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RESEARCH

An *Arabidopsis* Pentatricopeptide Repeat Is a Moonlighting Protein with Cross-talking In Vitro Adenylyl Cyclase and Kinase Activities

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Abstract

Downstream signalling involving adenylyl cyclases (ACs) and kinases is a key component of several processes in plants including cell division, growth, and response to stress. ACs are enzymes that generate the second messenger molecule, 3′,5′-cyclic adenosine monophosphate (cAMP) from 5′-adenosine triphosphate (ATP) while kinases are enzymes that catalyze the addition of a phosphate group to other molecules (trans-phosphorylation) or themselves (auto-phosphorylation). Apparently, while there has been an expanded record of various ACs and kinases identified in plants, no plant molecule to date has been shown to possess both the AC and kinase activities/functions and with such activities/functions having the characteristic of cross-talking interactions. Therefore, in an endeavor to find such a molecule, we searched the amino acid sequence of a known *Arabidopsis* AC, pentatricopeptide repeat (AtPPR) protein, and found a kinase-specific sequence signature (KSSS), which we speculated to be working in synergy with the AC center in this protein during downstream signalling. So, in order to test if this additional center is catalytically active and perhaps also having some cross-talking interactions with the AC center, we cloned, expressed, and affinity purified a truncated version of AtPPR, harboring both the AC and KSSS centers (AtPPR-AC/K). When tested in vitro, the recombinant AtPPR-AC/K showed a Mn^{2+}-dependent AC activity that is positively enhanced by Ca^{2+} and HCO_3^- and a trans-/auto-phosphorylation kinase activity capable of utilizing both ATP and GTP as substrates and specific to the serine, threonine, and tyrosine amino acids as target residues. In addition, the kinase activity of AtPPR-AC/K was found to be reduced by cAMP while at the same time, it was totally shut down by Ca^{2+}. This thus qualified both cAMP and Ca^{2+} as molecular switches or modulators, capable of regulating AtPPR functions through cross-talking interactions between the activities of its two domains. Our work, therefore, has essentially established AtPPR as the first member of a new class of moonlighting proteins with AC and kinase activities that have cross-talking interactions between themselves, conceivably presenting this protein as an ideal candidate for further explorations to improve plants, particularly agricultural crops.

Keywords *Arabidopsis thaliana* · Pentatricopeptide repeat · Moonlighting proteins · Adenylyl cyclase · Kinase · Cross-talking

Introduction

In plant biology, there is an unclarified but intriguing concept that is emerging and growing. This concept, termed "cross-talking" is defined as an interaction of two or more signalling pathways at different levels to ensure appropriate downstream responses (Klimecka and Muszyńska 2007). The interaction or integration of various signalling pathways via common components can either result in the generation of a similar response to different stimuli or alternatively having different signalling pathways affecting each other's outcome(s) negatively or positively (Klimecka and Muszyńska 2007). Due to this new emerging concept

Article Note AtPPR was identified as the first-ever moonlighting protein in plants with AC and kinase activities that have intramolecular cross-talking.

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of cross-talking, it is now slowly becoming clear and more apparent that the various pathways, previously studied as linear and/or isolated systems, are actually part of a more complex signalling network, with several overlaps among each other and also within their own individual branches (Zaccolo et al. 2005). For instance, it is now clear that many genes are inducible by more than one stimulus, and this is crucial because in reality, plants do concurrently encounter different stresses in various combinations and at different times, and therefore, must present an integrated response (Zaccolo et al. 2005). Fortunately, it has also been realized that in plants, there are proteins that exist as multi-domain and multi-functional molecules, thus opening opportunities for further studying and better understanding the concept of cross-talking. All that is required is to identify such protein molecules, experimentally confirm their catalytic activities, and then further characterize their biological functions.

Within the plant nucleotide cyclase (NC) family, i.e., adenylyl cyclases (ACs) and guanylyl cyclases (GCs), there is growing evidence that several distinctly different domain architectures (Chinkers et al. 1989) exist that tend to combine with other domains to make multi-domain enzymes or "moonlighting" proteins with multiple functions (Jeffery 2003). ACs and GCs generate the second messenger molecules, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), respectively, that have various important signalling and cell communication functions (Newton and Smith 2004). cAMP controls the cell cycle (Ehsan et al. 1998), transport of sodium ions via the voltage-independent channels (VICs) (Maathuis and Sanders 2001), stomatal movements (Jin and Wu 1999), growth and re-orientation of pollen tubes (Tezuka et al. 1993), and stress response (Choi and Xu 2010) while cGMP has a role in nitric oxide (NO)-dependent signalling (Moreau et al. 2010), salt stress and drought stress (Donaldson et al. 2004), ozone stress (Ederli et al. 2008), and response to pathogens (Meier et al. 2009).

In GCs, the GC domain co-exists and/or co-functions with canonical domains such as the kinase domain in AtWAKL10 (Meier et al. 2010), AtPepR1 (Qi et al. 2010), AtPSKR1 (Kwezi et al. 2018), AtPNPR1 (Turek and Gehring 2016), AtBRI1 (Kwezi et al. 2018), AtDGK4 (Vaz Dias et al. 2019), OsWAKL21.2 (Malukani et al. 2019), and SlGC17 and SlGC18 (Rahman et al. 2020), the natriuretic peptide-binding domain in AtPNPR1 (Turek and Gehring 2016), and the nitric oxide-binding domain in AtNOGC1 (Mulaudzi et al. 2011) and AtDGK4 (Vaz Dias et al. 2019). In ACs on the other hand, the AC domain combines and co-functions with signature domains such as the phosphodiesterase domain in MpCAPE (Kasahara et al. 2016); the phosphodiesterase and dehydrogenase domains in BnFolD (Kwiatkowski et al. 2022); the phosphodiesterase and permease domains in AtKUP5 (Al-Younis et al. 2018; Kwiatkowski et al. 2021); the permease domain in AtKUP7 (Al-Younis et al. 2015); multiple AC domains in AtLRRAC1, ZmRPP13-LK3, and BnFolD (Ruzvidzo et al. 2019; Yang et al. 2021; Kwiatkowski et al. 2021); the dioxygenase domain in AtNCED3 (Al-Younis et al. 2021); the hydrolase domain in MdTTM1 and MdTTM2 (Yuan et al. 2022); and the auxin-binding domain in AtTIR1, AtAFB1, AtAFB5, PpAFB1, PpAFB2, PpAFB3, and PpAFB4 (Qi et al. 2022; Wong et al. 2022).

