The *Arabidopsis thaliana* K^+-uptake permease 7 (AtKUP7) contains a functional cytosolic adenylate cyclase catalytic centre

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ABSTRACT Adenylate Cyclases (ACs) catalyze the formation of the second messenger cyclic adenosine 3′, 5′-monophosphate (cAMP) from adenosine 5'-triphosphate (ATP). Although cAMP is increasingly recognized as an important signaling molecule in higher plants, ACs have remained somewhat elusive. Here we used a search motif derived from experimentally tested guanylyl cyclases (GCs), substituted the residues essential for substrate specificity and identified the *Arabidopsis thaliana* K^+-uptake permease 7 (AtKUP7) as one of several candidate ACs. Firstly, we show that a recombinant N-terminal, cytosolic domain of AtKUP7$^{1-100}$ is able to complement the AC-deficient mutant *cyaA* in *Escherichia coli* and thus restoring the fermentation of lactose, and secondly, we demonstrate with both enzyme immunoassays and mass spectrometry that a recombinant AtKUP7$^{1-100}$ generates cAMP *in vitro*.

Keywords: cAMP; Adenylate cyclase; Second messenger; Arabidopsis thaliana

1. Introduction

Adenylate cyclases (ACs) (EC 4.6.1.1) catalyze the formation of the universal second messenger cyclic adenosine 3′, 5′-monophosphate (cAMP) from adenosine 5'-triphosphate. Cyclic AMP participates in key signal transduction pathways in all living organisms ranging from the simple prokaryotes such as *Escherichia coli* to complex multicellular organisms including *Homo sapiens*. Cyclic AMP was first discovered in eukaryotic cells as the factor that mediates the effects of hormones [1] while in lower eukaryotes including slime molds and fungi, cAMP regulates signaling pathways [2] that are critical for adaptation and survival [3-5]. In higher plants, cAMP has a role in many biological processes such as the activation of protein kinases in the leaf of rice [6] and the promotion of cell division in tobacco BY-2 cells [7]. More recently, cAMP has also been implicated in plant stress responses and defense [8, 9]. Furthermore, in *Vicia faba*, exogenously applied cAMP causes stomatal opening [10] and modulates ion transport through cyclic nucleotide gated channels (CNGC) [11-13].

The initial debate about the existence of cAMP in plants and its role as an authentic plant signaling molecule has been resolved not least because increasingly sensitive detection methods afford accurate *in vivo* measurements of cAMP. In addition, the discovery of components of cAMP signaling pathways, as well as cAMP-interacting proteins (i.e. ACs, phosphodiesterase (PDE) and protein kinase A (PKA)) have been reported [14, 15]. However, to-date, no gene encoding a PDE has been annotated in plants while a *Zea mays* protein that participates in polarized pollen tube growth remains the only experimentally confirmed AC in higher plants [16].

Here we report that the N-terminal cytosolic region of a K^+-uptake transporter 7 (KT/HAK/KUP7; AtKUP7, At5g09400) in *Arabidopsis thaliana* contains an AC catalytic centre and show that a recombinant AtKUP7$^{1\text{-}100}$ generates cAMP detectable by enzyme immunoassay and mass spectrometry, and can rescue an *E. coli* mutant that lacks the adenylate cyclase (*cyaA*) gene thus enabling lactose fermentation.

2. Materials and methods

2.1 *Generation of recombinant AtKUP7$^{1\text{-}100}$*

cDNA was synthesized from RNA extracted from leaf of Columbia 0 *Arabidopsis thaliana* using the RNeasy kit (Qiagen, Crawley, UK). The cDNA sequence of AtKUP7 was retrieved from The Arabidopsis Information Resource (TAIR) website (https://www.arabidopsis.org). The PCR product was amplified using the gene specific primers: *AtKUP7* Forward (5'-ATGGCGGAGGAAAGCAGTAT-3') and *AtKUP7* Reverse (5'-TTATTTCCTCCCAACGGTC-3', and cloned into the Gateway compatible pCR8 vector (Invitrogen, Carlsbad, USA) by TA cloning. The cytoplasmic domain of AtKUP7 was recombined into pDEST17 expression vector (Invitrogen, Carlsbad, USA) to create a pDEST17-*AtKUP7* fusion construct containing a C-terminal His tag for affinity purification. The purification of the recombinant protein was done under denaturing conditions using Ni-NTA agarose beads according to the manufacturer's instructions (The QIA expressionist, Qiagen, USA) and as detailed elsewhere [17] and in the Supplementary file.

