synthesis was done by PCR on first strand cDNA from above with 0.4 μM Primer GC1fwd and 0.4 μM Primer GC1rev using the expand high fidelity PCR system kit (Roche Diagnostics South Africa Pty. Ltd.) as instructed by the manufacturer, except that the deoxynucleotide triphosphate concentration was changed to 100 μM for each deoxynucleotide triphosphate. The *AtGC1* cDNA was cloned as a *Bam*HI/*Xho*I fragment into pBluescript SK(+/−) (Stratagene) to construct pBS:AtGC1 and sequenced. The *AtGC1* cDNA was subcloned from the pBS:AtGC1 construct into the *Bam*HI/*Xho*I sites of the glutathione *S*-transferase (GST) fusion expression vector pGEX-6P-2 (Amersham Biosciences UK Ltd.) to make the GST:AtGC1 fusion expression construct pGEX:AtGC1. *E. coli* BL21 (DE3) pLysS cells (Invitrogen Ltd., Paisley, UK) were transformed with pGEX:AtGC1 for the expression of the recombinant protein and with pGEX-6P-2 (Amersham Biosciences UK Ltd.) for a positive control experiment. *E. coli* BL21 (DE3) pLysS cells were grown at 30 °C to an OD_{600} of 0.8. The cells were then induced for expression of GST:AtGC1 or GST by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega Corp.) to a final concentration of 0.6 mM and growth at 30 °C for 60 min, with 3-isobutyl-1-methylxanthine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) added to a final concentration of 1 mM to inhibit resident bacterial phosphodiesterases. To determine NO dependence of AtGC1, the NO donor sodium nitroprusside was added to a final concentration of 1 mM to both uninduced and induced cells 10 min before harvesting of the cells. Cells were harvested by centrifugation at 4 °C at 10,000 rpm for 10 min, then resuspended in ice-cold phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Total *E. coli* proteins were extracted by three freeze-thaw cycles in PBS, with freezing done in liquid nitrogen and thawing done at 42 °C. Purification of both recombinant GST:AtGC1 and GST was performed on a glutathione-Sepharose 4B affinity column (Amersham Biosciences UK Limited) and eluted in glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) according to the protocol supplied by the manufacturer. The eluted proteins were desalted and concentrated using Amicon® Ultra 10,000 MWCO 15-ml centrifugal filter devices (Millipore Corp., MA).

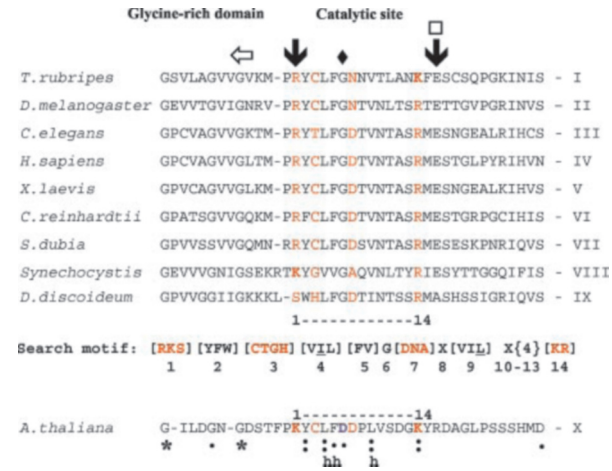

FIG. 1. **Alignment of GC catalytic domains.** Edited Clustal X alignment of catalytic domains of guanylyl cyclases is shown. The catalytic center is delineated by *two solid arrows*, and the glycine-rich motif is delineated by an *open arrow*. The deduced 14-amino acid-long search motif is in *bold*, and substitutions are in *square brackets*; *X* represents any amino acid, and *curly brackets* define the number of amino acids. *Red* amino acids are functionally assigned residues of the catalytic center, the *blue* amino acid is the replacement of the conserved glycine (♦), and the *underlined* amino acids in positions 4 and 9 are the third branched aliphatic amino acid not appearing in the alignment. The glutamic acid (*E*) implied in Mg^{2+} respectively Mn^{2+} binding (□) is not included in the search motif. *Asterisks* mark conserved residues, *colons* are conservative, and *periods* are semiconservative substitutions, and the letter *h* stands for hydrophobic residues forming the hydrophobic pocket. Accession numbers of aligned sequences are as follows: I, BAB60905; II, NP_524603; III, NP_494995; IV, NP_000171; V, BAA83786; VI (expressed sequence tag), BI717053; VII (expressed sequence tag), AL132834; VIII, NP_440289; IX, CAB42641; X, AY118140.