With regard to moonlighting GCs, it has been shown that, in AtPSKR1, AtBRI, AtPepR1, and AtDGK4, cGMP produced by the GC domain reduces the kinase activity, pointing to some form of an intramolecular allosteric modulation of the activity of the kinase domain by the activity of the GC domain or a cross-talking interaction between the two domains (Wheeler et al. 2017; Kwezi et al. 2018; Wong et al. 2020). In addition, calcium was also shown to operate as a switch between the GC and kinase activities in AtPSKR1 (Muleya et al. 2014). With regard to moonlighting ACs, it has been shown in AtKUP5, BnFolD, and AtTIR1 that a functional AC center is essential for the proper functioning of these three proteins as K^+ transporter, folate oxidizer, and auxin receptor, respectively (Al-Younis et al. 2018; Kwiatkowski et al. 2022; Qi et al. 2022; Wong et al. 2022), further pointing to the occurrence of intramolecular interactions between different domains of moonlighting proteins.

Surprisingly, while various NCs with co-functional and/or cross-talking GC/kinase domains (or AC and other domains) have been reported in plants, no experimental evidence to date, on the existence of a plant NC with co-functional and/or cross-talking AC/kinase domains, is available. Therefore, and following the identification of a kinase catalytic center termed kinase-specific sequence signature (KSSS) (Muleya and Irving 2016) in a known *Arabidopsis* AC, pentatricopeptide repeat (AtPPR) protein (Ruzvidzo et al. 2013), we decided to study this protein molecule as a potential candidate of moonlighting proteins with probable cross-talking characteristics between the AC and kinase activities.

Results

In Vitro AC and Auto-phosphorylation Activities

The identification of kinases in plants involved a systematic querying of protein sequences with a kinase motif (Fig. 1a), searching for the presence of at least one of the three kinase-specific sequence signatures (KSSS) of the motif (Muleya and Irving 2016). On the other hand, the identification of ACs mostly involved querying protein sequences with an AC motif (Fig. 1b) derived from a GC search motif (Ludidi and Gehring 2003) through modification at position 3, changing [CTGH] to [DE] (Gehring 2010). This modification was primarily based on previous findings, which indicated

