The *Arabidopsis thaliana* Brassinosteroid Receptor (AtBRI1) Contains a Domain that Functions as a Guanylyl Cyclase *In Vitro*

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**Background.** Guanylyl cyclases (GCs) catalyze the formation of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) from guanosine 5′-triphosphate (GTP). Cyclic GMP has been implicated in an increasing number of plant processes, including responses to abiotic stresses such as dehydration and salt, as well as hormones. **Principle Findings.** Here we used a rational search strategy based on conserved and functionally assigned residues in the catalytic centre of annotated GCs to identify candidate GCs in *Arabidopsis thaliana* and show that one of the candidates is the brassinosteroid receptor AtBRI1, a leucine rich repeat receptor like kinase. We have cloned and expressed a 114 amino acid recombinant protein (AtBRI1-GC) that harbour the putative catalytic domain, and demonstrate that this molecule can convert GTP to cGMP *in vitro*. **Conclusions.** Our results suggest that AtBRI1 may belong to a novel class of GCs that contains both a cytosolic kinase and GC domain, and thus have a domain organisation that is not dissimilar to that of atrial natriuretic peptide receptors, NPR1 and NPR2. The findings also suggest that cGMP may have a role as a second messenger in brassinosteroid signalling. In addition, it is conceivable that other proteins containing the extended GC search motif may also have catalytic activity, thus implying that a significant number of GCs, both in plants and animals, remain to be discovered, and this is in keeping with the fact that the single cellular green alga *Chlamydomonas reinhardtii* contains over 90 annotated putative CGs.

**RESULTS**

**Extending the search GC search motif and identification of AtBRI1**

The original GC search motif [RKS][YFW][GCTH][VIL][FV]-X[DNA][X][VIL][X][X][KR] [12] (Figure 1A) yielded seven Arabidopsis candidate proteins including AtGC1 that has been demonstrated to have GC activity *in vitro* [12]. Two of seven proteins are annotated kinases and one of the two (At1g79680) belongs to the group of wall associated kinase-like proteins (WAKLs). In a quest to identify further candidate GCs in plants we mutated the position 7 in the original search motif. This position is assigned as having a role in dimerisation that may not be a critical requirement for GC functionality. When [D] in position 7 is substituted by [L] in a 100 amino acid recombinant AtGC1(1–100), a domain that is encoded by the first exon of AtGC1 (Figure 1B), no significant loss in catalytic GC activity occurs (Fig. 1D). This implies firstly that a 100 amino acid domain is sufficient for activity and secondly, that [D] in position 7 is not essential for AtGC1 GC activity. These observation may hold for a number of GCs. Consequently we added [L] to make position 7 [DNAL] (Fig. 2A). This extended motif retrieves 123 *Arabidopsis thaliana* proteins including the brassinosteroid receptor AtBRI1 (*Arabidopsis thaliana* brassinosteroid insensitive 1 [NP_195650.1, At1g39400.1]). Furthermore, since the catalytic asparagine [N] in position 10, while quite conserved in some classes of nucleotide cyclases is substituted by [AILSTYHDE] in many annotated GCs [13], we further modified the motif to [RKS][YFW][GCTH]-[VIL][FV][X][X][VIL][X][X][KR]. All three motifs are specific for GC domains and can be used to identify candidate GCs in many diverse classes of nucleotide cyclases in prokaryotes and eukaryotes where they catalyse the formation of cGMP from GTP.

**Academic Editor:** Ivan Baxter, Purdue University, United States of America

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**Funding:** This work was supported by the NRF (South Africa) and the ARC (Australia).

**Competing Interests:** The authors have declared that no competing interests exist.

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rather than adenylyl cyclases (ACs) since they contain the residues [GCTH] in position 3 facing the purine and determining substrate specificity for GTP rather than ATP [13–15] and GenBank contains no annotated ACs that conform to either of the motifs.

The third and most relaxed motif appears in 171 Arabidopsis proteins of which 88 contain [EQCTDRVHY] at position 16 or 17 which is presumed responsible for metal ion binding [13]. Metal binding is normally associated with aspartic or glutamic acid ([DE]). Within the group of 88 proteins; 27 contain a PPi-binding arginine [R] and the C-terminal putative metal binding site is highlighted in aquamarine. The green triangles point to exon borders and the solid arrow shows the border of the fragment that we have tested for GC activity. (C) SDS-PAGE of the 3 purification steps of the recombinant protein AtGC1(1–100); “M” is the molecular weight marker, “FT” is the protein in the flow-through, “W” is the wash and “E” is the eluted recombinant protein. (D) In vitro GC activity assay. The control (cont.; empty bar) was obtained by omitting protein in the reaction mix and the concentration of GTP was 1 mM and that of Mn²⁺ or Mg²⁺ was 5 mM. The values for the wild-type protein (N-terminal fragment of 100 amino acids containing a [D] in position 7 of the catalytic centre) and the mutated protein (N-terminal fragment of 100 amino acids containing [L] in position 7 of the catalytic centre) are represented with solid bars. The bar values represent the mean cGMP (+/– SEM) generated in 15 minutes in three samples and the response pattern is representative of 3 independent experiments.

