Original Research

Brachypodium distachyon ERECTA-like1 protein kinase is a functional guanylyl cyclase

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1. Abstract

The plant proteins called ERECTA family play important role in inflorescence architecture, stomatal patterning and phloem-xylem organization. ERECTA proteins belong to the moonlighting proteins family containing the guanylyl cyclase (GC) catalytic center embedded within the intracellular kinase domain. This characteristic architecture of ERECTA proteins prompted us to experimentally confirm of enzymatic activity of one of these, BdERL1 (ERECTA-like1 from *Brachypodium distachyon*). We have shown that BdERL1 is dual-function protein with both kinase and GC activity. Moreover, our mutagenesis studies also revealed the catalytic roles of key conserved amino acid residues at the GC center and importantly, probing of the kinase and GC with Ca^{2+} and/or cGMP, shed light on the intramolecular regulations of BdERL1.

2. Introduction

The *ERECTA* family of genes, including *ERECTA (ER) and ERECTA-LIKE1,2 (ERL1,2)*, encode leucine-rich repeat receptor-like kinases (LRR-RLKs) in plants. The most studied *ERECTA* gene family from *Arabidopsis thaliana* plays important roles in many physiological processes. ERECTA kinases are involved in stomatal development, the regulation of longitudinal growth of aboveground organs, reproductive development and plant

responses to biotic and abiotic environmental stress factors [1]. The interaction between the ERECTA family and plant hormone signaling (such as auxin, gibberellins or ethylene) has also been confirmed [2–5]. Interestingly, a few years ago, three receptor kinases belonging to the ERECTA family from *A. thaliana*, At2g26330 ERECTA (ER), At5g62230 ERECTA-like1 (ERL1), and At5g07180 (ERL2), were bioinformatically selected as potential guanylyl cyclases (GCs) [6]. *In silico* identification of a 14 amino acid (aa) long catalytic center, [KS]X[SCG]X(10)[KR]X(0,3)[DHSE], within these proteins suggests that they are able to convert GTP into cGMP. However, biochemical proof of the GC activity of these kinases still needs to be discovered. The amino acid sequence of ERECTA proteins indicates that they belong to the moonlighting family of proteins containing a GC catalytic center embedded within the intracellular kinase domain. Many of these moonlighting proteins are receptor kinases that show a cytosolic dominant function, kinase functionality, an additional cryptic function, cyclase activity, and more importantly, regulatory roles between and within the proteins localized to their cellular microenvironments, which are poorly understood [7, 8]. As such, many of the biological processes and responses affected by this class of second messengers have not been fully elucidated.

In this report, we describe the biochemical characterization of BdERL1 from *Brachypodium distachyon* (NCBI accession no. XP_003561134.3). The purpose of our study was to establish the moonlighting function of the GC in BdERL1 and to determine the catalytic roles of the two functionally assigned and highly conserved amino acids at positions 3 and 14 within the GC catalytic center of BdERL1, as well as to investigate how a functional GC in BdERL1 might affect its kinase activity.

3. Materials and methods

3.1 Expression, purification and electrophoretic analysis of the GST-BdERL1 fusion protein

The studied fragment of *BdERL1* cDNA (NCBI no. XM_003561086.4) was amplified by PCR using the following primers: 5′-GGGGCCCCTGggatccATGAGGCTGACAGAGAATTTGAGC-3′ (forward) and 5′-AGTCACGATgcggccgcTCACATTGTGTGCTTGGATATGACC-3′ (reverse) (lower case letters indicate the *BamHI* and *Not*I restriction sites, respectively). The sequence of the studied cDNA fragment encodes the intracellular kinase domain with a conserved GC motif (648-982 aa).

The PCR product was introduced into the pGEX-6P-1 expression vector linearized with *BamHI* and *Not*I restriction enzymes using In-Fusion cloning technology (In-Fusion HD Cloning Kit, Clontech). The correctness of the cloned cDNA sequence was verified by sequencing. The *E. coli* BL21 strain, transformed with the resulting plasmids, was used to produce GST-tagged proteins. Cells were grown in LB medium supplemented with 2% glucose at 37 °C under vigorous aeration. Fusion protein production was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and incubated at 22 °C for 4 h in glass vessels connected to a BioFlo 120 bioprocess control station (Eppendorf). The pH was controlled at 6.5 ($\pm$ 0.2), the DO (dissolved oxygen) parameter was set to 30%, and the agitation speed was 300 rpm. Bacteria were collected by centrifugation, and the pellet was suspended in lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, protease inhibitor cocktail and 0.2 mg/mL lysozyme) and disrupted by 5×15 s of sonication. The soluble fraction was separated by centrifugation at $12\,000 \times g$ for 35 min at 4 °C, and the GST-tagged proteins were adsorbed onto glutathione–Sepharose 4B beads. After the column was washed with buffer containing 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl, the GST-BdERL1 complex was eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0). For a control expression vector, pGEX-6-P1 was used, or GST alone was purified as described above.

The homogeneity and purity of the protein fractions were analyzed by 10% (v/v) SDS–PAGE, and the gels were stained with Coomassie blue.

3.2 Site-directed mutagenesis of the BdERL1 protein

It was previously described that functionally assigned residues of the GC motif can be replaced with another amino acid to estimate the importance or relevance of these residues to maintain the functional configuration of the catalytic center [7]. The G residue localized at position 3 within the GC catalytic motif has been predicted to determine substrate specificity for GTP. We mutated the G residue (842) in the catalytic center to D (mutant $G_{842}D$). Additionally, the K residue located at position 14 in the GC center is responsible for stabilizing the transition state from GTP to cGMP. In our experiment, we mutated the K residue (853) to A (mutant $K_{853}A$).