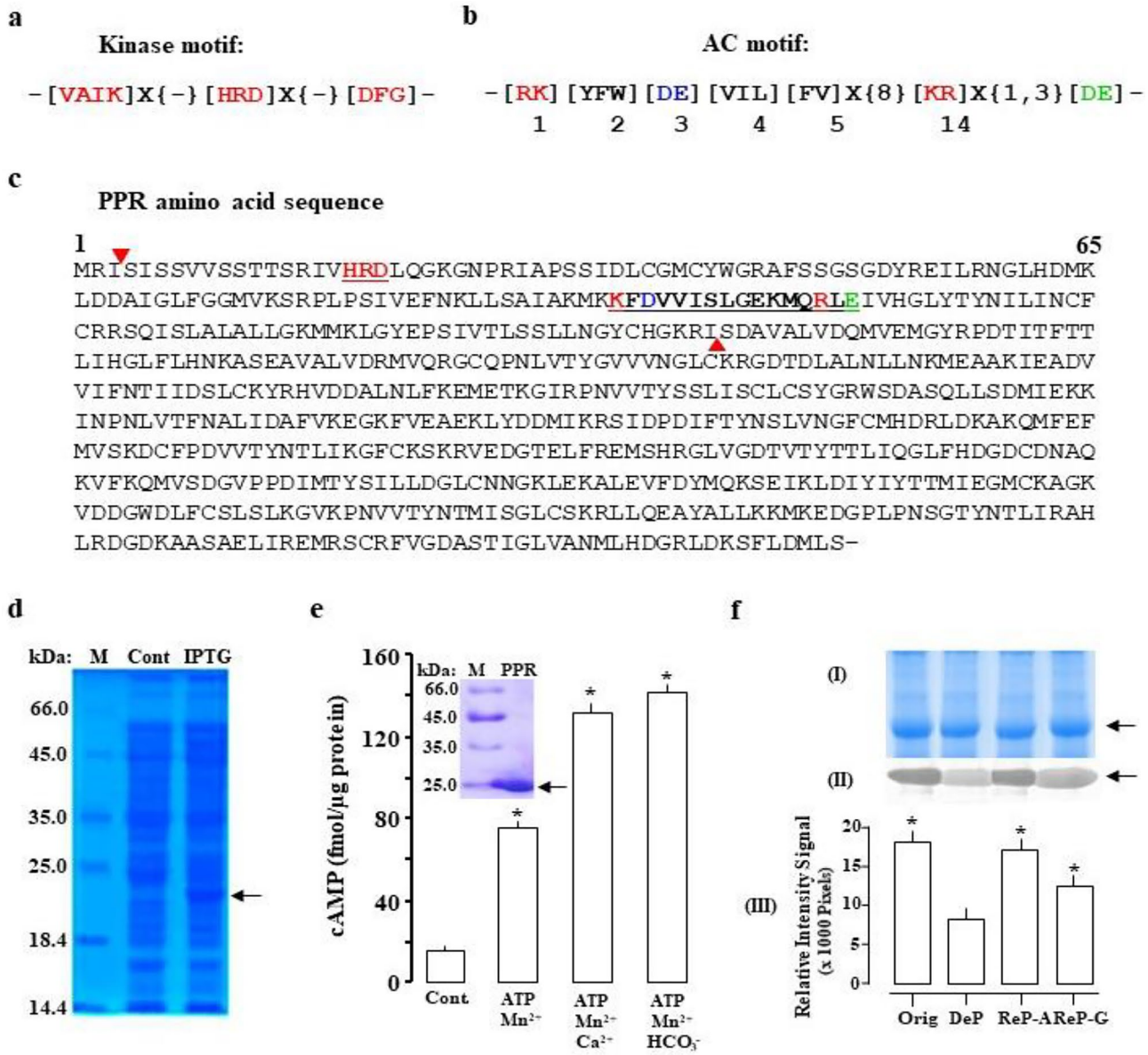

Fig. 1 Determination of the in vitro AC and auto-phosphorylation activities of AtPPR-AC/K. **a** The triad kinase motif showing its three kinase-specific sequence signatures (KSSS) in red. **b** The 14 amino acid AC search motif derived from annotated and experimentally tested GC and AC catalytic centers. The residue forming hydrogen bonding with purine at position 1 is highlighted in red, and the residue conferring substrate specificity in position 3 is highlighted in blue, while the amino acid in position 14, stabilizing the transition state from ATP to cAMP, is highlighted in red. The amino acid [DE] at 1–3 residues downstream from position 14 participates in Mg^{2+}/Mn^{2+} binding and is colored green. **c** The complete amino acid sequence of AtPPR with a KSSS between amino acids 17 and 21 (highlighted in red, bold, and underline) and the AC catalytic center between amino acids 99 and 116 (highlighted in different colors, bold, and underline), and the 168-amino acid sequence fragment, tested for AC and kinase activities, indicated within the inverted red triangles. **d** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of protein fractions (stained with Coomassie brilliant blue) from the induced (IPTG) and un-induced (Cont) cell cultures, where M is the molecular weight marker and the arrow marking the expressed recombinant AtPPR-AC/K protein. **e** cAMP generated by 5 µg recombinant AtPPR-AC/K in the presence of 1 mM ATP and 5 mM Mn^{2+}, or 1 mM ATP and 250 µM Ca^{2+}, or 50 mM HCO_3^- when 5 mM Mn^{2+} ion is the co-factor. Control reaction contained all other components except the protein, Ca^{2+} and HCO_3^-. Inset: A Coomassie brilliant blue stained gel after resolution of the affinity purified His-tagged recombinant AtPPR-AC-K (arrow) by SDS-PAGE. **f** Relative intensity signals generated by phosphoprotein bands (arrows) of 10 µg recombinant AtPPR-AC/K before de-phosphorylation (Orig), after de-phosphorylation (Dep), and after re-phosphorylation with either ATP (Rep-A) or GTP (Rep-G). The resolved bands are shown after staining with Coomassie brilliant blue (I) and Pro-Q Diamond (II) and in relation to their recorded relative intensity signals (III). Data are mean values ($n = 3$) and error bars show standard error (SE) of the mean. Asterisks denote values significantly different from those of control ($p < 0.05$) determined by the analysis of variance (ANOVA) and post hoc Student–Newman–Keuls (SNK) multiple range tests

that the conversion of GCs into ACs and vice versa could be easily achieved through a single mutation in the amino acid that confers substrate specificity in the catalytic center (Tucker et al. 1998; Roelofs et al. 2001). When the protein sequence of AtPPR was queried by both motifs, one KSSS was detected between amino acids 17 and 21 while the AC motif was detected between amino acids 99 and 116 (Fig. 1c). A fragment sequence of the At1g62590 gene (amino acids 4–171), harboring both motifs (Fig. 1c), was then cloned into a prokaryotic system and expressed into a 21.980 kDa His-tagged recombinant AtPPR-AC/K protein (Fig. 1d). To confirm and partly characterize the previously established in vitro AC activity of AtPPR (Ruzvidzo et al. 2013), the affinity purified version of AtPPR-AC/K (Fig. 1e, inset) was assayed in a reaction mixture containing ATP as substrate, Mn^{2+} as co-factor, and Ca^{2+} or HCO_3^- as modulator, followed by measurement of cAMP by enzyme immunoassay. Maximum activity was reached after 20 min of the reaction system, generating about 74 fmols/µg protein of cAMP in a reaction mixture containing protein only, approximately 131 fmols/µg protein of cAMP in a reaction mixture containing protein and Ca^{2+} and around 142 fmols/µg protein of cAMP in a reaction mixture containing protein and HCO_3^-, compared to only about 16 fmols/µg protein of cAMP of the control reaction (Fig. 1e).

To check if AtPPR can phosphorylates itself (auto-phosphorylation) in vitro, the purified recombinant AtPPR-AC/K was incubated in a reaction system that first de-phosphorylated it, followed by the addition of either ATP or GTP to allow for re-phosphorylation. Protein fractions were then resolved on a gel followed by reading of relative intensity signals of their respective phosphoprotein bands. As a result, a relative intensity signal of 8.5 × 103 pixels was detected on the de-phosphorylated protein compared to 18.7 × 103 pixels before de-phosphorylation and then intensity signals of 17.4 × 103 and 12.5 × 103 pixels after the de-phosphorylated protein was re-incubated with ATP and GTP, respectively, to re-phosphorylate itself (Fig. 1f). This thus signified an auto-phosphorylation activity for the AtPPR protein.

In Vitro Trans-phosphorylation Activity and Reaction Kinetics

To check if AtPPR can phosphorylates other proteins (trans-phosphorylation) in vitro, the purified recombinant AtPPR-AC/K was incubated in a reaction system containing ATP or GTP as substrate, serine/threonine (Ser/Thr), or tyrosine (Tyr) as target residues and cAMP or Ca^{2+} as possible modulators, followed by measurement of activity by fluorescence assay. Maximum activity was reached after 5 min of the reaction system, generating around 9 200 relative fluorescence units (RFU) in reaction mixtures containing the AtPPR-AC/K and ATP or GTP compared to only about 700 RFU of the control reaction containing AtPPR-AC/K only, when Ser/Thr was the target residue (Fig. 2a). In addition, the activity of AtPPR-AC/K was reduced from 9 200 to 5 250 RFU when the reaction mixture was supplemented with cAMP and to total diminishing when the reaction mixture was supplemented with Ca^{2+} (Fig. 2a). Furthermore, a maximum activity of around 9 350 RFU was reached for a reaction mixture containing the AtPPR-AC/K and Ser/Thr compared to approximately 7 700 RFU for a reaction mixture containing AtPPR-AC/K and Tyr, when ATP was the substrate (Fig. 2b). When the reaction kinetics of AtPPR-AC/K was calculated, using ATP as substrate, a K_m constant of 2.07 µM and V_{max} constant of 2794.87 nmoles/min/mg protein were obtained when Ser/Thr was the target residue (Fig. 2c) while a K_m constant of 2.96 µM and V_{max} constant of 2276.92 nmoles/min/mg protein were obtained when Tyr was the target residue (Fig. 2d).