The harvested cells were resuspended in 8 mL of lysis buffer (10 mM NaH_2PO_4, 10 mM Tris-Cl, 6 M guanidine hydrochloride; pH 8) for each 1 g of cell pellet, then mixed on a rotary mixer and centrifuged at 2300 *g* for 15 min. The resulting supernatant was collected and mixed with 1 mL of 50% (w/v) Ni-NTA beads (Qiagen, USA) for each 10 mL lysate on a rotary mixer for 30 min. The lysate-resin mixture was loaded into a gravity column and allowed to settle and the flow-through discarded. The resin was washed three times with 60 mL wash buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl and 8 M urea; pH 6.3). The mass of the $AtKUP7^{1-100}$ was estimated using the ProtParam tool on the ExPasy Proteomics Server (http://au.expasy.org/tool/.protpatram.html). The purified protein was then used for *in vitro* enzymatic assays. The purification methods and the refolding protocol are further detailed in the Supplementary file.

2.2 *Computational assessment of the AtKUP7 AC centre*

A full-length AtKUP7 model was generated using the iterative threading assembly refinement (I-TASSER) method [18]. The full-length AtKUP7 amino acid sequence was submitted to the I-TASSER server available on-line at: http://zhanglab.ccmb.med.umich.edu/I-TASSER/ and the model with the highest quality based on their C-score was downloaded from the server. The AtKUP7 model was visualized and analyzed, and the images were created using UCSF Chimera (ver. 1.10.1) [19]. Docking of ATP to the AC centre of AtKUP7 model was performed using

AutoDock Vina (ver. 1.1.2) [20]. The ATP docking pose was analyzed and docking images were created using PyMOL (ver 1.7.4) (The PyMOL Molecular Graphics System, Schrödinger, LLC).

2.3 *In vitro adenylate cyclase enzymatic assay and detection of cAMP*

In the enzyme immunoassay cAMP levels were measured using the Biotrak enzyme immunoassay kit (GE Healthcare, USA) system as described by the manufacturer using the acetylation protocol. Liquid chromatography tandem mass spectrometry (LC-MS/MS; Thermo LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Pittsburgh, PA, USA)) was used to detect cAMP generated from reaction mixtures containing 10 µg of recombinant protein in 50 mM Tris-Cl; 2 mM isobutylmethylxanthine (IBMX; Sigma), 5 mM $MgCl_2$ or $MnCl_2$ and 1 mM ATP in a final volume of 100 µL. All methods are more extensively detailed elsewhere [21].

2.4 *Complementation of cyaA mutation in E. coli AC-deficient strain*

The *E. coli cyaA mutant* SP850 strain (lam-, el4-, relA1, spoT1, cyaA1400 (:kan),thi-1) [22], deficient in the adenylate cyclase (*cyaA*) gene, was obtained from the *E. coli* Genetic Stock Centre (Yale University, New Haven, USA) (accession No. 7200). Then pDEST17-$AtKUP7^{1\text{-}100}$ construct was used to transform the *E. coli cyaA* mutant strain by heat shock (2 min at 42 °C). Bacteria were grown at 37 ^{o}C in LB media containing ampicillin and kanamycin (100 µg/mL) until they reached an OD_{600} of 0.6 and then incubated with 0.5 mM isopropyl-beta-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) for transgene induction for 4 hours prior to streaking on MacConkey agar.