Site-directed mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (Agilent). A nonmethylated double strand was synthesized using 125 ng of forward (5'-gagcaactctagaagaacgatatcaaagctgtatacatcagattt-3') and 125 ng of reverse (5'-aaatctgatgtatacagctttgatatcgttcttctagagttgctc-3') primers for mutant $G_{842}D$ and 125 ng of forward (5'-gaatcattgtcaacagccgccttccccgtgagcaactc-3') and 125 ng of reverse 5′-gagttgctcacggggaaggcggctgttgacaatgattc-3') primers for mutant $K_{853}A$ from a plasmid DNA containing the *BdERL1* gene. The sites where mutagenesis occurred are underlined. PfuUltra High Fidelity DNA Polymerase (Agilent) was used in accordance with the manufacturer's instructions to amplify the plasmids. The original methylated template plasmids were digested with

the *DpnI* restriction enzyme, leaving the amplified plasmid, which was transformed into *E. coli* XL10-Gold ultracompetent cells (Agilent). Single colonies were selected, and the clones were analyzed by DNA sequencing.

3.3 Determination of BdERL1 WT and mutant guanylyl cyclase activity

The guanylyl cyclase activity of the studied proteins was determined by estimating the rate of cGMP formation. For the BdERL1 WT and mutant assay, the reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM $MnCl_2$, GTP as a substrate at concentrations between 0.25–2 mM and 5 μg of the recombinant fusion enzyme (in some variants supplied with 1 μM Ca^{2+} ions and/or calmodulin (AtCaM1; 0.5–10 μg)) in a final volume of 100 μL. After incubation at 30 $°$C for 15 min, the reaction was stopped by boiling at 100 $°$C for 10 min, and then the samples were centrifuged at 16 000 $\times$ g for 10 min.

Analogically, the cross reactivity of the studied proteins with ATP as a substrate at concentrations between 0.25–2 mM was analyzed.

A negative control with the pure GST protein was performed to exclude its cyclase activity.

3.4 Mass spectrometry analysis of cyclic nucleotides (cGMP and cAMP)

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used as the method for the GC/AC activity assay. The enzymatic activity was defined as the amount of cGMP/cAMP produced by 1 mg of protein per minute.

LC-MS/MS experiments were performed using the Nexera UHPLC and LCMS-8045 integrated system (Shimadzu Corporation). The ionization source parameters were optimized in positive ESI mode using pure cGMP or cAMP dissolved in HPLC-grade water (Sigma). Samples were separated using a reversed-phase C18 column (150 $\times$ 2.1 mm, 2.6 μm, Kinetex) in 10% methanol with 0.1% (v/v) formic acid) (solvent A (water with 0.1% (v/v) formic acid), solvent B methanol with 0.1% (v/v) formic acid) with a flow rate of 0.3 mL/min. The interface voltage was set at 4.0 kV for positive electrospray (ES+). Data acquisition and analysis were performed with the LabSolutions workstation for LCMS-8045.

The calculation of cyclic nucleotide content was based on standard curves for cGMP and cAMP ranging from 0 to 200 pg (0–0.7 pmol).

3.5 Kinase activity assay

For quantification of the BdERL1 protein kinase activity, a Kinase-Glo plus luminescent kinase assay (V3771, Promega) was used according to the manufacturer's protocol. Briefly, purified intracellular domain of BdERL1 (1 μg) was added to a 50 μL reaction mixture (25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 μg/μL Histone Type III-S, and 10 μM ATP supplied with 0.1 or 1 μM cGMP or 1 μM Ca^{2+} ions and/or calmodulin (At-CaM1; 0.5–10 μg)). Reactions were performed at 30 $°$C for 15 min and stopped by adding Kinase-Glo reagent. After equilibrating the mixture at room temperature for 10 min, the luminescence was monitored using a Synergy HT Multi-Mode Microplate Reader. The results are expressed as relative luminesce units, and experiments were performed in triplicate.

3.6 Computational modeling

Domain predictions were performed using Inter-Pro (https://www.ebi.ac.uk/interpro/) and LRRfinder (http://www.lrrfinder.com/lrrfinder.php). The $Met^{648} \rightarrow Val^{918}$ fragment of BdERL1 was modeled against the AtBRI1 crystal structure (PDB ID: 5LPV) [9] using MODELLER (ver. 9.25) software [10]. Docking simulations of cGMP to the GC domain of BdERL1 were performed using AutoDock Vina (ver. 1.1.2) [11]. All structures, binding poses, and images were analyzed and created using UCSF Chimera (ver. 1.10.1) [12].

4. Results and discussion

4.1 Identification of a GC catalytic motif in BdERL1

A highly conserved and stringent GC search motif for higher plants [KS]X[SCG]X(10)[KR]X(0,3)[DHSE] was constructed, and its continuous refinement in recent years has allowed for the discovery of a number of functional candidate GCs in the plant kingdom [13–20]. Based on bioinformatics prediction of the GCPred tool (http://gcpred.com), the BdERL1 protein was identified as a potential new guanylyl cyclase harboring a conserved GC motif that is localized within the cytosolic C-terminus of the studied protein (BdERL1 GC center$_{840-853}$) [21]. It is a common feature for all identified transmembrane plant guanylyl cyclases that a conserved GC center is embedded at a moonlighting site within an intracellular kinase domain. The GC motif present within BdERL1 [840-<u>S</u>FG<u>I</u>VLLELLTGK<u>K</u>-853] possesses all three highly conserved functional amino acids localized at positions 1, 3 and 14 (Fig. 1A), where serine at position 1 forms a hydrogen bond with guanine, glycine at position 3 confers substrate specificity for GTP and lysine at position 14 stabilizes the transition state from GTP to cGMP. Additionally, two amino acids away from the C-terminal end of the GC sequence is aspartic acid [D], which is responsible for interacting with Mg^{2+}/Mn^{2+} ions [7, 8, 19, 20] (Fig. 1B). Moreover, bioinformatics analysis of the amino acid sequence of BdERL1 revealed that the calmodulin-binding site is localized within the kinase domain of the protein and did not show the presence of the EF hand motif (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html; https://www.uniprot.org/uniprot/I1H187) (Fig. 1B).