Discussion

Moonlighting proteins are classified as single-chain polypeptides that perform two or more physiologically relevant and distinct functions, which do not result from gene fusions, splice variants, or multiple isoforms (Liu and Jeffery 2020). These proteins can also be cytosolic enzymes with other catalytic activities and capable of migrating to other different intracellular locations (Liu and Jeffery 2020). Moonlighting proteins are key to various cellular signalling systems in most organisms including plants (Turek and Irving 2021). In *Arabidopsis thaliana*, nucleotide cyclases (NCs) are an example of moonlighting proteins with cross-talking characteristics, which among them are several receptor kinases such as AtWAKL10, AtPepR1, AtPSKR1, AtBRI1, and AtDGK4 with intracellular guanylyl cyclases (GC) and kinase activities (Irving et al. 2012).

GCs generate 3′,5′-cyclic guanosine monophosphate (cGMP) from 5′-guanosine triphosphate (GTP) that acts as a second messenger, initiating various signal transduction pathways (Schaap 2005) while kinases are highly important and well-established enzymes that catalyze the addition of a phosphate group to other molecules (trans-phosphorylation) or themselves (auto-phosphorylation), resulting in localized increases in negative charges, which affect substrate conformation, forming switches in signalling cascades of all living cells (Hanks and Hunter 1995; Shiu and Bleecker 2001). Both enzymes are crucial in a variety of cellular processes such as disease resistance, self-incompatibility, hormone perception, growth, reproduction, and development (Stein et al. 1996; Clark et al. 1997). Apparently, while several moonlighting proteins with GC and kinase activities have been reported in plants (Irving et al. 2012), no protein in general, with adenylyl cyclase (AC) and kinase

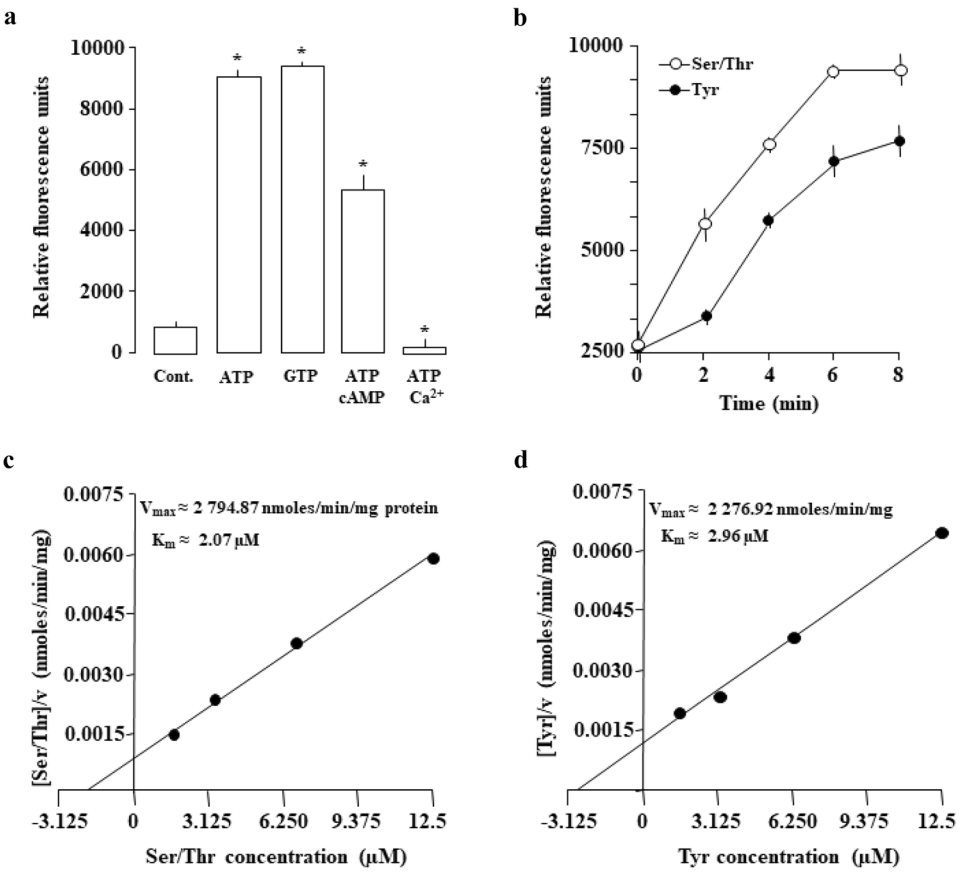

Fig. 2 Determination of the in vitro trans-phosphorylation activity and reaction kinetics of AtPPR-AC/K. Trans-phosphorylation activity of AtPPR-AC/K when **a** ATP or GTP is substrate either supplemented or not supplemented with cAMP or Ca^{2+} and when **b** Ser/Thr or Tyr is the target residue. Reaction kinetics of AtPPR-AC/K when **c** Ser/Thr or **d** Tyr is the target residue and ATP the sole substrate. Control reaction contained all other components except the protein. Data are mean values ($n = 3$), and error bars show SE of the mean. Asterisks denote values significantly different from those of control ($p < 0.05$) determined by ANOVA and post hoc SNK multiple range test

activities, exists. An AC is an enzyme capable of generating 3′,5′-cyclic adenosine monophosphate (cAMP) from 5′-adenosine triphosphate (ATP), which then acts as a second messenger for various downstream cellular processes such as control of the cell cycle (Ehsan et al. 1998), transport of Na^+ ions via the voltage-independent channels (VICs) (Maathuis and Sanders 2001), stomatal movements (Jin and Wu 1999), growth and re-orientation of pollen tubes (Tezuka et al. 1993), and response to stress (Choi and Xu 2010).