3. Results and discussion

3.1 *Identification of an AC catalytic centre at the cytosolic region of* $AtKUP7^{1\text{-}100}$

Recently, a search motif derived from functionally assigned residues in the GC catalytic centres of molecules across species has led to the identification of a number of candidate GCs in plants [23, 24]. In plant GCs, the functionally tested motif is comprised of 14 amino acids (aa). The amino acid in position 1 [R, K or S] does the hydrogen bonding with guanine, the amino acid in position 3 [CTGH] confers substrate specificity and the amino acid in position 12, 13 or 14 [K, R]

stabilize the transition state from GTP to cGMP. The amino acid [D, E] at 1-3 residue downstream from position 14, participates in Mg^{2+}/Mn^{2+}-binding site [25]. The core [YFW] is situated between the assigned residue that does the hydrogen bonding (position 1) and the amino acid that confers substrate specificity (position 3) (Fig. 1A). Since the catalytic centres of ACs and GCs differ only in the amino acid that confers substrate specificity [15, 24-26], we modified the GC motif by changing the third position of the 14 aa search term from [CTGH] to [DE] (Fig. 1A) to identify candidate ACs in plants. This substitution was proposed based on the findings of previous studies that showed the conversion of GCs into ACs and *vice versa* through site directed mutagenesis of the residue implicated in substrate recognition [25, 26]. We then queried the Arabidopsis proteome using this rationally modified AC motif ([RKS][YFW][DE][VIL]X(8,9)[KR]X(1,3)[DE]) and retrieved 341 proteins. We further narrowed the search by including the residues [VIL] typical for experimentally tested plant GCs [17, 21, 23, 27, 28] in position 9 and [R] between 5 and 20 aa upstream of position 1 since an N-terminal arginine is essential for pyrophosphate binding [29]. The extended AC motif ([R]X(5,20)[RKS][YFW][DE][VIL]X(4)[VIL]X(4)[KR]X(1,3)[DE]) identifies 14 candidate proteins (Supplementary file) one of which is the AtKUP7 (At5g09400) that is annotated as a vacuolar K^+ transporter. This protein is predicted to harbour 12 transmembrane domains with the AC catalytic centre located in the N-terminal cytosolic domain (Fig. 1B) spanning from aa 76 to 91. This AC catalytic centre also appears in other plant orthologues including *Vitis vinifera*, *Solanum lycopersicum and Sorghum bicolor* (Fig. 1C) and importantly, the functionally assigned residues in this AC centre are also present in the only experimentally confirmed AC in higher plants, the PSiP protein from *Zea mays* (accession No. AJ307886). The PSiP has been reported to have a role in pollen tube growth and fertilization [16].

In addition to the identification of an AC catalytic centre in AtKUP7 using a rationally designed AC motif, we also assessed the feasibility of the putative AC centre to bind the substrate ATP and catalyze the subsequent conversion into cAMP using computational methods. We have modeled the AtKUP7 by iterative threading and show in a model that the AC catalytic centre is solvent exposed thus allowing for unimpeded substrate interactions and presumably also for catalysis (Fig. 2). Further probing of the AC centre by molecular docking of ATP suggests that ATP can dock at the AC centre with a good free energy and a favorable binding pose. Specifically, the negatively charged phosphate end of ATP points towards the lysine residue

while the adenosine end is surrounded by negatively charged residues (Fig. 2) much like in structurally resolved GC centres [24, 30].

3.2 *AtKUP7$^{1-100}$ rescues an AC deficient E. coli mutant strain*

In order to investigate if the AtKUP7 AC can rescue an *E. coli* AC deficient mutant, the AtKUP7$^{1-100}$ was cloned and expressed in an *E. coli* SP850 strain lacking the AC (*cyaA*) gene that in turn prevents lactose fermentation. As a result of the *cyaA* mutation, the AC deficient *E. coli* and the un-induced transformed *E. coli* remain colourless cells when grown on MacConkey agar. In contrast, the AtKUP7 transformed *E. coli* SP850 cells, when induced with 0.5 mM IPTG, form red colored colonies much like the wild type *E. coli* (Fig. 3) thus indicating a functional AC centre in the recombinant AtKUP7$^{1-100}$ that has rescued the *E. coli cyaA* mutant growing on MacConkey agar.

