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Chapter

The Knowledge Landscape of Adenylyl Cyclases in Model Plant, *Arabidopsis thaliana*

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Abstract

One strategy for improving responses and adaptation systems of plants to stress is to target molecules involved in signaling and transduction of the stimuli effected by stresses. One such molecule is adenylyl cyclase (AC) – an enzyme that catalyzes the conversion of adenosine 5'-triphosphate (ATP) to the second messenger, 3',5'-cyclic adenosine monophosphate (cAMP). cAMP, in turn, transduces signals in response to the various biotic and abiotic stress factors. Surprisingly, as far as five decades ago, attempts to isolate ACs and/or detect cAMP from the research model plant, *Arabidopsis thaliana*, were inconclusive or a matter of serious debates due to the absence of appropriate techniques or advanced technologies. This chapter, therefore, herein takes the reader on a journey from the 1970s to the present day, unraveling the challenges encountered, developments made, and successes realized in efforts and attempts to identify and characterize ACs in *A. thaliana*. The chapter covers from the early age of unsuccessful attempts to the more recent and successful advanced technologies such as the motif search approach, omics analysis and homologous cloning. Perspectives on the direction that future knowledge-building around this important group of plant proteins are also shared.

Keywords: *Arabidopsis thaliana*, adenylyl cyclase, plant signaling, cAMP, motif search

1. Introduction

In this chapter, we present part of our work that involves the identification and characterization of a special group of plant enzymes termed adenylyl cyclases (ACs) in the model plant *Arabidopsis thaliana*. These enzymes essentially catalyze the conversion of adenosine 5'-triphosphate (ATP) to the second messenger, 3',5'-cyclic adenosine monophosphate (cAMP) [1–3]. The two molecules (ACs and cAMP) play crucial roles in many diverse and downstream biological processes of all living

organisms, ranging from prokaryotes (e.g., *Escherichia coli*) to complex multicellular eukaryotes (e.g., *Homo sapiens*). In plants, ACs and cAMP play crucial roles linked to highly complex systems that assist in the maintenance and progression of cellular homeostasis. This is quite important considering the sessile nature of plants in the current rapidly fluctuating environments associated with a wide range of biotic and abiotic stress factors. Ideally, these two signaling molecules mediate several metabolic and physiological processes in plants including control of the cell cycle [4], activation of the phenylalanine ammonia-lyase (PAL) enzyme [5] and regulation of the phenylpropanoid pathway [6]. In addition, the molecules are also involved in stomatal movements [7], cation transport via voltage-independent channels (VICs) [8], pollen tube growth [9–12] as well as response to stressful conditions via the cyclic nucleotide-gated channels (CNGCs) [7, 13–16].

The chapter begins by introducing *A. thaliana* as a model organism for plant research followed by a narration of the journey that was followed from the 1970s to the present day, on the details of the challenges encountered, developments made, and successes realized with regard to the identification/discovering and characterization of ACs in this plant. The chapter also goes on to present the impact of such an adventure by highlighting related achievements made in other plants. Finally, the chapter closes by looking at future prospects by proposing possible engagements that could be pursued in this kind of an intriguing area of study in Plant Sciences.

2. *A. thaliana* as a model organism for plant research

A. thaliana is a weed-like plant that belongs to the Cruciferae family, commonly known as the mustard family. It is a dicot. The plant has become an essential model for plant scientists since the inception of the mid-1980s due to the availability of its whole genome sequence, molecular genetic markers and a large collection of its sequence-indexed DNA-insertion mutants [17]. Its different ecotypes have been collected from natural populations and are available for experimental analysis. The Columbia (Col-0) and Landsberg (Ler) ecotypes are the scientifically accepted standards for genetic and molecular studies [18]. The plant has a relatively small genome, which has led to a large base of mutants, genetic mapping and gene sequencing [19]. The plant is small in size, it self-pollinates and has a fast-growing capacity with a generation time of only 6 weeks (from germination to the first seeds under continuous light). A single plant can produce thousands of seeds within 2 months that can easily be screened in a single standard petri dish. Large numbers of the plants can also be grown in a very small space (as much as 100 plants in a pot or a small chamber) and such plants also being able to be easily or conveniently mutagenized by chemical mutagens or radiation [20]. As a result of all these attributes that are positive and amenable, *A. thaliana* has become an ideal model organism for plant research, particularly dicots, in laboratories.