In this study, we considered the significance of ACs and kinases in plants and searched for a kinase catalytic motif in a known *Arabidopsis* AC, pentatricopeptide repeat (AtPPR) protein (Ruzvidzo et al. 2013), followed by assessment of its catalytic activity and the possible cross-talking interaction of such activity with the AC activity. We then apparently identified this motif in the form of a kinase-specific sequence signature (KSSS) (Muleya and Irving 2016) (Fig. 1a) co-existing with the AC catalytic motif (Gehring 2010) (Fig. 1b) of AtPPR (Fig. 1c) and then further tested this additional motif for activity. Ideally, ACs carry a rationally designed catalytic motif that was discovered through a systematic approach, which involved the identification of key amino acid residues in the catalytic center of known and experimentally tested NCs (Ludidi and Gehring 2003; Gehring 2010). Kinases, on the other hand, possess at least one of the three KSSS of its triad motif (HRD, VAIK, and DFG), necessary for phosphorylation as has already been exemplified in AtBRI1, AtWAKL10, AtPSKR1, and AtPepR1 (Muleya and Irving 2016).

We then began our analysis of the AtPPR protein by first confirming its known AC activity through an in vitro assaying of the expressed (Fig. 1d) and affinity purified (Fig. 1e, inset) form of its truncated version, harboring the AC motif and KSSS (AtPPR-AC/K) (Fig. 1c). In that assay, the AtPPR-AC/K showed a Mn^{2+}-dependent activity that was positively modulated by Ca^{2+} and HCO_3^- (Fig. 1e). This suggested that AtPPR could be a soluble AC (sAC) because sACs strictly utilize Mn^{2+} as co-factor and are specifically activated by both Ca^{2+} and HCO_3^- ions (Braun and Dods 1975; Steer and Levitzki 1975; Chen et al. 2000). When tested for kinase activity in vitro, the purified AtPPR-AC/K exhibited a wide range of attributes typical of other closely related plant moonlighting kinases with cross-talking characteristics. Firstly, AtPPR-AC/K could auto-phosphorylate itself (Fig. 1f) as well as trans-phosphorylating other protein targets (Fig. 2) just like AtPSKR1 and AtBRI1 (Muleya et al. 2016; Oh et al. 2000). Secondly, AtPPR-AC/K could utilize ATP and GTP as substrates (Fig. 2a) as was the case with AtPSKR1 and AtWAKL10 (Meier et al. 2010; Kwezi et al. 2011). Thirdly, AtPPR-AC/K phosphorylated the serine (Ser), threonine (Thr), and tyrosine (Tyr) residues as amino acid targets (Fig. 2b) as

was the case with AtPSKR1 and AtWAKL10 (Meier et al. 2010; Muleya et al. 2016). Lastly, AtPPR-AC/K displayed reaction kinetics parameters (Fig. 2d) closely related to those of AtWAKL10 (K_m = 2.7 µM and V_{max} = 2269.74 nmoles/min/mg protein) (Meier et al. 2010) and AtPSKR1 (K_m = 7.5 µM and V_{max} = 1800 nmoles/min/mg protein) (Kwezi et al. 2011).

In addition to the above-mentioned attributes, the kinase activity of AtPPR-AC/K was also found to be reduced by cAMP (a product of its AC motif), while at the same time, it was totally shut down by Ca^{2+} (that activated the AC activity) (Fig. 2a). This thus qualified both cAMP and Ca^{2+} as molecular switches or modulators, capable of regulating AtPPR functions through cross-talking interactions between the activities of its two domains. Notably, this scenario whereby the activity of a kinase domain in a moonlighting NC is reduced by the product of its associated cyclase domain has been previously observed in GCs such as AtPSKR1, AtBRI, AtPepR1, and AtDGK4, whereby cGMP reduced kinase activity, pointing to some form of an intramolecular allosteric or cross-talking interaction between the two domains (Wheeler et al. 2017; Kwezi et al. 2018; Wong et al. 2020). In addition, calcium was also previously shown to operate as a switch between the GC and kinase activities in AtPSKR1 (Muleya et al. 2014). With regard to moonlighting ACs, reports on cross-talking interactions between the cyclase domain and its other associated domains are also slowly beginning to emerge. For instance, it has been shown that a functional AC center in AtKUP5 and BnFolD is essential for their proper functioning as enzymes in the transportation of K^+ ions and metabolism of folate, respectively (Al-Younis et al. 2018; Kwiatkowski et al. 2022). In addition to this, the AC activity of AtTIR1 was also found to be tightly linked to its auxin perception and binding capabilities, thereby also ultimately tightly controlling the protein's main biological function of regulating root growth (Qi et al. 2022; Wong et al. 2022). Therefore, our results obtained in this study indicated that there is cross-talking interaction between the AC and kinase motifs of AtPPR as a moonlighting protein, whereby cAMP is centrally involved and Ca^{2+} possibly acting as a switch between the AC and kinase activities as is the case in AtPSKR1 (Muleya et al. 2014).