The full-length amino acid sequence of BdERL1 belonging to the leucine-rich-repeat receptor kinase fam-

A.

LRR domain   kinase domain

TM domain

GC motif

[KS]X[SCG]X(10)[KR]X(0,3)[DHSE]

B.

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648  MRLTENLSEKYIIGYGASSTVYKCVLKNGKAIAVKRLYSQYNHGAREFET

698  ELETVGSIRHRNLVSLHGFSLSPHGNLLFYDYMENGSLWDLLHGPSKKVK

748  LDWDTRLRIAVGAAQGLAYLHHDCNPRIVHRDVKSSNILLDEHFEAHLSD

798  FGIAKCVPAAKTHASTYVLGTIGYIDPEYARTSRLNEKSDVYSFGIVLLE

848  LLTGKKAVDNDSNLHQLILSRADDNTVMEAVDSEVSVTCTDMGLVRKAFQ

898  LALLCTKRHPMDRPTMHEVARVLLSLMPAPALKPSYTTASKTVDYTRYLA

948  TTPDLNHDGTDIGNNSSSDEQWFVRFGEVISKHTM-
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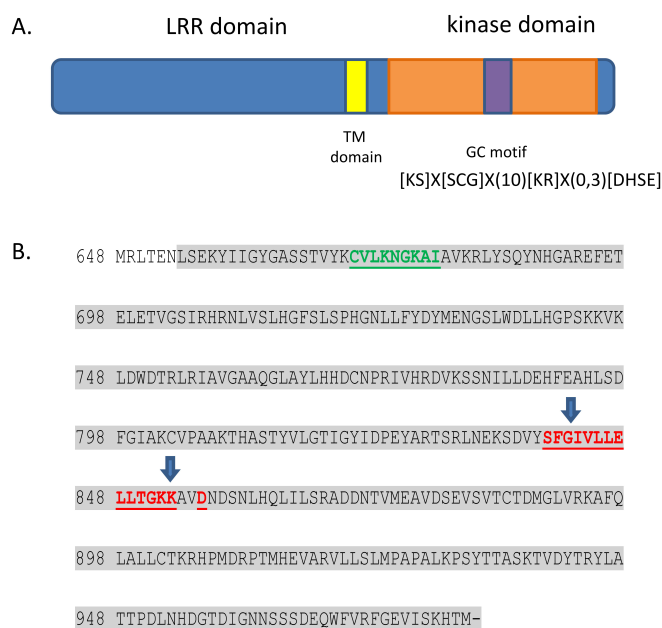
Fig. 1. Structural features of GC catalytic domain of BdERL1. (A) Representation of domain organization of BdERL1 containing a leucine-rich repeats (LLRs), transmembrane domain (TM), and fourteen amino acid long search motif GC motif embedded in kinase domain. (B) Amino acid sequence of BdERL1 fragment showing the kinase domain (grey shaded) with GC center (underlined and red) and potential CaM binding domain (underlined and green). Arrows indicate two functional residues in the GC catalytic center at 3 and 14 positions mutated for functional studies of BdERL1.

ily shows very high homology to other kinases of monocotyledonous plants, such as *Hordeum vulgare* (92% amino acid sequence identity), *Aegilops tauschii* (92%), *Triticum aestivum* (92%), *Oryza brachyantha* (91%), *Panicum miliaceum* (91%), and *Sorghum bicolor* (90%). Lower similarity (~70%) was observed between BdERL1 and LRR-RLKs belonging to dicotyledonous plants such as *Camellia sinensis* (76%), *Eucalyptus grandis* (76%), *Vitis vinifera* (76%), *Ricinus communis* (73%) or *A. thaliana* (72%). However, a high level of identity among the plant ERECTA-like protein family is noticeable regardless of their divergent phylogenetic evolution.

Alignment of the BdERL1 aa sequence with the three most experimentally studied ERECTA proteins from *A. thaliana* (AtER At2g26330; AtERL1 At5g62230; AtERL2 At5g07180) revealed the highest (72%) identity with the AtERL2 protein. All of these proteins possess a 14 aa long GC motif with highly conserved functional residues and architecture characteristics for moonlighting kinases. The significant level of similarity suggests common (moonlighting) functions for these proteins in both plant species. It was revealed that AtERL2 is involved in the determination of epidermal cell division, i.e., whether the cell should divide proliferatively to produce pavement cells or divide asymmetrically to generate stomatal complexes [22]. The

GC activity has not been studied in any ERECTA protein from *A. thaliana* thus far; therefore, BdERL1 is the first experimentally confirmed guanylyl cyclase belonging to the ERECTA family.

4.2 Computational assessment of the BdERL1 GC domain

We performed computational assessments using structural and molecular docking strategies to determine the catalytic feasibility of the BdERL1 GC domain. Since the BdERL1 kinase domain has 44% amino acid identity to AtBRI1 and an even higher identity at the corresponding GC center (78%), we modeled BdERL1 against the AtBRI1 crystal structure (PDB ID: 5LPV) [9]. We show in this model that the GC catalytic center is solvent exposed, allowing for substrate interactions and also presumably for catalysis (Fig. 2A). Furthermore, the GC center of BdERL1 contains a characteristic alpha-helix barrel followed by a loop secondary fold that is an innate feature of experimentally validated plant GCs and ACs identified by this motif-based approach [20]. Further probing of the GC center by molecular docking of GTP suggested that GTP can dock at the GC center with a good free energy value (–5.6 kcal/mol) and a favorable binding pose where the negatively charged phosphate end of GTP points toward K853 (position 14 of the motif) while the guanine end is surrounded by negatively charged residues including S840 (position 1 of the motif) (Fig. 2), similar to the structurally resolved GC centers [23]. We also performed the same docking simulation with ATP and found that the mean binding affinity was lower than that obtained with GTP (data not shown).