doi:10.1371/journal.pone.0000449.g001

Figure 1. Site-directed mutagenesis and functional testing of AtGC1(1–100). (A) Original 14 amino acid search motif for GCs [12]; substitutions are in square brackets, X represents any amino acid and curly brackets define the number of amino acids. (B) AtGC1 (At5g05930): The position of the GC catalytic centre is marked in red; the underlined aspartic acid [D] is the amino acid that has been changed into a leucine [L] by site directed mutagenesis. The open arrow marks the conserved PPi-binding arginine [R] and the C-terminal putative metal binding site is highlighted in aquamarine. The green triangles point to exon borders and the border of the fragment that we have tested for GC activity.

AtBRI1 (Fig. 2). In AtBRI1, the putative catalytic core was identified within the cytosolic kinase domain.

The choice of for further testing AtBRI1 was informed by several factors including the fact that brassinosteroids are physiologically well characterised growth regulators that await further elucidation of their signal transduction networks as well as the availability of a number of AtBRI1 mutants that can support these investigations. Brassinosteroid receptors have been identified in several other species and these also contain the conserved GC motif (Fig. 2D).

The recombinant protein that we decided to synthesise and test for in vitro activity contains the predicted GC catalytic centre of AtBRI1 (At4g39400) and 50 additional amino acids on both the N- and the C-terminus (Fig. 2C). This peptide (AtBRI1-GC) is part of the cytoplasmic domain containing the N-terminal part aspartic acid ([D] at –33 from the catalytic centre) implicated in
metal binding [16] as well as a metal binding [D] in position 17 relative to the C-terminus of the motif.

Testing a recombinant GC domain (AtBRI1-GC) for activity

The capacity of the recombinant putative GC domain of AtBRI1 (AtBRI1-GC) to generate cGMP from GTP was assessed with two independent methods. Firstly, we used an enzyme immunoassay to check if a reconstituted recombinant AtBRI1-GC could function as a GC in vitro. The results indicate that the recombinant protein can cyclase GTP and does so preferably in the presence of Mg2+/Mn2+ (Fig. 3A).

In order to verify the result obtained with this anti-body based detection method we also used mass spectrometry. Firstly, we established that the Q-TOF mass chromatogram could detect cGMP at fmol concentrations (Fig. 3D, right inset) much like the enzyme immunoassay. We detected neither cGMP in the solution containing the recombinant protein only (Fig. 3B) nor in the reaction mix in the absence of the protein (Fig. 3C). Our sample generates cGMP in a time dependent way (Fig. 3D). We calculated that after 5 min. incubation in the presence of 1 mM GTP 100 fmoles cGMP/μg protein were generated and after 20 min. >3 pmoles cGMP/μg protein (Fig. 3D). We noted that values of the amount of cGMP generated obtained with the mass spectroscopy read higher than those obtained with the enzymatic assay and this observation has been made consistently in independent in vitro experiments with recombinant proteins (data not shown). In addition, it is noteworthy that plant GC activities are reportedly low and certainly not at the levels observed for some soluble animal GCs [17]. The main reason for this is that higher activities might require co-factors (e.g. Ca2+, chaperones or co-proteins) or post-translational modifications that are not present in the recombinant tested in vitro.

We also used mass spectrometry to test AtBRI1-GC for adenylyl cyclase activity in the presence of 1 mM ATP as the substrate and could not detect significant amounts of cAMP generated obtained with the mass spectroscopy read higher than those obtained with the enzymatic assay and this observation has been made consistently in independent in vitro experiments with recombinant proteins (data not shown). In addition, it is noteworthy that plant GC activities are reportedly low and certainly not at the levels observed for some soluble animal GCs [17]. The main reason for this is that higher activities might require co-factors (e.g. Ca2+, chaperones or co-proteins) or post-translational modifications that are not present in the recombinant tested in vitro.