3. The long journey to discover ACs and cAMP signaling in plants

3.1 Doubts and controversy on the possible existence of ACs or cAMP signaling in plants

Since the identification of cAMP in 1957 by Earl Wilbur Sutherland and TW Rall [21], wherein they studied the effect of adrenaline on the decomposition of glycogen

into glucose in hepatocytes, a number of studies on ACs in animals and microorganisms have been performed [22–24]. Such studies included classification, sequencing, crystal structure analysis and functional characterization, ultimately leading to an unmasking of the catalytic mechanisms of this special group of enzymes [22–24]. However, despite all this impressive progress being made in other living organisms, the existence and/or functioning of ACs or cAMP in plants, still remained a matter of strong debate and serious controversy [25]. Firstly, because higher plants differ from other eukaryotic organisms in that they lack neurotransmitters and possess cell walls, therefore, the presence of plant cell walls would seem to compromise the believed and accepted mammalian AC/cAMP model [26]. Secondly, early studies of higher plant ACs were hampered by the fact that the proteins under study were mostly membrane-bound rather than soluble ones and therefore, their recovery and detection were somewhat very sneaky and difficult [27]. Thirdly, the attendant presence in crude biological extracts of active cyclic nucleotide phosphodiesterases, which hydrolyze cAMP also was a huge drawback [27]. Lastly, ACs represented a relatively very low proportion of plant cellular proteins for firm conclusions to be reached [27].

3.2 Hopes and continued debates

By the mid-1970s, both ACs and cAMP had been firmly established as important signaling molecules in animals and lower eukaryotes [1, 2, 28, 29]. Given this realization, it is not surprising that plant scientists continued to believe that this very same eukaryotic AC/cAMP signaling system was universal and therefore, operating in plants too [30]. Therefore, more work on plant AC/cAMP signaling continued. Nonetheless, further controversy and debates still ensued on these efforts, which perhaps can be best summarized by a concluding remark in one of the reviews of 1977, which stated as follows: “Our present knowledge, or rather ignorance, of cAMP in higher plants does not permit us to indulge in speculation on its function and thus to increase the disparity between available facts and conclusions, which are based solely on the conviction that plants, with respect to cAMP, should behave like animals or certain bacteria” [31]. As a result of this, plant scientists were therefore, strongly cautioned not to jump into conclusions even though they strongly believed that an AC/cAMP system existed in plants. The main reasons for this restraint were mostly technical. For instance, one criticism was mainly based on the fact that the reported results were merely either presumptive deductions from observed physiological effects of endogenously supplied cAMP or cAMP analogs, or that the conclusions were based solely onto insufficiently rigorous chromatographic identifications [31].

For instance, the demonstration of higher plant AC/cAMP activities had typically and traditionally been conducted through the use of either histochemical or biochemical procedures, whereby the histochemical procedures were predominantly based on the standard Wachstein-Meisel lead phosphate precipitation technique, which by that time, gave early indications of AC activity in *Zea mays*, *Pisum sativum*, *Alnus glutinosa*, *Vicia faba*, *Phaseolus vulgaris* and *Populus* species; while on the other hand, the biochemical approach proposed the presence of AC activity in *Medicago sativa*, *Spinacea oleracea*, *Ricinis communis*, *P. sativum*, *Verticillium albo-atrum* and *P. vulgaris* [26]. Moreover, some mass spectrometry-based analytical techniques of that period also indicated the presence of an AC/cAMP system in several plant species but still, the actual solid functional evidence was lacking [32].