4.3 *In vitro* analysis of the GC activity of wild-type BdERL1

To test whether BdERL1 is able to generate cGMP *in vitro*, the fragment (BdERL1$_{648-982}$) containing the predicted GC center was first expressed in *E. coli* and affinity purified. The molecular mass of recombinant GST-BdERL1 protein predicted *in silico* was 64.3 kDa (https://web.expasy.org/cgi-bin/compute_pi). The prediction was consistent with the molecular weight of the GST-BdERL1 protein band observed on the polyacrylamide gel (Fig. 3).

Experimental analysis confirmed the bioinformatics prediction of the webtool GCPred [21] as well as the subsequent computational assessments involving both structural assessments and molecular docking simulations, thus suggesting that BdERL1 can act as a functional guanylyl cyclase. The highest GC activity of the BdERL1 protein was observed in the presence of 1.5 mM GTP, magnesium, and manganese ions, where the cGMP concentration reached its maximum of ~70 pmol mg protein^{-1}·min^{-1} at 30 °C. The enzyme exhibited a K$_m$ of 0.294 mM for GTP, and the V$_{max}$ was 72 fmol cGMP μg protein^{-1}·min^{-1} (Fig. 4A, Fig. 5). The BdERL1 activity was similar or higher compared to that of the results previously determined for other plant so-called moonlight proteins with guanylyl cyclase
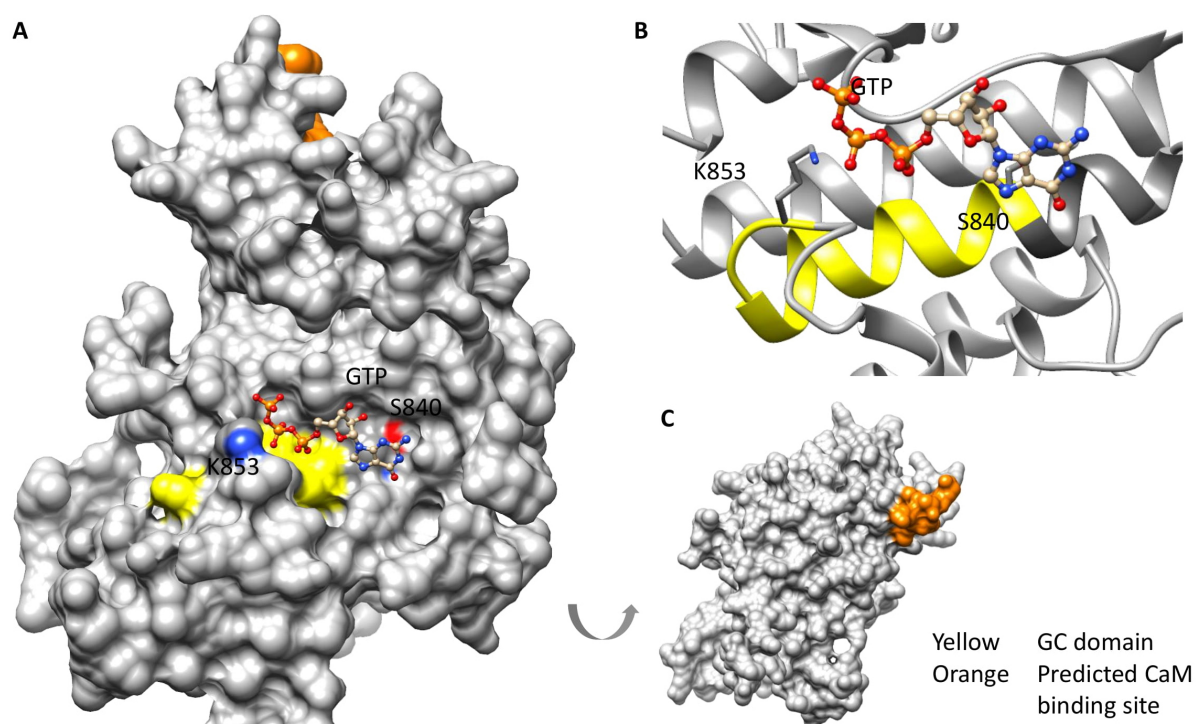
Fig. 2. Computational assessment of the BdERL1 GC domain. Docking of GTP to the GC center of BdERL1 and the interaction of GTP with the key residues at the catalytic center (yellow) is shown as (A) surface and (B) ribbon models, respectively. Conserved key amino acids at position 1 (S840) and position 14 (K853) of the motif are shown as individual residues and labelled accordingly in the models. (C) The predicted CaM binding site (orange) is located at the rear of the kinase and GC domains.
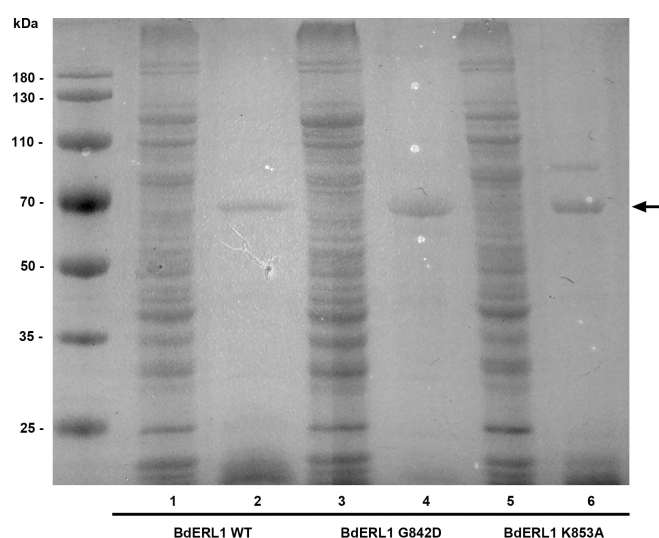