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and potential BRI1 kinase substrates have been identified such as signal component. BRI1 is ubiquitously expressed in Arabidopsis leucine rich repeat extracellular domain, BRI1 has been identified in membrane [28,29]. Based on the binding of the ligand BR to the a leucine rich repeat receptor like kinase [27] located in the plasma membrane 

Brassinosteroids (BRs) are polyhydroxylated plant steroid hormones with an essential role in co-regulating many processes including embryogenesis, cell elongation and vascular differentiation [25,26]. Brassinosteroid Insensitive-1 (BRI1) was first identified from mutant analysis and then cloned and found to be a leucine rich repeat transmembrane kinase [27] located in the plasma membrane [28,29]. Based on the binding of the ligand BR to the leucine rich repeat extracellular domain, BRI1 has been identified as a BR receptor in Arabidopsis [29,30] and therefore a critical signal component. BRI1 is ubiquitously expressed in Arabidopsis and potential BRI1 kinase substrates have been identified such as transphthalamine-like protein which is phosphorylated in vitro by the kinase domain of BRI1 [31]. Several models have been developed to describe the signaling events following perception of BR by BRI1 (see [32]) involving other membrane associated proteins and activation of transcription factors. The observation that AtBRI1 does harbor a functional GC domain within the cytosolic part of the molecule might suggest that cGMP is a second messenger in some BR dependent processes. However, this hypothesis remains to be tested. Several genes that regulate physiological functions are stimulated by BR as well as being influenced by cGMP. An example for this dual dependence is plant cell elongation [26]. Microarray studies revealed that genes involved in cell wall expansion such as expansins and pectinesterases are up-regulated by both BR [32] and membrane permeable cGMP treatments [4].

Both BR and gibberellin interact to regulate plant growth. Some of these interactions are antagonistic but in other cases, BR can potentiate gibberellin activity [33]. Gibberellin, itself, stimulates increases in cGMP [7]. It is conceivable that in some instances the GC domain of BRI1 could stimulate cGMP production and so potentiate gibberellin activity. On a speculative note, there may be key molecules within specific cells that specify decreased cytoplasmic kinase activity and enhance the GC activity of the AtBRI1.

There are several recessive alleles of AbBRII in the cytoplasmic kinase domain. Of these mutants, bri-101 is the only mutant in the GC catalytic region ([E] 1078 to [L]) and it is insensitive to BR and also has reduced kinase activity when tested in a heterologous system [27,28]. Interestingly, this mutation should not affect the GC activity as it occurs at position 8 which can be any amino acid. Three other mutants have been found in the region that we show confers GC activity in vitro, being: bri-103,104 [A] 1031 to [T], bri-105-107 [Q] 1059 to stop (which would exclude the GC catalytic domain from the truncated protein) and bri-115 [G] 1048 to [N] [28]. The domain that we have identified (Fig. 2) occurs within the kinase domain [28]. We demonstrate that the isolated 114 amino acid recombinant peptide (AtBRI1-GC) has GC activity in vitro (Fig. 3). The relative importance of the two functions in the action of the receptor remains to be demonstrated bearing in mind that previously work has focused on the kinase domain as the GC domain had not been identified. Interestingly, a number of enzymes have recently been identified as “moonlighting” proteins with dual functions [34]; the kinase and GC activity of AtBRI1 could be yet another example.

On a more general level, the finding implies that functional GC domains may be part of a large variety of different multifunctional signaling molecules and receptors in particular. It is noteworthy that the atrial natriuretic peptide receptors NPR1 and NPR2 both signal through cGMP and have an AtBRI1-like domain organisation that the atrial natriuretic peptide receptors NPR1 and NPR2 both signal through cGMP and have an AtBRI1-like domain organisation.

Finally, the fact that two recombinant proteins (AtGC1(1–100) and AtBRI1 - GC) of less than 120 amino acids have GC activity in vitro begs a reexamination of the minimal catalytic requirement for GCs and may suggest that the number of different GC domains is significantly higher than currently assumed. This is in keeping with the fact that the single cellular green alga Chlamydomonas reinhardtii contains a surprisingly large number (>90) of annotated putative GCs [1] and with the increasing number of biological processes discovered that are modulated by the second messenger cGMP [2,3].

| Table 1. Unique Arabidopsis proteins retrieved with the search pattern: [R](X5,20)[RKS][YFW][GCTH][VIL][FV][X3][VIL][X4][KR][X1,2][D] |
|---|
| I.D. | Annotation |
| At1g90950 | Function unknown |
| At1g14370 | Protein kinase APK2a |
| At1g7750 | Leucine-rich repeat transmembrane protein kinase |
| At1g28440 | Leucine-rich repeat transmembrane protein kinase |
| At1g48220 | Serine/threonine protein kinase, similar to Ptk kinase interactor |
| At1g69270 | Leucine-rich repeat family protein, protein kinase family |
| At1g69910 | Protein kinase family protein |
| At1g73080 | Nucleotide binding leucine-rich repeat RK, immune response |
| At1g76370 | Protein kinase |
| At2g01860 | Pentatricopeptide (PPR) repeat-containing protein |
| At2g02800 | Protein kinase APK2b |
| At2g26330 | Leucine-rich repeat protein kinase, putative ERECTA |
| At2g32800 | Kinase family protein with dual protein kinase domains |
| At3g02130 | Leucine-rich repeat transmembrane protein kinase |
| At3g02810 | Protein kinase family protein |
| At3g07070 | Protein kinase family protein |
| At3g24790 | Protein kinase family protein |
| At3g46340 | Leucine-rich repeat protein kinase, similar to light repressible receptor PK |
| At3g46350 | Leucine-rich repeat protein kinase |
| At3g46400 | Leucine-rich repeat protein kinase, similar to light repressible receptor PK |
| At4g20270 | Leucine-rich repeat transmembrane PK, CLAVATA1 kinase |
| At4g39400 | BRI1 (ATBRI1-GC) |
| At5g09390 | AtGC1 |
| At5g07180 | Leucine-rich repeat protein kinase, putative ERECTA |
| At5g10530 | Lectin protein kinase, similar to receptor lectin kinase 3 |
| At5g16500 | Protein kinase family protein |
| At5g62330 | Leucine-rich repeat protein kinase, putative ERECTA |