Even though not very conclusive, the procedures discussed above principally revealed that cAMP levels in higher plants were relatively very low compared to other organisms, e.g., <20 pmol/g fresh weight compared to >250 pmol/g wet weight in animals [30, 33]. Hence, because of the low AC activity and barely detectable amounts of cAMP as well as the questionable experimental procedures used to identify cAMP and/or AC activity, the significance of cAMP in higher plants remained strongly doubted and/or even overlooked [34]. For this reason, it has also been so difficult to discern function(s) of ACs or cAMP in plants [35]. Consequently, in 1995, Assmann even went on as far as to declare that no plant AC gene had ever been cloned and, moreover, in 2006, Linder also asserted that no AC molecule had ever been conclusively identified in higher plants [30, 36]. In general, it has therefore, been commonly argued that until a plant AC is either cloned and/or its protein sufficiently purified to allow for microsequencing and a complete enzymological characterization, the relevance and validity of the AC activity and/or cAMP signaling reported in plants would always be open to serious or rather very heated debates [30].

3.3 Leads and indications

Despite the uncertainty and controversy of the mid-1970s, surrounding the AC/cAMP signaling system in plants, the notion of its existence still continued to stay alive. Firstly, because of the fact that both cell-permeant 8-BrcAMP and stimulation of *albeit* unknown ACs with forskolin could elicit concentration- and time-dependent biological responses such as increases in Ca^{2+} influx across the plasma membrane [37, 38]. Secondly, biochemical evidence, which included the finding that crude alfalfa (*M. sativa* L.) root extracts could show a calmodulin-dependent AC activity, was reported [39]. Thirdly, data from the whole-cell patch-clamp recordings of *V. faba* mesophyll protoplasts, convincingly revealed that outward K^+ current increased in a dose-dependent fashion following the intracellular application of cAMP and not AMP, cGMP or GMP [40]. Lastly, cAMP-dependent up-regulations of a calcium-permeable conductance, activated by hyperpolarization, were reported in guard and mesophyll cells of *A. thaliana* and *V. faba* [41].

3.4 Setting up the platform

The advent and era of high-throughput technologies (twenty-first century) eventually made it possible to assign functions to genes based on their homology, using the Basic Local Alignment Search Tool (BLAST). Apparently and according to Wong and Gehring in 2013 [42], higher plant ACs could not be identified using BLAST homology searches based on annotated ACs from prokaryotes, lower eukaryotes or animals because plant ACs are often part of complex multifunctional proteins with different domains and functions that are not conserved. Thus, BLAST searches with the known and experimentally confirmed ACs from other organisms could not return any plausible candidate(s). In addition, it was also noted that the pro-site signatures for class I and II ACs ((EYFG[SA]X(2)LWXLYK) and (YRN[XW[NS]E[LIVM]RTLHFXG) respectively) are not present in the Arabidopsis proteome even if 2 mismatches were allowed [33]. Fortunately, between 2003 and 2011, six functional guanylyl cyclases (GCs) (structural analogs of ACs that convert guanosine 5'-triphosphate (GTP) to another second messenger, 3',5'-cyclic guanosine monophosphate (cGMP)) from Arabidopsis were positively identified [43–47], using a 14 amino acid long search motif deduced from an alignment of conserved and functionally assigned amino acids

3.5.1 Confirmation and validation of the presence of the AC motif in targeted protein candidates

Before any step was taken, it was necessary and important to first check and confirm presence of the AC motif in each of the targeted AC protein candidates. To do this, complete copy DNA (cDNA) and amino acid sequences of the targeted candidate were retrieved from The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org/>) followed by analysis of the amino acid sequence for presence of the motif [33], using the PROSITE database located within the Expert Protein Analysis System (ExPASy) proteomics server (<https://www.expasy.org/>). In addition, both the presence and location of the motif in each targeted candidate were also confirmed and validated by ACPred, available at <http://gcpred.com/acpred/> [49]. (See example in **Figure A1**, Appendix A).