Fig. 3. SDS-PAGE of BdERL1 WT, BdERL1 $G_{842}D$ and BdERL1 $K_{853}A$. Lane 1 - protein fractions from IPTG-induced *E. coli* BL21 containing the construct pGEX-6P1-BdERL1 WT. Lane 2- purified GST-BdERL1 WT fusion protein. Lane 3 - protein fractions from IPTG-induced *E. coli* BL21 containing the construct pGEX-6P1-BdERL1 $G_{842}D$. Lane 4- purified GST-BdERL1 $G_{842}D$ fusion protein. Lane 5 - protein fractions from IPTG-induced *E. coli* BL21 containing the construct pGEX-6P1-BdERL1 $K_{853}A$. Lane 6- purified GST-BdERL1 $K_{853}A$ fusion protein.

activity, BdPepR2 [24], HpPepR1 [18], AtPepR1 [25], At-BRI1 [26]. However, there have been some concerns about the biological significance of the low amounts of cGMP and reduce GC activity compared to their animal counterparts. The role of guanylyl cyclase activity in moonlighting proteins has been discussed and questioned as other studies using less sensitive methods have not detected cGMP in case of AtBRI1 [9, 27].

Moreover, the BdERL1 protein was also able to convert ATP to cyclic AMP, but the catalytic activity was approximately two times lower than that with GTP as the substrate (Fig. 4B). This suggests a significant reduction in affinity for ATP at the catalytic center, which is consistent with the data obtained from the molecular docking simulations, which also predicted a lower binding affinity for ATP compared to GTP at the catalytic center. Cross-reactivity is not uncommon for plant GCs and ACs since substrate discrimination is normally poor for moonlighting centers, and this innate characteristic has been described in previous reports [13, 16, 17]. Notably, the addition of an equivalent amount of ATP and GTP (1:1 ratio) into the reaction mixture generated cGMP and cAMP in amounts that were both lower than those achieved by their respective substrates alone, although the concentration of the cAMP product remained two times lower than that of cGMP, thus clearly showing a preference for GTP over ATP as the substrate (Fig. 4B). Since nucleotide triphosphates share very
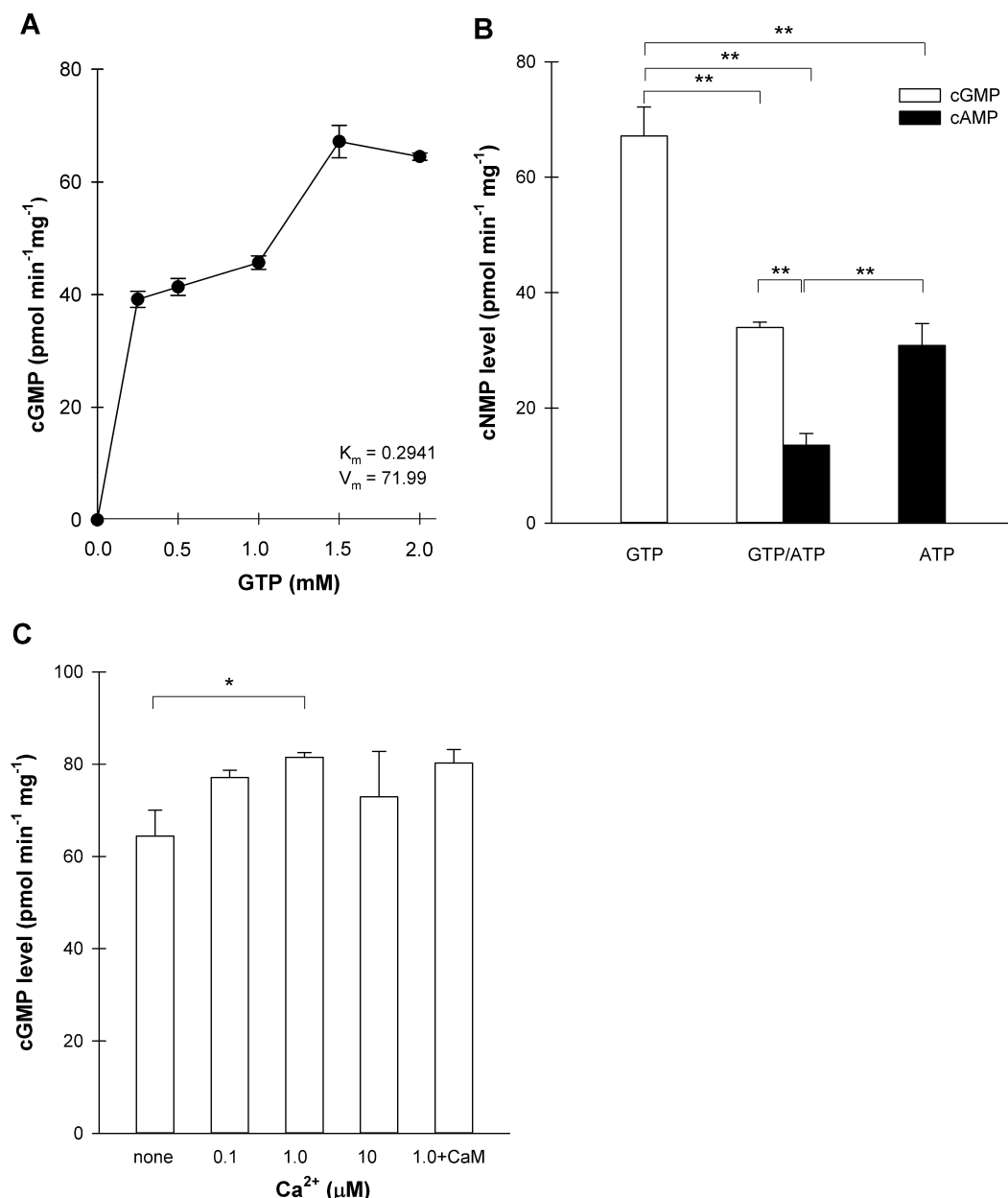
A