Cyclic Nucleotide Assays—cGMP levels were measured by radioimmunoassay using the cGMP (^{125}I) assay system kit (Amersham Biosciences UK Ltd.) as described in the supplier's manual for the acetylation protocol. Purified GST:AtGC1 recombinant fusion protein (140 μg) was used to determine AtGC1 guanylyl cyclase activity *in vitro* (21). This was followed by measurements of cGMP produced from the reaction using the cGMP (^{125}I) assay system kit. Cyclic AMP generated *in vitro* by GST:AtGC1 was determined using the cAMP Biotrak enzyme immunoassay (Amersham Biosciences UK Ltd.).

RESULTS

Sequence alignment of representatives of annotated catalytic domains of GCs from prokaryotes and eukaryotes are shown in Fig. 1. The residues implicated in catalysis (18, 19) are indicated in *red*, and a 14-amino acid-long search motif spanning the catalytic center was deduced (Fig. 1). An isoleucine (Ile) and leucine (Leu) was added to positions 4 and 9, respectively, to include all three aliphatic amino acids with non-polar side chains; this is in keeping with requirements of these positions, since they are part of the hydrophobic pocket where the purine moiety binds (18). The glutamic acid (Glu) implied in Mg^{2+} binding (18) that is conserved in the present alignment (Fig. 1) was not included in the search motif, since it is not conserved in all GCs (19). The GC catalytic domain alignments also revealed that GCs contain a glycine-rich domain N-terminal of the catalytic center (Fig. 1), and this is the case in all currently annotated GCs (not shown). This glycine-rich domain can be expressed as (G-{*X*}3,4-G-*X*{2,3}-G) and will subsequently be referred to as glycine-rich motif and used as secondary search parameter.

A pattern search of PIR with the catalytic center motif identifies 263 protein sequences of which 208 are annotated GCs and the rest are either hypothetical proteins or annotated proteins with different functions. A pattern search of the *A. thaliana* genome data base with no substitution allowed re-

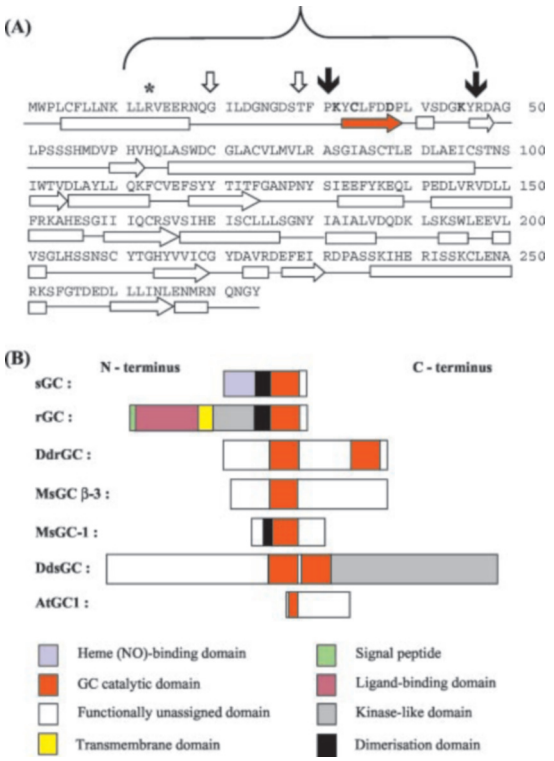

FIG. 2. **Secondary structure and domain organization of AtGC1.** *A*, predicted secondary structure of AtGC1 with the designated catalytic domain under the *bracket*, the catalytic center between *solid arrows*, the glycine-rich motif delineated by *open arrows*, and the putative PPi-binding arginine (*R*) residue marked by an *asterisk*. A *line* under the amino acid symbols signifies a predicted coil, *open boxes* are α-helices, and *open arrows* are β-sheets. The *red arrow* marks the designated purine-binding hydrophobic pocket (18, 19). *B*, comparison of domain arrangements between different types of GCs.