3.5.2 Computational analysis of the targeted protein candidates

Next, computational analysis of each of the targeted AC protein candidates was also undertaken to assess and determine the ability of its AC center to bind ATP and catalyze its subsequent conversion into cAMP. To do that, a 3-dimensional (3D) model of the targeted protein candidate was constructed by artificial intelligence using its AlphaFOLD beta version with low predicted error and very high confidence (pLDDT >90) [54]. This software uses a neural network-based model of artificial intelligence to predict protein structures from their amino acid sequences at an atomic level of accuracy. It first aligns the amino acid sequence input with sequences of known structures for pair-wise representation. The representation is then used to produce atomic coordinates for each residue, thus predicting the necessary rotation and then assembling a structured chain of amino acid residues. Its developers freely provide the source code for access to trained modelers and a script for predicting structures of novel input sequences [54]. In our case, the full-length amino acid sequence of each of the targeted protein candidates was submitted to the AlphaFOLD database followed by downloading of the model with the highest quality (based on C-scores). The downloaded model was then visualized and analyzed using UCSF ChimeraX next-generation molecular visualization program (v.1.10.1.) [55]. SeeSAR 3D (v.12.0.1) desktop modeling platform was then next used to perform docking of ATP (PubChem ID: 5957) to the AC center of the selected model via FlexX docking functionality [56, 57]. A structural alignment was then conducted by fragment assembly simulations based on iterative templates using the iterative threading assembly refinement (I-TASSER) server to match the selected model to an experimentally confirmed structure in the PDB library [56]. The model with the highest C-score was then analyzed using PyMOL (v.1.7.4.) (Schrödinger LLC, New York, USA) and ultimately adopted in the study. (See example in **Figure A1**, Appendix A).

3.5.3 Cloning and expression of the recombinant AC proteins

After determining that the AC center in the structural model of each of the targeted AC protein candidates was solvent-exposed, thus allowing for unimpeded substrate interactions and ultimately catalysis [57], we then went on to generate the respective protein candidate as a recombinant product so as to test it biochemically. To clone a fragment of each of the targeted AC protein candidates harboring the AC motif, total RNA was extracted from six-week-old *A. thaliana* ecotype Col-0 seedlings

using the RNeasy plant mini kit, in combination with DNase 1 treatment, as instructed by the manufacturer (Qiagen, Crawley, UK). cDNA synthesis from total RNA and subsequent amplification of the targeted gene fragment from the cDNA were simultaneously performed in the presence of two sequence-specific primers for each protein candidate (**Table A1**, Appendix B), using a Verso 1-Step RT-PCR kit, in accordance with the manufacturer's instructions (Thermo Scientific, Rockford, USA). The resultant PCR product was then cloned into a pTrcHis2-TOPO expression vector via the TA cloning system (Invitrogen Corp., Carlsbad, USA) to make a pTrcHis2-TOPO:AC fusion expression construct with a C-terminus His purification tag.

To express the recombinant AC protein, competent *E. coli* BL21 Star pLysS cells (Invitrogen, Carlsbad, USA) were transformed (through heat shock at 42°C for 2 min) with the pCRT7/NT-TOPO:AC fusion construct and 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. Protein expression was induced through the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich Corp., Missouri, USA) at a final concentration of 1 mM and when the optical density (OD₆₀₀) of the cell culture had reached 0.5 (approximately 3 h). The culture was then left to grow for a further 3 h at 37°C. (See example in **Figure A1**, Appendix A).

3.5.4 Purification of the recombinant AC proteins

The resultant expressed recombinant AC protein (for each targeted AC candidate molecule) was purified by preparing a cleared cell lysate of the induced *E. coli* cells under non-native denaturing conditions (since most of those protein recombinants were primarily expressed in form of inclusion bodies (IBS)), whereby the harvested cells were resuspended in lysis buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl; pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, 7.5% (v/v) glycerol) at a quantitative ratio of 1 g pellet weight to every 10 ml buffer volume, mixed thoroughly using a mechanical stirrer at 24°C for 1 h and then centrifuged at 2500×g for 15 min. Supernatant was collected as the cleared lysate and transferred to 2 ml of 50% (w/v) nickel-nitriloacetic acid (Ni-NTA) slurry (Sigma-Aldrich Corp., Missouri, USA) that had been pre-equilibrated with 10 ml of lysis buffer and the lysate/slurry mixture then gently swirled on a rotary mixer for 1 h at 24°C. This step allowed for binding of the AC recombinant protein onto the Ni-NTA resin. The lysate-resin mixture was loaded into an empty XK16 column (Bio-Rad Laboratories Inc., California, USA) and allowed to settle and flow through discarded. The protein-bound resin was then washed three times with 30 ml of wash buffer (8 M urea, 100 mM NaH₂PO₄, 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.