To augment our findings, we also undertook some in silico analysis of the AtPPR protein. Firstly, since AtPPR was found to possess an auto-phosphorylation capacity, we searched for possible phosphorylation sites within this protein and identified at least 23 sites (Fig. S1; Appendix A) to which this very same protein or other kinases might target for phosphorylation. Notably, 7 of those predicted sites formed part of the recombinant AtPPR-AC/K protein that was cloned and tested in this study (Fig. S1; Appendix A). Secondly, since Ca^{2+} appeared to be the switch between the AC and kinase activities of AtPPR, we searched for the possible existence of calcium-binding sites within this protein and predicted a single site in form of a 13-residue calmodulin-binding sequence (FNKLLSAIAKMKK) (Fig. 2S; Appendix B). Incidentally, this predicted site happened to be part of AtPPR-AC/K, positioned between its AC and kinase motifs (Fig. S2; Appendix B). Ideally, the existence of a calcium-binding site within AtPPR suggests that the effect of Ca^{2+} on this protein could be through physical interaction rather than a mere ionic effect. Lastly, after determining that AtPPR is a moonlighting protein with cross-talking interactions between its AC and kinase activities, we sought to check if there could be any homologs of this protein existing in the plant proteome harboring the same domain signatures. From this evaluation, a total of 7 proteins were identified, among which 3 were paralogs and 4 orthologs of AtPPR (Fig. S3; Appendix C). The paralogs included RPF2, RPF4, and S-adenosyl-L-methionine-dependent methyltransferase while the orthologs were one each from *Thlaspi arvense*, *Eutrema salsugineum*, *Capsella rubella*, and *Camelina sativa*. All these homologs except one have at least two AC motifs and at least two kinase motifs compared to AtPPR, which only has one apiece. Regrettably, when closely reviewed, not even one of these 7 identified homologs has yet been characterized to at least allow us infer function(s) to AtPPR. Luckily, in one of our recent studies, we characterized a PPR protein from soybean (http://www.sbg.bio.ic.uk) as an AC (Bobo et al. 2022), and this protein (Glyma.07G251000, ID: XP_003529590), just like AtPPR, also harbors both the AC and kinase motifs (kinase motif not yet characterized). Functionally, Glyma.07G251000 is annotated to be involved in early plant development and stress response (https://www.uniprot.org/), a role that could possibly be inferred to AtPPR. Overall, the identification of homologs for AtPPR indicates firstly that these types of proteins are widespread in plants and secondly that their catalytic motifs/signatures are highly conserved among plant species, particularly dicots.

All in all, findings of this study open a platform or space for more work to be undertaken on this new group of proteins, to further interrogate them and elucidate their role and significance in plants.

Conclusion

Work of our study herein has unarguably established AtPPR as the first-ever member of a new class of moonlighting proteins with AC and kinase activities that have cross-talking interactions between themselves, conceivably presenting this protein as an ideal candidate for further explorations to improve plants, particularly crops in the agricultural sector.

Recommendations

From this study, it is worth noting that the rationale behind us working on the AtPPR protein was not only motivated by its possibility of being a moonlighting protein with cross-talking interactions between its two catalytic motifs but also as a result of its other associated and/or annotated attributes. For instance, the protein is known to be exclusively expressed in chloroplasts and mitochondria, where it essentially controls the biogenesis of these two key organelles, through regulation of translation and modification (Barkan and Small 2014; Williams and Barkan 2003). The protein is also known to play a key role in the restoration of cytoplasmic male sterility (CMS), which is a maternally inherited trait that results in an inability of a plant to produce functional pollens (Pring et al. 1995). In this regard, AtPPR ideally suppresses this type of sterility linked to the expression of some mitochondrially encoded sterility-associated genes termed Rf genes that are also commonly found in other important crop plants such as petunia (Bentolila et al. 2002), rice (Komori et al. 2004), maize (Cui et al. 1996), and radish (Koizuka et al. 2003).

Therefore, by considering the above-given attributes of AtPPR together with our findings in this study, AtPPR could perhaps be targeted for organelle-level gene manipulations aimed at generating crop plants with high productivity and enhanced reproductive vigor. Aspects such as the deliberate massification of cellular chloroplast and mitochondrion numbers in crop plants for increased photosynthetic capacities and energy yields, respectively, or a direct manipulation of the Rf genes for enhanced crop fertility could be explored. In this regard therefore, further engagements involving in vivo, in planta, in silico, and/or mutational studies of this important protein are thus strongly recommended.

Materials and Methods

Generation of the Recombinant AtPPR-AC/K Expression Construct

Total RNA was extracted from 6-week-old *Arabidopsis* ecotype Columbia-0 (Col-0) seedlings using the RNeasy plant mini kit, in combination with DNase 1 treatment, as instructed by the manufacturer (Qiagen, Crawley, UK). Copy DNA (cDNA) sequence of At1g62590 (AtPPR) was retrieved from The Arabidopsis Information Resource (TAIR) website (https://www.arabidopsis.org) and analyzed for presence of the AC catalytic motif (Gehring 2010) using the PROSITE database located within the Expert Protein Analysis System (ExPASy) proteomics server (https://www.expasy.ch/) and any of the three kinase-specific sequence signatures (Muleya and Irving 2016) using the PhosPhAt 4.0 software (https://phosphat.uni-hohenheim.de). AtPPR cDNA synthesis from total RNA and the subsequent amplification of the AtPPR-AC/K gene fragment from the cDNA were simultaneously performed in the presence of two sequence-specific primers (forward: 5′-CG**GGATCC**GAGCCAAATCGAGAAGATGAGG-3′ incorporating a *Bam*HI restriction site and reverse: 5′-C**GAATTC**AGCATTTCCACCATTTGATCAAC-3′ incorporating an *Eco*RI restriction site), using a Verso 1-Step RT-PCR Reddy Mix kit and in accordance with the manufacturer's instructions (Fermentas International Inc., Burlington, Canada). The produced PCR product was then restriction digested with the *Bam*HI and *Eco*RI enzymes before being cloned into a similarly digested pCRT7/NT-TOPO expression vector (Invitrogen, Carlsbad, USA), to generate a pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct with an N-terminus His purification tag.

Expression of the Recombinant AtPPR-AC/K Protein

For expression of the recombinant AtPPR-AC/K protein, the pCRT7/NT-TOPO:AtPPR-AC/K fusion expression construct was used to transform (through heat shock at 42 °C for 2 min) chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells (Lucigen Corporation, Middleton., USA). Cells were grown in double strength yeast-tryptone (2YT) media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, and 4 g/L glucose; pH 7.0) containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, on an orbital shaker (250 rpm) at 37 °C. To induce expression, 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich Corp, Missouri) was added to the media when the optical density (OD_{600}) of the cell culture had reached 0.5 (approximately 2 h). The culture was then left to grow for a further 3 h at 37 °C.