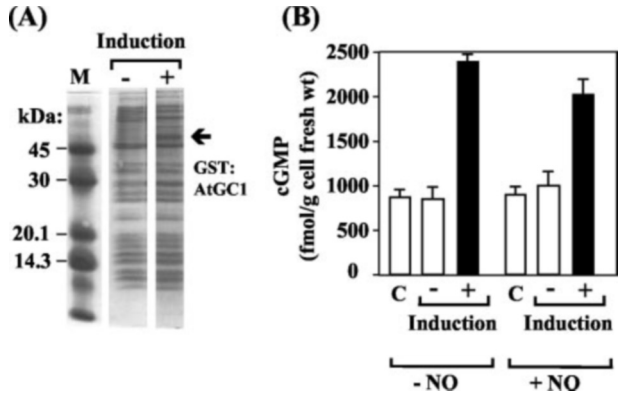

FIG. 3. **Expression of AtGC1 as a GST recombinant fusion protein in *E. coli*.** *A*, SDS-PAGE gel of uninduced (−) and induced (+) proteins from *E. coli* BL-21 (DE3) pLysS containing the expression construct (pGEX:AtGC1). The *solid arrow* marks the expressed recombinant protein (GST:AtGC1) after 60-min induction with 0.6 mM IPTG at 30 °C. *B*, cGMP levels in extracts from *E. coli* (0.1 g) determined with a cGMP (^{125}I) radioimmunoassay system (see "Experimental Procedures"). The control (*C*) is the cGMP value from *E. coli* extracts containing vector (pGEX-6P-2) only, induced with 0.6 mM IPTG for 60 min; − is the value of the uninduced and + of the induced cells. NO induction was achieved with 1 mM nitroprusside (see "Experimental Procedures"). cGMP levels of the control (*C*) and the non-induced (−) are not different, and both are significantly different from the induced (+) ($p < 0.01$). The *bars* represent a mean of quadruplicate data points, with S.D. indicated by *bars*. The data are representative of three independent experiments.

turns no candidates. If a replacement with any amino acid was permitted at the functionally unassigned but conserved glycine (Gly) residue in the catalytic center, seven candidates molecules are returned. Of the seven *A. thaliana* proteins returned only one also contains the N-terminal glycine-rich motif. This *A. thaliana* molecule of unassigned function was termed AtGC1. AtGC1 is 274 amino acids in length and contains the search motif in its N-terminal region between residues 32 and 46 (Fig. 2). The functionally unassigned but conserved glycine (Gly) in the catalytic center is replaced by an aspartic acid (Asp) (Fig. 1). Homology searches with AtGC1 return GCs, albeit with *e*-values $\geq$0.19, as well as more closely related ($<e^{-20}$) human, mouse, mosquito, and *Drosophila* proteins without commonly known GC domains and of currently unknown function.

AtGC1 contains the conserved arginine (Arg) or lysine (Lys) (Fig. 1) that participates in hydrogen bonding with guanine (18, 19) and the cysteine (Cys) that confers substrate specificity for GTP (18, 19, 25). Finally, one of the amino acids that has been reported to stabilize the transition state in the conversion of GTP to cGMP, namely arginine (Arg), is conservatively replaced by lysine (Lys) (18, 19). Such a replacement is also seen in the Fugu (*Takifugu rubripes*) GC (Fig. 1). AtGC1 contains a glycine-rich domain N-terminal of the catalytic center (Fig. 1) and 17 amino acids N-terminal of the motif an arginine (Arg) (Fig. 2*A*) that is implied in PP_i binding (18). This arginine is flanked by hydrophobic amino acids, and this together with the distance to the catalytic center is common in annotated class III nucleotide cyclases (18, 19).

The modeled secondary structure (26) (Fig. 2*A*) predicts a β-sheet spanning the purine-binding hydrophobic pocket, a feature that has been reported previously (18). Three-dimensional structure prediction (27) (not shown) indicates 18% identity of AtGC1 with the P-loop in nucleotide triphosphate hydrolases. A domain comparison (Fig. 2*B*) between classical GCs and AtGC1 reveals the unusual N-terminal position of the catalytic motif as well as the reduced size of the domain. Pairwise BLAST sequence comparison of AtGC1 against sequences from the dimerization domain, kinase-homology domain, transmembrane domain, ligand-binding domain, and heme-binding domain that interacts with NO indicated that AtGC1 does not have significant sequence similarity with any of these domains.