To refold the purified denatured recombinant AC protein, the washed protein-bound resin was equilibrated with 2 ml of gradient buffer (8 M urea, 200 mM NaCl, 50 mM Tris-Cl; pH 8.0, and 20 mM β-mercaptoethanol) before the column was connected to a Bio-Logic F40 Duo-Flow chromatography system (Bio-Rad Laboratories Inc., California, USA), programmed to run a linear refolding gradient. The refolding gradient was performed by linearly diluting the 8 M gradient buffer to 0 M urea concentration with a refolding buffer (200 mM NaCl, 50 mM Tris-Cl; pH 8.0, 500 mM glucose, 0.05% (w/v) polyethylene glycol, 4 mM reduced glutathione, 0.04 mM oxidized glutathione, 100 mM non-detergent sulfobetaine, and 0.5 mM phenylmethanesulfonylfluoride (PMSF)) over 10 h at a flow rate of 0.5 ml/min.

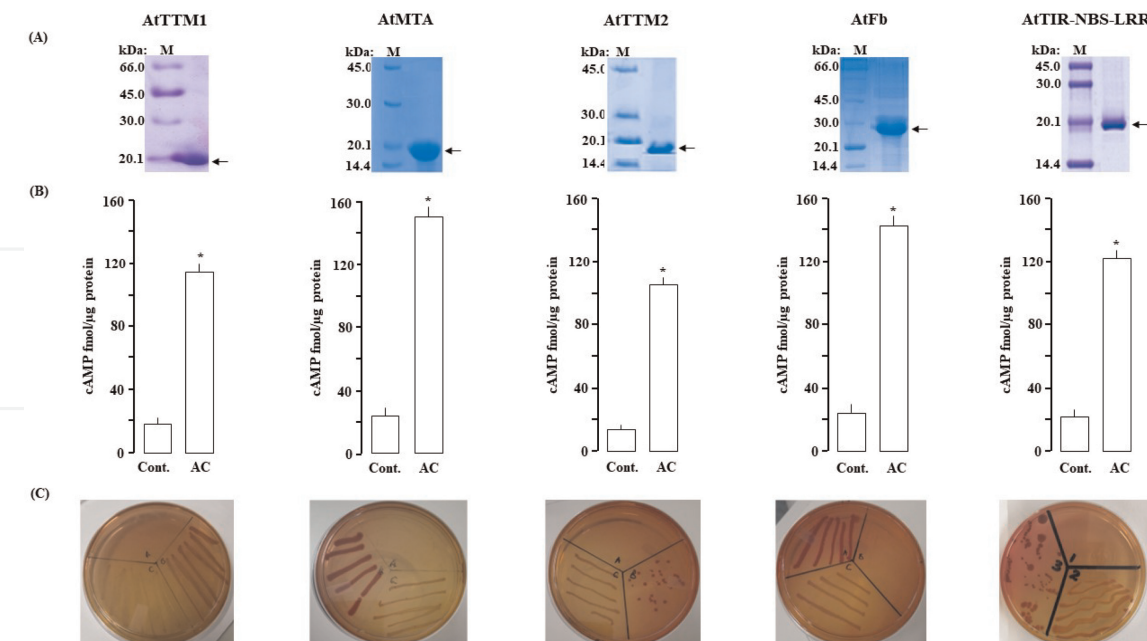


Figure 2. Determination of the AC activity of *A. thaliana* proteins. (A) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) profiles of the Ni–NTA purified versions of AtTTM1, AtMTA, AtTTM2, AtFb and AtTIR–NBS–LRR recombinants, where (M) is the molecular weight marker and the arrow marking the expressed proteins. (B) cAMP levels generated by 5 µg recombinant in the presence of 1 mM ATP and 5 mM Mn²⁺, and as determined by ELISA. Control reactions contained all other components except proteins. Data are mean values ($n = 3$) and error bars show the 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. (C) Ability of the recombinant AtTTM1, AtMTA, AtTTM2, AtFb and AtTIR–NBS–LRR proteins to complement *cyA* mutation in *E. coli* SP850 to ferment lactose. All protein-expressing SP850 *E. coli* cells showed a strong reddish color as if they were wild-type cells [59, 60] while *cyA* mutant cells yielded yellowish colonies.