3.3 *In vitro AC activity of recombinant AtKUP7$^{1-100}$*

To test if the AtKUP7 AC centre generates cAMP *in vitro*, the AtKUP7$^{1-100}$ was expressed in *E. coli* and affinity purified (Supplementary file). The AC activity of the fragment was tested in a reaction mixture containing ATP and with either Mg^{2+} or Mn^{2+} as the cofactor. A maximum activity was reached after 25 min of enzymatic reaction, generating 42.5 fmol/µg protein of cAMP in the presence of Mn^{2+} and 27 fmol/µg protein of cAMP in the presence of Mg^{2+} (Fig. 3B) while the amount of cAMP in the un-induced bacterial protein extract is not significant (not shown). Cyclic AMP levels were also measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) specifically identifying the presence of the unique product ion at m/z 136 $[M+H]^+$ that is fragmented in a second ionization step, in addition to the parent ion at m/z 330 $[M+H]^+$. This fragmented product ion was then used for quantitation. In the presence of Mn^{2+}, the recombinant AtKUP7$^{1-100}$ generates cAMP that increases with time achieving a maximum amount of 55 fmol/µg protein of cAMP at 25 min. We note that this activity is 10-50 times lower than the animal ACs and this may be due to the more localized micro-regulatory role of such AC centres that assume the roles of rapid molecular switches capable of diverting from one signaling network to another much like those observed in plant GCs e.g. PSKR1 [31]. A

representative ion chromatogram of cAMP showing both the parent and product ion peaks is shown in Fig. 4B.

Interestingly, in *Paramecium*, cAMP formation is stimulated by K^+ conductance, and this conductance in turn is an intrinsic property of the AC. This multi-domain protein acts as both an AC and a K^+ channel where a canonical S4 voltage-sensor occupies the N-terminal and a K^+ pore-loop sits in the C-terminus on the cytoplasmic side [32]. Incidentally, AtKUP7 also has such dual domain architecture as characterized by its K^+ transporter and a cytosolic AC centre although we note that KUP7 is likely a proton-coupled K^+ carrier rather than a K^+ channel. It will therefore be interesting to test if cAMP production is dependent on the K^+ conductance and/or if cAMP can modulate K^+ conductance. If so, it is conceivable that AtKUP7 may operate as a cAMP-dependent K^+ flux sensor.

In summary, we report the identification of an AC catalytic centre in the cytosolic domain of $AtKUP7^{1-100}$ discovered using rationally curated motif-based searches and supported by computational simulation on AtKUP7 models, and show that $AtKUP7^{1-100}$ generates cAMP detectable by immunoassay and mass spectrometry. Furthermore, we predict that many more ACs with varied domain organizations await discovery in higher plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at …..

References

1. Sutherland, E. W., Robison, G. A. & Butcher, R. W. (1968) Some aspects of biological role of adenosine 3',5'-monophosphate (cyclic AMP), *Circulation.* **37**, 279-306.