B

C

Fig. 4. Enzymatic guanylyl cyclase activity of recombinant BdERL1 WT. (A) Recombinant BdERL1 WT activity in response to various concentrations of GTP. Reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM MnCl$_2$, 5 mM MgCl$_2$, GTP (0.25–2 mM), and 5 μg of BdERL1 protein in a final volume of 100 μL. (B) Determination of BdERL1 WT substrate specificity. Reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM MnCl$_2$, 5 mM MgCl$_2$, 1.5 mM GTP or/and 1.5 mM ATP, and 5 μg of the purified protein in a final volume of 100 μL. (C) The effect of calcium and calmodulin AtCaM1 on the guanylyl cyclase activity of BdERL1 WT. Reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM MnCl$_2$, 5 mM MgCl$_2$, 1.5 mM GTP, Ca^{2+} (0.1–10 μM) or 0.5 μg of AtCaM1 recombinant protein supplemented with 1.0 Ca^{2+} μM and 5 μg of the purified protein in a final volume of 100 μL. Data are mean values (n = 3), and error bars show standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test (* $p < 0.05$; ** $p < 0.01$).

similar size and structural features, ATP could compete with GTP for the binding site at the GC center.

Previous reports have shown that the activity of plant transmembrane GCs called moonlight proteins can be modulated by calcium ions [28–30]. *In vitro* experiments have shown that the GC activity of the phytosulfokine receptor AtPSKR1 is significantly enhanced by calcium at physiological levels (0.1–10 μM), and more impor-

tantly, the same concentration of Ca^{2+} that activates GC activity also exerts an inhibitory effect on kinase activity [31]. The authors suggested that Ca^{2+} can play the role of a molecular switch for the guanylyl cyclase/kinase activity of select plant proteins. Based on previous studies, we therefore examined the effects of calcium (0–10 μM) on the GC activity of BdERL1. The results showed a subtle but statistically significant impact from Ca^{2+} (1 μM)
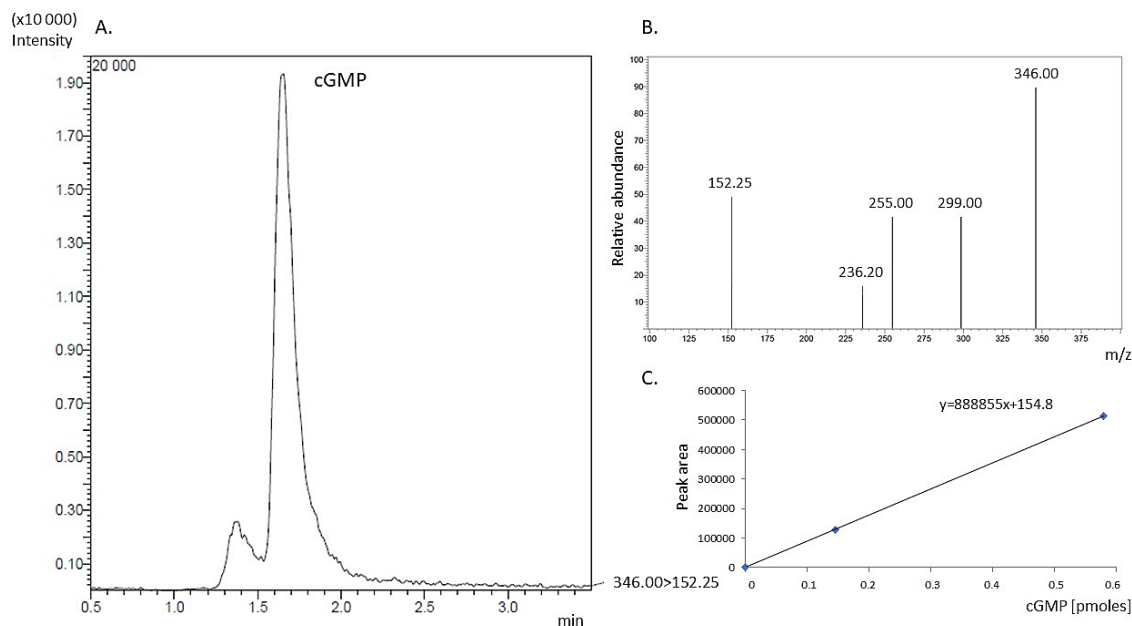
255

Fig. 5. Detection of cGMP generated by BdERL1 by LC-MS/MS. (A) Determination of guanylyl cyclase activity of the BdERL1 protein by LC-MS/MS. Ion chromatogram of cGMP was generated from a reaction mixture containing 5 μg of purified protein and GTP as a substrate in the presence of 5 mM MnCl$_2$ and 5 mM MnCl$_2$. (B) Inset showing parent cGMP ion at *m/z* 346.00 [M + H]+ and corresponding fragmented daughter ion at *m/z* 152.25 [M + H]+. Fragmented product ion was used for quantitation. (C) Inset showing the cGMP calibration curve performed with 0–0.7 pmol of pure cGMP on the column.
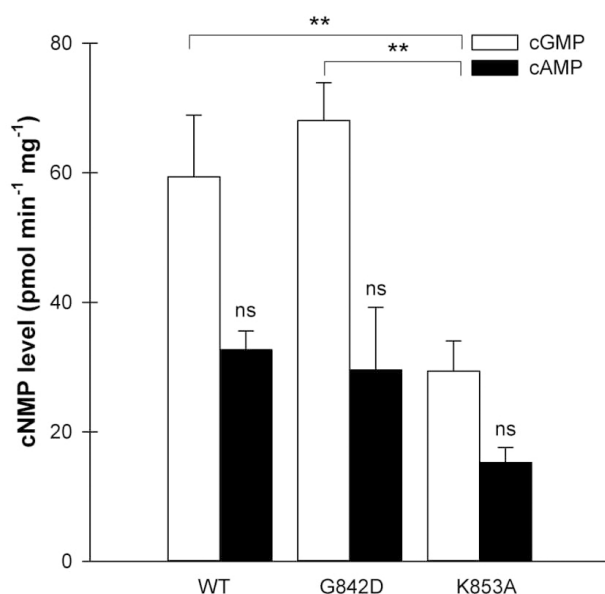