Purification of the Recombinant AtPPR-AC/K Protein

The recombinant AtPPR-AC/K protein was purified by preparing a cleared cell lysate of the induced *E. coli* cells under non-native denaturing conditions, where the harvested cells were resuspended in a urea lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl (pH 8.0), 500 mM NaCl, 20 mM β-mercaptoethanol, 10 mM imidazole, and 7.5% (v/v) glycerol) at a ratio of 1 g pellet weight to every 10 mL buffer volume and mixing thoroughly with a magnetic stirrer for 1 h at 24 °C. The mixture was then centrifuged at 2500 *g* for 15 min and the supernatant collected as cleared lysate. The collected cleared lysate was transferred to 2 mL of 50% (w/v) nickel-nitrilotriacetic acid (Ni–NTA) slurry (Catalog # P6611;

Sigma-Aldrich Corp., Missouri, USA) pre-equilibrated with 10 mL of lysis buffer, and the two components gently mixed on a rotary mixer for 1 h at 24 °C. This step allowed for binding of the protein onto the Ni–NTA resin. The cleared lysate-resin mixture was loaded into an empty XK16 column (Bio-Rad Laboratories Inc., California, USA), allowed to settle and the flow-through discarded. The protein-bound resin was then washed three times with 30 mL of wash buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl; pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, 7.5% (v/v) glycerol, and 40 mM imidazole) to remove unbound proteins.

Refolding of the Recombinant AtPPR-AC/K Protein

The washed protein-bound resin was pre-equilibrated with 3 mL of refolding buffer A (8 M urea, 200 mM NaCl, 50 mM Tris–Cl (pH 8.0), 500 mM glucose, 0.05% (w/v) poly-ethyl glycol (PEG), 20 mM β-mercaptoethanol), in preparation for protein refolding on a Bio-Logic F40 Duo-Flow chromatography system (Bio-Rad Laboratories, California, USA). The denatured and purified recombinant AtPPR-AC/K protein was then refolded into its native and soluble form using a controlled linearized gradient system, whereby the 8 M urea salt in the refolding buffer A was gradually diluted to 0 M concentration, using a refolding buffer B (200 mM NaCl, 50 mM Tris–Cl (pH 8.0), 500 mM glucose, 0.05% (w/v) poly-ethyl glycol (PEG), 4 mM reduced glutathione, 0.4 mM oxidized glutathione, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)). The refolding of the denatured AtPPR-AC/K protein was run for 13 h at a flow rate of 0.5 mL/min. After refolding, the renatured recombinant protein was eluted in 2 mL of the non-denaturing native elution buffer (200 mM NaCl, 50 mM Tris–Cl (pH 8.0), 250 mM imidazole, 20% (v/v) glycerol, and 0.5 mM PMSF). The eluted native protein fraction was then freed from its buffering salts and excess water using a Spin-X UF concentrator device with a molecular weight cut off (MWCO) limit of 5000 (Product # 431,482; Corning Life Sciences Corp., New York, USA) and in accordance with the manufacturer's instructions. Concentration of the resulting eluted AtPPR-AC/K protein was then determined by the Bradford method (Bradford 1976) and an ND2000 NanoDrop spectrophotometer (Thermo Scientific Inc., California, USA).

Confirmation of the AC Activity of the Recombinant AtPPR-AC/K Protein

The AC activity of the purified renatured recombinant AtPPR-AC/K protein was assessed in vitro by measuring the cAMP content generated from a 200 µL reaction mix containing 5 µg of AtPPR-AC/K, 50 mM Tris–Cl (pH 8.0), 2 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich Corp., Missouri, USA) (phosphodiesterase inhibitor), 5 mM Mn^{2+}, and 1 mM ATP and or not supplemented with 250 µM Ca^{2+} or 50 mM HCO_3^-. Background cAMP levels in control reactions were measured in tubes that contained the incubation mediums but no protein or Ca^{2+} or HCO_3^-. In all cases, reactions were incubated at room temperature (24 °C) for 20 min and then terminated by the addition of 10 mM ethylenediaminetetraacetic acid (EDTA) as well as boiling for 5 min and cooling on ice for 2 min before centrifugation at 2300 *g* for 3 min. The resulting supernatant was assayed for cAMP content using the cAMP-linked enzyme immunoassaying system, following its acetylation protocol and as is described by the supplier's manual (Code: CA201; Sigma-Aldrich Corp., Missouri, USA). In all cases, each experiment was performed in triplicate ($n=3$) using three different protein extracts that had been independently prepared.

In Vitro Auto-phosphorylation Activity Assaying of the Recombinant AtPPR-AC/K Protein

Auto-phosphorylation kinase activity of the purified recombinant AtPPR-AC/K protein was assessed by first setting a de-phosphorylation reaction system, whereby 10 µg of AtPPR-AC/K protein was mixed with 400 units of the lambda phosphatase enzyme in the presence of 100 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazeethanesulfonic acid (HEPES), 2 mM dithiothreitol (DTT), 0.1 mM $MnCl_2$, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 50% glycerol, and 0.01% (v/v) Brij 35 (pH 7.5) (Merck Group, Darmstadt, Germany). The reaction mix was then incubated for 2 h at room temperature before either termination with 2X SDS loading buffer (125 mM Tris–HCl pH 6.8, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 4% (w/v) SDS, and 200 mM DTT) or a re-phosphorylation treatment following the addition of a 4X PhosStop reagent (Roche Products, Basel, Switzerland). In the second step, a re-phosphorylation reaction was initiated by adding 1 mM ATP or 1 mM GTP to the de-phosphorylated samples in the presence of 20 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, and 0.2 mM DTT and incubating for 30 min at room temperature. The reactions were then terminated with the addition of a 2X SDS loading buffer and boiling for 5 min at 95 °C. After boiling, the samples were allowed to cool down and then centrifuged at 10 000 *g* for 1 min.