2. Francis, S. H., Blount, M. A. & Corbin, J. D. (2011) Mammalian cyclic nucleotide phosphodiesterases: Molecular mechanisms and physiological functions, *Physiol Rev.* **91**, 651-690.
3. Bretschneider, T., Vasiev, B. & Weijer, C. J. (1999) A model for *Dictyostelium* slug movement, *J Theor Biol.* **199**, 125-136.
4. Bahn, Y. S., Molenda, M., Staab, J. F., Lyman, C. A., Gordon, L. J. & Sundstrom, P. (2007) Genome-wide transcriptional profiling of the cyclic AMP-dependent signaling pathway during morphogenic transitions of *Candida albicans*, *Eukaryot Cell.* **6**, 2376-2390.
5. Biswas, A., Bhattacharya, A. & Das, P. K. (2011) Role of cAMP signaling in the survival and infectivity of the protozoan parasite, *Leishmania donovani*, *Mol Biol Inter.* **2011**, 782971.
6. Komatsu, S. & Hirano, H. (1993) Protein-kinase activity and protein-phosphorylation in rice (*Oryza sativa*) leaf, *Plant Sci.* **94**, 127-137.
7. Ehsan, H., Reichheld, J. P., Roef, L., Witters, E., Lardon, F., Van Bockstaele, D., Van Montagu, M., Inze, D. & Van Onckelen, H. (1998) Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells, *FEBS Lett.* **422**, 165-169.
8. Gottig, N., Garavaglia, B. S., Daurelio, L. D., Valentine, A., Gehring, C., Orellano, E. G. & Ottado, J. (2009) Modulating host homeostasis as a strategy in the plant-pathogen arms race, *Commun Integr Biol.* **2**, 89-90.
9. Thomas, L., Marondedze, C., Ederli, L., Pasqualini, S. & Gehring, C. (2013) Proteomic signatures implicate cAMP in light and temperature responses in *Arabidopsis thaliana*, *J Proteomics.* **83**, 47-59.
10. Curvetto, N., Darjania, L. & Delmastro, S. (1994) Effect of 2 cAMP analogs on stomatal opening in *Vicia faba* - possible relationship with cytosolic calcium concentration, *Plant Physiol Biochem.* **32**, 365-372.
11. Maathuis, F. J. M. & Sanders, D. (1996) Mechanisms of potassium absorption by higher plant roots, *Physiol Plantarum.* **96**, 158-168.
12. Lemtiri-Chlieh, F. & Berkowitz, G. A. (2004) Cyclic adenosine monophosphate regulates calcium channels in the plasma membrane of Arabidopsis leaf guard and mesophyll cells, *J Biol Chem.* **279**, 35306-35312.
13. Zelman, A. K., Dawe, A., Gehring, C. & Berkowitz, G. A. (2012) Evolutionary and structural perspectives of plant cyclic nucleotide-gated cation channels, *Front Plant Sci.* **3**, 95.
14. Assmann, S. M. (1995) Cyclic AMP as a 2nd messenger in higher plants - Status and future prospects, *Plant Physiol.* **108**, 885-889.
15. Gehring, C. (2010) Adenyl cyclases and cAMP in plant signaling - past and present, *Cell Commun Signal.* **8**.
16. Moutinho, A., Hussey, P. J., Trewavas, A. J. & Malho, R. (2001) cAMP acts as a second messenger in pollen tube growth and reorientation, *Proc natl Acad Sci U.S.A.* **98**, 10481-10486.
17. Meier, S., Ruzvidzo, O., Morse, M., Donaldson, L., Kwezi, L. & Gehring, C. (2010) The Arabidopsis wall associated kinase-like 10 gene encodes a functional guanylyl cyclase and is co-expressed with pathogen defense related genes, *PloS One.* **5,** 8904.
18. Zhang, Y. (2008) I-TASSER server for protein 3D structure prediction, *BMC Bioinformatics.* **9**.

19. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C. & Ferrin, T. E. (2004) UCSF chimera - A visualization system for exploratory research and analysis, *J Comput Chem.* **25**, 1605-1612.
20. Trott, O. & Olson, A. J. (2010) software news and update AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, *J Comput Chem.* **31**, 455-461.
21. Kwezi, L., Ruzvidzo, O., Wheeler, J. I., Govender, K., Iacuone, S., Thompson, P. E., Gehring, C. & Irving, H. R. (2011) The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signaling in plants, *J Biol Chem.* **286**, 22580-22588.
22. Shah, S. & Peterkofsky, A. (1991) Characterization and generation of *Escherichia coli* adenylate cyclase deletion mutants, *J Bacteriol.* **173**, 3238-42.
23. Ludidi, N. & Gehring, C. (2003) Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*, *J Biol Chem.* **278**, 6490-6494.
24. Wong, A. & Gehring, C. (2013) The *Arabidopsis thaliana* proteome harbors undiscovered multi-domain molecules with functional guanylyl cyclase catalytic centers, *Cell Commun Signal.* **11**, 48.
25. Tucker, C. L., Hurley, J. H., Miller, T. R. & Hurley, J. B. (1998) Two amino acid substitutions convert a guanylyl cyclase, RetGC-1, into an adenylyl cyclase, *Proc natl Acad Sci U.S.A.* **95**, 5993-5997.
26. Roelofs, J., Meima, M., Schaap, P. & Van Haastert, P. J. M. (2001) The Dictyostelium homologue of mammalian soluble adenylyl cyclase encodes a guanylyl cyclase, *EMBO J.* **20**, 4341-4348.
27. Kwezi, L., Meier, S., Mungur, L., Ruzvidzo, O., Irving, H. & Gehring, C. (2007) The *Arabidopsis thaliana* brassinosteroid receptor (AtBRI1) contains a domain that functions as a guanylyl cyclase *in vitro*, *PloS One.* **2**, e449.
28. Qi, Z., Verma, R., Gehring, C., Yamaguchi, Y., Zhao, Y., Ryan, C. A. & Berkowitz, G. A. (2010) Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels, *Proc natl Acad Sci U.S.A.* **107**, 21193-21198.
29. Liu, Y., Ruoho, A. E., Rao, V. D. & Hurley, J. H. (1997) Catalytic mechanism of the adenylyl and guanylyl cyclases: modeling and mutational analysis, *Proc natl Acad Sci U.S.A.* **94**, 13414-13419.
30. Wong, A., Gehring, C. & Irving, H. R. (2015) Conserved functional motifs and homology modeling to predict hidden moonlighting functional sites, *Front Bioeng Biotechnol.* **3**, 82.
31. Muleya, V., Wheeler, J. I., Ruzvidzo, O., Freihat, L., Manallack, D. T., Gehring, C. & Irving, H. R. (2014) Calcium is the switch in the moonlighting dual function of the ligand-activated receptor kinase phytosulfokine receptor 1, *Cell Commun Signal.* **12**, 60.
32. Weber, J. H., Vishnyakov, A., Hambach, K., Schultz, A., Schultz, J. E. & Linder, J. U. (2004) Adenylyl cyclases from *Plasmodium*, *Paramecium* and *Tetrahymena* are novel ion channel/enzyme fusion proteins, *Cell Signal.* **16**, 115-125.