Fig. 6. Detection of cGMP and cAMP generated by BdERL1 WT, BdERL1 G$_{842}$D and BdERL1 K$_{853}$A. LC–MS/MS quantification of cGMP/cAMP levels generated by BdERL1 WT recombinant and mutated proteins in presence of 1.5 mM GTP or 1.5 mM ATP, 5mM MnCl$_2$ and MgCl$_2$ and 5 μg of purified proteins. Data are mean values (n = 3), and error bars show standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test (* $p < 0.05$; ** $p < 0.01$).

on the cGMP level produced by BdERL1 (Fig. 4C). Since no known direct calcium ion binding sites have been detected for plant GCs, we decided to examine the possibility of BdERL1 regulation via the Ca^{2+}/CaM calmodulin pathway [28, 31]. Bioinformatics analysis of the amino acid sequence of BdERL1 revealed that the calmodulin-binding site is localized within the kinase domain of the protein (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html) (Fig. 1B). Therefore, we experimentally tested the effect of calmodulin isoform 1 (AtCaM1) from *A. thaliana* on the GC activity of BdERL1. However, the GC activity of BdERL1 was not affected by calmodulin (Fig. 4C). Additionally, the amount of AtCaM1 (0.5–10 μg) also had no effect, the activity remained the same level (data not shown). The 3D model of BdERL1 also revealed the absence of a notable cleft or cavity that may accommodate CaM. It is therefore likely that Ca^{2+} modulation of moonlighting plant GCs is not achieved through the Ca^{2+}/CaM calmodulin pathway (Fig. 2C).

4.4 *In vitro* analysis of the GC activity of BdERL1 mutants

The wild-type (WT) BdERL1 protein (without amino acid sequence changes) and two BdERL1 proteins with mutations to the GC catalytic center were generated. It was previously described in an animal retinal guanylyl cyclase that substitution of the amino acid that confers substrate specificity can convert a GC into an AC [32]. In the case of plant GCs, the amino acid at position 3 of
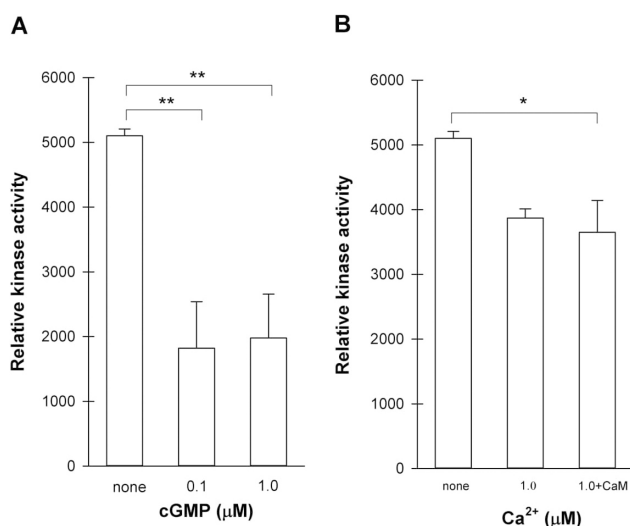
A

B

Fig. 7. Determination of kinase activity of recombinant BdERL1 WT by Kinase–Glo plus luminescent kinase assay. (A) BdERL1 WT kinase activity in response to various concentrations of cGMP. Reaction mixture contained 25 mM Tris/HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 $\mu g/\mu L$ Histone Type III-S, 10 μM ATP (control), supplemented with 0.1 or 1 μM cGMP and 1 μg of BdERL1 protein in a final volume of 50 μL. (B) The effect of calcium and calmodulin AtCaM1 on the kinase activity of BdERL1 WT. Reaction mixture contained 25 mM Tris/HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 $\mu g/\mu L$ Histone Type III-S, 10 μM ATP (control), supplemented with 1 μM Ca^{2+} alone or 1 μM Ca^{2+} with 0.5 μg AtCaM1 with 1 μg of BdERL1 protein in a final volume of 50 μL. Data are mean values (n = 3), and error bars show standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test (* $p < 0.05$; ** $p < 0.01$).

the motif was hypothesized to confer substrate specificity [20, 33], and therefore, we mutated this amino acid (G_{842}) in BdERL1 to the negatively charged aspartic acid residue (D) to investigate whether this mutation affects the GC and AC catalytic activities. As previously described, site-directed mutagenesis by substituting amino acids at the positions responsible for substrate specificity can turn a GC into an AC and *vice versa* [8, 32, 34]. However, our results showed that mutation at position 3 of BdERL1 (G_{842}D) did not affect GC and AC activities, as the cGMP and cAMP levels produced by the mutant protein were comparable to those produced by WT BdERL1 (Fig. 6). This result implies that the catalytic activity of BdERL1 is regulated by other means, such as localized substrate concentrations, which could in turn behave transiently to precisely modulate the GC/AC activities of BdERL1 in response to upstream signals, much like in human interleukin-1 receptor associated kinase 3 (IRAK3) GC, which was also identified by this motif-based approach [35]. Interestingly, a similar result was observed with the ortholog AtBRI1, in which BdERL1 shares high identity with both the kinase and GC regions. In AtBRI1, mutating glycine at position 3 of the catalytic center to a positively charged lysine did not yield any change in GC activity compared to the wild-type en-