To test biological activity of the candidate molecule, we have obtained the cDNA by reverse transcriptase-PCR and expressed it in a prokaryotic system (Fig. 3*A*). Only in protein extracts from transformed induced bacteria do we observe a fusion protein (GST:AtGC1) of the predicted molecular mass of 57 kDa. When equal amounts of cells were extracted and assayed for cGMP with a radioimmunoassay, it was observed that the cGMP levels were >2.5-fold elevated in extracts from transformed induced bacteria as compared with both extracts from non-induced cells and the control (Fig. 3*B*). The findings would suggest that the *A. thaliana* protein is either a *bona fide* GC or stimulates resident *E. coli* GCs.

Fig. 4 shows the purified recombinant AtGC1 and results from *in vitro* testing of the recombinant molecule. The GC activity of total extracted protein from untransformed, but IPTG-induced, *E. coli* is less than 10 fmol/μg of protein and higher in the presence of Mn^{2+} than Mg^{2+}. The purified fusion protein (Fig. 4*A*) has an activity of >20 fmol/μg in the presence of Mg^{2+} and a drastically reduced activity (>50×) in the presence of Mn^{2+} (Fig. 4*B*). Both control extract from un-transformed induced *E. coli* obtained after glutathione-Sepharose 4B column purification and GST on its own show no activity, thus indicating that the cGMP measured in the recombinant protein preparation is solely due to the catalytic function of AtGC1. A time course of GST:AtGC1-dependant cGMP formation shows that >80% of the product was generated in less than 10 min and that no significant amount of cGMP was produced from heat-inactivated recombinant protein under the same ex-

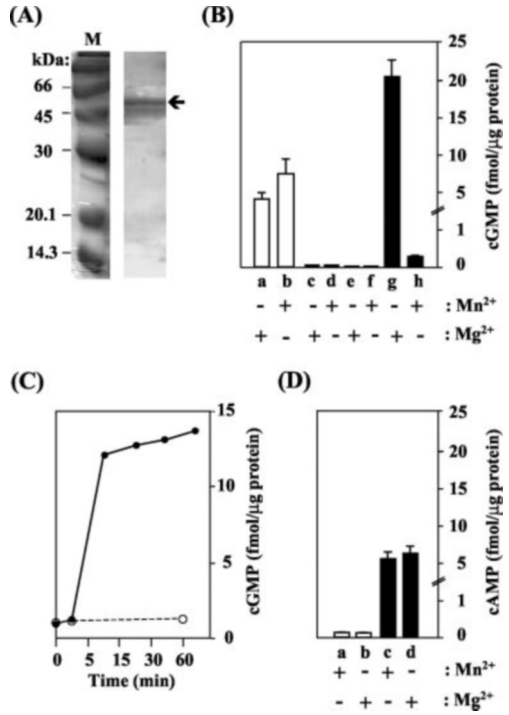

FIG. 4. **Expression of AtGC1 as a GST recombinant fusion protein and *in vitro* testing.** *A*, SDS-PAGE gel of recombinant GST:AtGC1. *B*, cGMP levels in fmol per μg of protein in the presence of 5 mM Mg^{2+} or Mn^{2+} and 1 mM 3-isobutyl-1-methylxanthine. The substrate was 1 mM GTP, and the incubation time was 30 min. *Open bars* (*lanes a* and *b*) represent cGMP values from untransformed, IPTG-induced *E. coli* protein extracts (see "Experimental Procedures"). *Solid bars* are cGMP values from GST column-purified extracts of non-transformed, but induced, *E. coli* (*lanes c* and *d*), a GST only control (*lanes e* and *f*), and GST:AtGC1 recombinant protein (*lanes g* and *h*). The *bars* represent a mean of quadruplicate data points, with S.D. indicated by *bars*. The data are representative of three independent experiments. *C*, time course of GST:AtGC1 GC activity performed *in vitro*. cGMP levels were measured from reactions that were allowed to proceed for 0, 5, 15, 30, and 60 min. Control reactions were performed in the same way except that GST:AtGC1 was heat-inactivated. Data points are means of duplicate measurements. *D*, cAMP levels in fmol per μg of recombinant GST:AtGC1 in the presence of 5 mM Mg^{2+} or Mn^{2+}. The substrate was 1 mM ATP, and the incubation time was 30 min. *Open bars* (*lanes a and b*) represent cAMP values from purified GST. *Solid bars* are cAMP values from GST:AtGC1 recombinant protein (*lanes c* and *d*). The *bars* represent a mean of triplicate data points, with S.D. indicated by *bars*.

perimental conditions (Fig. 4*C*). The results obtained from *in vitro* testing are thus consistent with monomeric or homo-oligomeric catalytic function that is independent of ATP, activating proteins or co-factors other than Mg^{2+}. Finally, GST:AtGC1 was also tested for adenylyl cyclase activity *in vitro* (Fig. 4*D*). The result indicates that with ATP as substrate and in the absence of GTP, AtGC1 has adenylyl cyclase activity that is >3 times lower than the GC activity and not significantly different in the presence of either Mg^{2+} or Mn^{2+}.