Figure legends:

Figure 1: Structural feature of the adenylate cyclase catalytic centre of AtKUP7 transporter. A, the 14 amino acid motif of annotated and experimentally tested GCs and ACs catalytic centres. B, amino acid sequence of the AtKUP7 transporter. The complete sequence of the AtKUP7 transporter is shown and the AC domain is located at the cytosolic N-terminal position. AC catalytic centre is in bold and the 104 amino acid fragment tested for AC activity is underlined. The 12 transmembrane domains are highlighted in blue colour. C, alignment of the AC centres of AtKUP7 and its orthologues including *Vitis vinifera* (*V.v.*), *Solanum lycopersicum* (*S.l.*) and *Sorghum bicolor* (*S.b.*). A *Zea mays* pollen signalling protein with adenylate cyclase activity, PSiP (acession No. AJ307886) represents the only confirmed AC in higher plants.

Figure 2: The AtKUP7 models. A, Docking of ATP at the AC centre and the interaction of ATP with the key residues in the catalytic centre is as shown in the surface and B, ribbon AtKUP7 models. C, The full-length AtKUP7 model showing the location of the AC centre at the solvent-exposed cytosolic region. The AC centre and the metal-binding residues are highlighted in yellow and cyan respectively. The residues implicated in interaction with ATP are coloured according to their charges in the surface models and shown as individual atoms in the ribbon model. AtKUP7 was modeled using the iterative threading assembly refinement (I-TASSER) method on the on-line server: http://zhanglab.ccmb.med.umich.edu/I-TASSER/ [18] and ATP docking simulation was performed using AutoDock Vina (ver. 1.1.2) [20].

Figure 3: Functional characterization of AtKUP7$^{1-100}$. A, the recombinant AC domain of AtKUP7$^{1-100}$ complemented the *cyaA* mutant *E. coli* (SP850). The wild type *E. coli* shows strong red colour while both the *cyaA* mutant and the *cyaA* mutant with un-induced recombinant AtKUP7$^{1-100}$ yielded colourless colonies. B, Cyclic AMP generated by recombinant AtKUP7$^{1-100}$ at different time points in reaction mixtures containing 10 µg protein, 2 mM IBMX, 1 mM

ATP and 5 mM Mn^{2+} or Mg^{2+}. The inset shows an SDS-PAGE gel of the affinity purified recombinant protein.

Figure 4: Detection of cAMP generated by $AtKUP7^{1-100}$ by liquid chromatography tandem mass spectrometry (LC-MS/MS). A, cAMP was generated from reaction mixture containing 10 µg of the purified recombinant protein, 50 mM Tris-Cl; 2 mM IBMX, 5 mM $MnCl_2$ and 1 mM ATP. HPLC elution profile of cAMP and a calibration curve is shown in the inset. The calculated amount of cAMP after 25 min of enzymatic reaction is 55 fmol/µg protein. B, Representative ion chromatogram of cAMP showing the parent and daughter ion peaks (see arrows and inset for the structures).