PK, protein kinase; RK, receptor kinase. Note: The listed proteins constitute a significant overrepresentation (p<1e-1) in the Fatigo (level 4) categories of phosphorus metabolic processes, protein metabolic process, cellular macromolecular metabolic process and biopolymer metabolic process. doi:10.1371/journal.pone.0000449.t001

DISCUSSION
Brassinosteroids (BRs) are polyhydroxylated plant steroid hormones with an essential role in co-regulating many processes including embryogenesis, cell elongation and vascular differentiation [25,26]. Brassinosteroid Insensitive-1 (BRI1) was first identified from mutant analysis and then cloned and found to be a leucine rich repeat receptor like kinase [27] located in the plasma membrane [28,29]. Based on the binding of the ligand BR to the leucine rich repeat extracellular domain, BRI1 has been identified as a BR receptor in Arabidopsis [29,30] and therefore a critical signal component. BRI1 is ubiquitously expressed in Arabidopsis and potential BRI1 kinase substrates have been identified such as transphthalamine-like protein which is phosphorylated in vitro by the kinase domain of BRI1 [31]. Several models have been developed to describe the signaling events following perception of BR by BRI1 (see [32]) involving other membrane associated proteins and activation of transcription factors. The observation that AtBRI1
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. Search motifs were also used to query the Arabidopsis genome via the Arabidopsis server (www.arabidopsis.org) using the "Pat-match" function.

**Site-directed mutagenesis**

A non-methylated double strand was synthesized using 0.5 μM Forward (5’-ATACGCTATCCGATCTCAGT-3’) and 0.5 μM Reverse (5’-CATCGTACGACCAAGGGAAGATCGAATAGGCAGGAT-3’) primers from a clone
containing the AtGC1 gene (At5g05930). The site where the mutagenesis occurred is underlined. Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used in accordance with the manufactures instructions to amplify the plasmid. The original methylated template plasmid was digested using DpnI (New England Biolabs) leaving the amplified plasmid which was transformed into E. coli Topo 10F competent cells (Invitrogen). Single colonies were selected and the clones were analyzed by DNA sequencing.

**Synthesis of recombinant protein**
Since no introns are in the putative GC domain of AtBRI1, genomic DNA from Arabidopsis thaliana (Col) was used as the PCR template. PCR amplifications of AtBRI1 GC domain (At4g39400.1: 3132–3638) (Forward primer with BamHI site: 5'-GGATCCCAAGATTCGTGAAGCTGAGGTTTG-3', reverse primer with EcoRI site: 5'-TCCAAGATTCGTCAGCAACGTGTTTGAAATGT-3') were performed on a Mastercycler personal (Eppendorf, 22339 Hamburg Germany), in five 50 μL reaction volumes. Each reaction contained 1.5 mM MgCl2, 200 μM dNTPs, 0.5 μM reverse and forward primer, 7.4 ng of genomic DNA, and 2.5 units of Taq polymerase (Fermentas GmbH, St. Leon-Rot, Germany). The thermal cycling parameters were: initial denaturation at 96 °C for 3 min., followed by 30 sec. at 96 °C, 50 °C for 45 sec. and 72 °C for 1 min, for 32 cycles, followed by a final extension at 72 °C for 10 min. A fragment of 340 bp was excised from the gel and purified using the GFX purification kit as per manufacturer’s instruction (Amersham Biosciences, Little Chalfont, UK). The fragment was cloned into the pCR® Topo®-TOPO®-NT vector (Invitrogen Ltd., Paisley, UK) and used to transform E. coli BL21 (DE3) pLysS cells (Invitrogen Ltd., Paisley, UK); colonies were selected and inserted variants verified by sequencing.

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**cGMP measurements**

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**Author Contributions**

Conceived and designed the experiments: CG HI. Performed the experiments: LK SM OR LM. Analyzed the data: LK SM. Wrote the paper: CG HI.

**ACKNOWLEDGMENTS**

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