DISCUSSION

Since there is compelling evidence that plants operate cGMP-dependent signaling pathways, we decided to use the resource of the complete *A. thaliana* genome sequence to search for GCs. The approach was based on the observation that known GC catalytic domains appear highly conserved between highly diverged organisms. We reasoned that plant GCs may contain at least some of the amino acid residues that are conserved in the catalytic center of known GCs, specifically the residues that are directly implicated in catalytic activity. Furthermore, the obligatory presence of the glycine-rich motif found adjacent to the N terminus of the catalytic center of all known GCs suggested inclusion of this feature as additional search criterium. In GCs the glycine-rich motif may play a similar role as in the P-loop of GTP-binding proteins where it gives flexiblity to the part of protein that interacts with the phosphate group in GTP, thus facilitating substrate binding (28). We anticipated that plant GCs would be significantly evolutionarily distinct and different from known GCs, since homology searches have failed to identify functional molecules in plants. Consequently, a search motif was constructed to allow conservative and semiconservative replacements of amino acid residues in the catalytic center. The glutamic acid (Glu) implied in Mg^{2+} binding (Fig. 1) (18) was not included in the search motif, since it is substituted by wide range of different amino acids (Asp, Ala, Gln, Cys, Thr, Arg, and Tyr) in prokaryotic guanylyl cyclases (19). In addition, the glutamic acid (Glu) is also replaced by aspartic acid (Asp) in some type I and type II adenylyl cyclases (18). This substitution pattern would thus suggest that polarity and charge of the amino acid side chain in this position is not restrictive for catalysis.

The fact that no *A. thaliana* protein contains the unsubstituted search motif prompted a decrease of the stringency of the motif allowing for a replacement of the functionally unassigned but highly conserved glycine in the catalytic center (Fig. 1). The modified search parameters yield seven *A. thaliana* proteins, and only one, AtGC1, fulfils the secondary criteria of the presence of the adjacent glycine-rich motif. It is concluded that the motif search with one substitution in the catalytic center is not highly selective for GCs, since 20 proteins out of 260 proteins identified in PIR that contain the motif are implicated in functions not related to known GC activity. However, the combination of the two search parameters, the catalytic center search motif and the glycine-rich motif, identify annotated GCs only. This would suggest that the use of both parameters combined is a reliable tool for the identification of candidate GCs.

AtGC1 contains the conserved residues that participate in hydrogen bonding with guanine (18, 19) and the cysteine (Cys) that confers substrate specificity for GTP rather than ATP (18, 19). This structural feature is reflected in the *in vitro* activity of AtGC1 which shows significantly more guanylyl than adenylyl cyclase activity. The arginine (Arg) that stabilizes the transition state in the conversion of GTP to cGMP is conservatively replaced by lysine (Lys) (18, 19), and such a substitution is also observed in Fugu (*T. rubripes*) (Fig. 1). AtGC1 also contains the hydrophobic pocket to which nucleotide triphoshate purine moieties can bind (18), and the secondary structure modeling predicts that this pocket is part of a β-sheet (Fig. 2*A*). A further N-terminal feature of GCs is the presence of a pyrophosphate (PPi) binding motif that consists of an arginine (Arg) flanked by aliphatic amino acids (18). AtGC1 contains such a motif 22 amino acids from the catalytic center and hence in a position that is conserved in PPi-binding sites of known GCs (Fig. 2*A*). The similarity between the predicted three-dimensional structure of AtGC1 and that of the P-loop structure contained in nucleotide triphosphate hydrolases (28, 29) is noteworthy, since nucleotide triphosphate hydrolases can convert GTP to both GDP and GMP (30, 31). In triphosphate hydrolases, the P-loop interacts with Mg^{2+} for GTP binding and hydrolysis, and it may be argued that the P-loop-like structure in AtGC1 has an analogous function, and this is supported by the observed dependence of the catalytic activity of AtGC1 on Mg^{2+} (Fig. 4*B*).