zyme [23], although this mutation in AtPSKR1 GC resulted in reduced GC activity [14], an effect that was confirmed in a separate subsequent study by [29]. Notably, another study on the GC in AtPNP-R1 (a natriuretic peptide receptor from *A. thaliana*) revealed that threonine at position 3 of the motif, when substituted with the negatively charged glutamic acid, resulted in a nearly 4-fold reduction in the cGMP level but had no effect on AC activity; however, mutating threonine to aspartic acid did increase the cAMP generation of this AtPNP-R1 GC by as much as threefold [16]. Clearly, mutation to highly conserved amino acids at the GC catalytic center can alter the tertiary structure and surface charges or the hydrophobicity of the cavity responsible for substrate binding to different extents, and as such, candidate GC or AC proteins must be evaluated both structurally and experimentally for substrate preference and substrate binding affinities [8].

In a second mutated BdERL1 protein, the amino acid lysine (K) at position 14 was mutated to alanine (A) (K_{853}A). Based on the 3D model of BdERL1, it can be assumed that lysine at position 14 plays a very important role in the interaction with the negatively charged phosphate of GTP. Computational simulations of GTP docking with the GC catalytic centers of four previously identified plant guanylyl cyclases (AtPSKR1, AtPEPR1, AtBRI1, AtWAKL10) all revealed that mutation of the amino acid at position 14 to leucine resulted in reduced affinity for GTP [8]. Indeed, *in vitro* analysis of the enzymatic activity of the BdERL1(K_{853}A) mutated protein showed that both the cGMP (~30 pmol mg protein$^{-1} \cdot$min^{-1}) and cAMP (~15 pmol mg protein$^{-1} \cdot$min^{-1}) levels generated by the BdERL1(K_{853}A) mutant protein were two times lower than those generated by WT BdERL1 (Fig. 6). Consistently, previous experimental findings have shown that mutating these highly conserved positively charged residues to AA in AtBRI1 resulted in a marked decrease in GC activity [23]. Therefore, both *in silico* and *in vitro* analyses suggest that a positively charged amino acid [K or R] at position 14 of the GC catalytic center is crucial for optimal GTP/ATP catalysis.

4.5 *In vitro* analysis of the kinase activity of wild-type BdERL1

The architecture of previously characterized LRR-RLKs reveals that they are moonlighting proteins with GCs embedded within the primary kinase domain [36, 37], and sequence analysis has also disclosed the same domain organization for BdERL1. Here, we showed that BdERL1 is also a functional kinase, and more importantly, its activity is inhibited by cGMP (Fig. 7A). The addition of cGMP at concentrations of 0.1 or 1 μM to the reaction mixture resulted in a twofold reduction in BdERL1 kinase activity. This result affirmed our hypothesis that cGMP, as a product of the moonlighting GC domain, can directly modulate kinase activity, an intramolecular regulatory feature that is consistent with moonlighting domains in complex proteins

[17, 35, 38, 39]. This regulatory effect has also been observed in previously characterized GC kinases AtPSKR1 and AtBRI1 [14, 23].

Calcium ions were previously proposed to be a bimodal switch between GC and the kinase activity of AtPSKR1 [29]. The kinase activity of AtPSKR1 was directly inhibited by free calcium ions, and its guanylyl cyclase activity was enhanced at similar calcium concentrations. Consistent with the findings of AtPSKR1, the molecular switch function of calcium also operates in BdERL1, where Ca^{2+} reduces the kinase activity while acting as an activator of the moonlighting GC domain, which will in turn generate more cGMP to reduce its kinase activity even further (Fig. 7A). In plants, calcium may directly or indirectly regulate protein kinases [30, 40], while bioinformatics and *in vitro* analyses have also revealed the presence of a calmodulin-binding motif within the kinase domains of AtPSKR1 and AtBRI1; thus, it is conceivable that calcium may modulate the cyclase/kinase activities indirectly via CaM isoforms [23, 31]. However, although Ca^{2+} significantly inhibited the kinase activity of BdERL1, there was no significant difference in its activity with the addition of calcium alone or with the addition of both calcium and calmodulin, as both of these conditions inhibited the kinase to a similar extent (Fig. 7B). Moreover, the amount of AtCaM1 (0.5–10 μg) also had no effect, the activity remained the same level (data not shown). Although bioinformatics analysis has suggested the presence of a calmodulin-binding site in the kinase domain, the structure lacks a clear cavity for the accommodation of CaM, as revealed by our structural analysis with the BdERL1 model (Fig. 2C).

5. Conclusions

In conclusion, we employed a combination of computational and experimental approaches to identify a functional GC that is embedded within the kinase of BdERL1. Moreover, our mutagenesis studies revealed the catalytic roles of key conserved amino acid residues at the GC center. Importantly, probing the kinase and GC with Ca^{2+} and/or cGMP shed light on the intramolecular regulation of BdERL1, thus confirming the dual activity of its cytoplasmic region. Given the involvement of ERL1 in inflorescence architecture, stomatal patterning and phloem-xylem organization [41–43], our results not only reveal an important signaling component in the LRR-RLK family of proteins but can also guide research that focuses on ERECTA-dependent receptor-ligand signaling during plant development and responses to biotic and abiotic stresses [42, 44].

6. Author contributions

All authors have read and approved the final manuscript. Conceptualization—BŚB and ASJ; methodology—BŚB and MD; formal analysis—BŚB, MD,

KH and AW; investigation—BŚB; writing—original draft preparation—BŚB; writing—review & editing—MD, AW, KJ and ASJ; visualization—KJ; supervision—KJ and ASJ; funding acquisition—BŚB, MD, AW.

7. Ethics approval and consent to participate

Not applicable.

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10. Conflict of interest

The authors declare no conflict of interest.

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