The de-phosphorylation and re-phosphorylation as well as the untreated (control) protein samples were all resolved by SDS-PAGE on a 12% (v/v) gel, followed by staining with a Coomassie solution (20% (v/v) ethanol, 20% (v/v) methanol, 20% (v/v) acetic acid, and 0.5% (w/v) Coomassie stain) for 1 h. The stained gel was de-stained for 1 h (or until the bands were clear enough to be visualized) in a de-staining solution (20% (v/v) ethanol, 20%

(v/v) methanol, 20% (v/v) acetic acid, and 0.5% (w/v)). The de-stained gel was snap-photographed on a Chemi-Doc™ Imaging system (Bio-Rad Laboratories Inc.,) using the Bio-Image Lab™ software. The gel was then completely de-stained overnight in another de-staining solution (80% (v/v) acetic acid, 20% (v/v) ethanol, 20% (v/v) methanol). This de-staining solution, besides removing all the Coomassie stain from the gel, also fixed the gel by ensuring a complete removal of the SDS buffer that could later on interfere with the phosphorylation staining and signalling. In the next morning, the de-stained gel was washed three times (10 min per wash) with distilled water before being stained with the Pro-Q Diamond phosphoprotein G solution (Life Technologies Inc., Carlsbad, USA) in the dark for 90 min. The stained gel was then de-stained three times (30 min per each step) with the de-staining solution (20% (v/v) acetonitrile and 50 mM sodium acetate; pH 4.0) in the dark. The de-stained gel was finally washed twice (5 min per wash) with distilled water. The resultant phosphoprotein bands were then analyzed on a Chemi-Doc™ Imaging system (Bio-Rad Laboratories Inc.,) using Bio-Image Lab™ software at 488 nm blue laser (BL) and 520 nm band-pass (BP) and under a 40 nm emission filter. Their resultant relative intensity signals were then quantified into pixels, using ImageJ software (http://rsbweb.nih.gov/ij/). In all cases, each experiment was performed in triplicate ($n = 3$) using three different protein extracts that had been independently prepared.

In Vitro Trans-phosphorylation Activity Assaying of the Recombinant AtPPR-AC/K Protein

Trans-phosphorylation kinase activity of the purified recombinant AtPPR-AC/K protein was assessed in vitro by measuring its ability to direct the phosphorylation of special substrate peptides (Ser, Thr, and Tyr), using an Omnia™ Ser/Thr/Tyr-Recombinant system (Catalog # KNZ1241/KNZ3101; Life Technologies, Carlsbad, USA). A 50 µL reaction mixture containing 10 µg of AtPPR-AC/K, 1X reaction buffer, 1 mM ATP or GTP, 0.2 mM DTT, and 25 µM Ser/Thr or Tyr-peptide supplemented or not supplemented with 0.1 µM cAMP (Kwezi et al. 2011) or 0.1 µM Ca^{2+} (Bers et al. 2010; Muleya et al. 2014) was prepared in a Black FluoroNunc Maxisorp 96-well plate (AEC Amersham, Little Chalfont, UK) and incubated at 30 °C for 5 min. The reaction mix was then immediately excited at a wavelength of 360 nm (λ_{ex} 360) and fluorescence measured at the emission wavelength of 485 nm (λ_{em} 485) every minute for 20 min at 30 °C. Readings were recorded as RFU, using a Fluoroskan Ascent FL fluorometer (AEC Amersham, Little Chalfont, UK). In all cases, each experiment was performed in triplicate ($n = 3$) using three different protein extracts that had been independently prepared.

Reaction Kinetics of the Trans-phosphorylation Activity of AtPPR-AC/K

Individual reaction setups of increasing concentrations of the Omnia™ substrate peptide (0.0, 1.56, 3.125, 6.25, 12.5, and 25.0 µM) and 1 mM ATP or GTP as the sole substrate of phosphorylation were prepared and their activities recorded. Initial velocity for each of the used peptide concentration was then determined followed by a sketching of the Hanes plot (Irving et al. 2012). Kinetic constants (K_m and V_{max}) for the recombinant AtPPR-AC/K were then derived from this plot, where K_m was determined as the negative value of the x-intercept ($x = -K_m$, when $y = 0$) of a linear fit of the data while V_{max} was calculated from the y-intercept ($y = K_m/V_{max}$, when $x = 0$) of the same linear fit. Each experiment was performed in triplicate ($n = 3$) using three different protein extracts that had been independently prepared.

Searching for Phosphorylation Sites in AtPPR

The predicted phosphorylation sites in AtPPR were determined using the Group-based Prediction System Version 6 (https://gps.biocuckoo.cn/) (a server for prediction of phosphorylation sites in eukaryotes) with the AtPPR protein sequence as the query sequence at medium threshold (Wang et al. 2020). This system uses three machine learning algorithms together with evolutionary information to hierarchically make predictions with high accuracy.

Searching for Calcium-Binding Sites in AtPPR

The Calmodulin Target Database prediction program (http://calcium.uhnres.utoronto.ca/) was used to search for presence and frequency of the 17-residue calmodulin-binding site: KLWKKLLKLLKKLLKLG (Kauer et al. 1986; Ruzvidzo and Chatukuta 2023) in the AtPPR protein sequence. The program predicts the calmodulin-binding site sequences based on criteria such as hydropathy, α-helical propensity, residue weight, residue charge, hydrophobic residue content, helical, and occurrence of particular residues. In this regard, sequences are scored from 0 to 9, with the most likely binding site assigned a series of 9 s (Yap et al. 2000).

Searching for AtPPR Homologs with AC and Kinase Motifs

BLASTp search at NCBI (https://blast.ncbi.nlm.nih.gov/) was carried out using AtPPR protein sequence as the query sequence (Altschul 1993). Returned hits were then aligned by ClustalW, using its MegaX software to determine

homologs (Kumar et al. 2018). A search for presence of the relaxed AC motif ([RKS]X[DE]X(9,11)[KR]X(1,3)[ED]) and the triad kinase motif ([VAIK]X{-}[HRD]X{-}[DFG]) in each of the obtained homolog was then carried out on Prosite, using the ScanProsite tool (https://prosite.expasy.org/scanprosite/), and the number and frequencies of each motif were then determined (Sigrist et al. 2013).

Statistical Analysis

All assay data was subjected in triplicate sets ($n=3$) to one-way ANOVA (Super-Anova, Statgraphics Version 7, 1993; Statgraphics Corp., The Plains, VI, USA). Where ANOVA revealed significant differences between treatments, means were separated by the post hoc SNK multiple range test ($p<0.05$).

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Author Contribution OR conceived the idea and designed the study; TBD, KSS, EDB, AS, PC, and DTK conducted the experiments; OR and TBD wrote the manuscript; and all authors read, edited, and approved the manuscript.

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Declarations

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