AtGC1 contains a number of features that are unusual in currently annotated GCs. First, the catalytic center motif is found close to the N-terminal rather than the C-terminal or central part of the molecule where it is located in both particulate and soluble guanylyl cyclases (32–34). C-terminal loca-

tion is the norm in particulate GCs where the N terminus functions as receptor and the C-terminal GC domain generates the cytosolic signal. In addition, in AtGC1 there is no evidence of a signal sequence for direction to the membrane and the designated PPi-binding arginine (Arg) is only 12 amino acids removed from the N-terminal end of the molecule. Other features typical of particulate GCs, the ligand-binding domain, transmembrane domain, kinase homology domain, and dimerization domain are also absent.

Contrary to the homodimeric particulate GCs, the soluble GCs form α/β heterodimers (33, 35). Soluble GCs are the principal effector of the gaseous messenger NO. NO dependence of soluble GCs in turn requires a heme-binding region, and this region is N-terminal of the catalytic domain. NO binds covalently via two cystine-thioether bonds that are provided by a conserved C-*X*-*X*-C-H motif in soluble GCs (36). In AtGC1 there is no evidence of a heme-binding motif that is required for NO binding. The absence of such a heme-binding motif is thus consistent with the lack of NO inducibility observed in AtGC1. From the sequence analysis, one would thus predict that AtGC1 is not a membrane protein, not similar in domain structure to known receptor or soluble GCs, and its lack of a heme-binding group implies activity that is insensitive to NO, while catalytic activity *in vitro* implies that it is active either as a monomer or homo-oligomer.

The assay result obtained from transformed induced *E. coli* demonstrates a significant increase in cGMP (Fig. 3). Since in this assay cGMP was generated in intact bacteria and in the absence of added substrate, it can be concluded that at least the N terminus of the recombinant was present in the cytosol and thus supports the predictions based on structural features. Since AtGC1 is likely to be located in the cytosol, we tested whether the activity *in vivo* in *E. coli* cells was affected by the NO donor sodium nitroprusside, and the observed NO insensitivity is consistent with the structural features of AtGC1. NO independence in soluble GCs does not appear to be the norm; however, an unusual soluble GC from *Manduca sexta* (MsGC-β3) that does not require dimerization has been reported (37). In this molecule amino acids thought to participate in heme binding are substituted with non-similar amino acids (37). AtGC1 and MsGC-β3 are thus both soluble GCs that do not depend on heterodimer formation for activity. Taken together, our results imply that AtGC1 is active as a monomer, homodimer, or homo-oligomer, with no obligatory dependence on membrane associations, activation by ligands, ATP, or other co-factors with the exception of Mg^{2+}.

While homodimeric and oligomeric activity is common in particulate GCs (1, 17, 34), monomeric activity in a soluble GC has only recently been reported (38). Monomeric GC activity has also been demonstrated in a soluble GC (DdsGC) from *Dictyostelium discoideum* (39) that has in fact been identified as a homologue of an adenylyl cyclase. It is in keeping with this ancestry that DdsGC has two catalytic domains within a single molecule. The discovery of another GC from *M. sexta* (MsGC-I) has further complicated attempts to categorize GCs (40, 41). MsGC-I shows highest sequence identity with receptor GCs throughout its catalytic and dimerization domains but does not contain the ligand-binding, transmembrane, or kinase-like domains typical receptor GCs (40). Both AtGC1 and MsGC-I contain C-terminal extensions that are not present in other known guanylyl cyclases. MsGC-I shows no similarity to domains typical of soluble GCs but appears to exist as a soluble homodimer insensitive to NO stimulation (40); however, despite the absence of a transmembrane domain an as yet undefined membrane association *in vivo* has been suggested (40).

AtGC1 is adding to the steadily growing number of structurally diverse molecules with GC activity and it appears that many more and diverse GC-dependent physiological responses await discovery. We are currently in the process of elucidating the biological role of AtGC1 with particular emphasis on cGMP dependent promotion of stomatal opening (12, 13) and responses to plant hormones such as kinetin and immunoreactant plant natriuretic peptides (13–15).

Acknowledgments—We thank Amanda Lochner (University of Stellenbosch) for help with the cGMP assay and Cathal Seoighe (South African National Bioinformatics Institute) for critical discussion.

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