After refolding, the resultant renatured recombinant AC protein was eluted in 2 ml of 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 recombinant was then de-salted and concentrated using a Spin–XUF filtration/concentration device with a molecular weight cut-off (MWCO) point of 3000 Da, in accordance with the manufacturer’s instructions (Corning Corp., New York, USA). Protein concentration was then determined by both the Bradford method [58] and an ND2000 nanodrop spectrophotometer (Thermo Scientific Inc., Massachusetts, USA) before the recombinant protein was stored at -20°C for further downstream uses. (Refer to **Figure 2** below).

3.5.5 In vitro assaying of the recombinant AC proteins

The probable *in vitro* AC activity of the purified recombinant version of each of the targeted AC candidate molecules was tested by incubating 5 µg of the protein in 200 µl of 50 mM Tris–Cl (pH 8.0), containing 2 mM isobutylmethylxanthine (IBMX, Sigma–Aldrich Corp., Missouri, USA) (to inhibit phosphodiesterases), 5 mM Mn²⁺ (as co-factor) and 1 mM ATP (as substrate), followed by measurement of the generated cAMP by immunoassay. Background cAMP levels in control reactions were measured in tubes containing all the other components but no protein. All incubations were performed at room temperature (24°C) for 20 min and terminated by the addition of 10 mM EDTA followed by boiling for 3 min and cooling on ice for 2 min before centrifugation at $2500\times g$ for 3 min. The resulting supernatants were in essence

assayed for cAMP content using the cAMP-linked enzyme-linked immunosorbent assay (ELISA) kit, following its acetylation protocol and as described by the supplier's manual (Sigma-Aldrich Corp., Missouri, USA; code: CA201). The anti-cAMP antibody in this assaying system is highly specific for cAMP with an approximately 10^6 times lower affinity for cGMP. In all cases, each experiment was performed in triplicate ($n = 3$) using three different protein extracts that had been independently prepared. Data generated were then subjected to one-way analysis of variance (ANOVA) (-Super-Anova, Statsgraphics Version 7, 1993; Statsgraphics Corp., The Plains, VI, USA) and wherever ANOVA revealed significant differences between treatments, means were separated by *post hoc* Student–Newman–Keuls (SNK) multiple range test ($p < 0.05$). (Refer to **Figure 2**).

3.5.6 Mass spectrometric analysis of the AC activities

To validate findings obtained by enzyme immunoassay, another additional method, tandem liquid chromatography-mass spectrometry (LC–MS/MS), was used. This method is capable of specifically and sensitively detecting cAMP levels at femtomolar concentrations. To do this, the acetylated cAMP samples from the immunoassay step were introduced into a Waters API Q-TOF Ultima mass spectrometer (Waters Microsep, Johannesburg, RSA) with a Waters Acquity UPLC at a flow rate of 180 ml/min. Separation was achieved in a Phenomenex Synergi (Torrance, CA) 4 μ m Fusion-RP (250 $\times$ 2.0 mm) column when a gradient of solvent “A” (0.1% formic acid) and solvent “B” (100% acetonitrile) was applied over 18 min. During the first 7 min, the solvent composition was kept at 100% “A” followed by a linear gradient of up to 80% “B” for 3 min, and then a re-equilibration to the initial conditions. An electrospray ionization in the negative (W-) mode was used at a cone voltage of 35 V, to detect molecules and generate chromatograms. (See example in **Figure A1**, Appendix A).

3.5.7 Complementation testing of the recombinant AC proteins

To further confirm and validate the AC activities of the targeted and tested AC protein candidates, complementation testing was used. In this process, the *E. coli* mutant strain, SP850, (*lam*-, *el4*-, *relA1*, *spoT1*, *cyaA1400* (:kan), *thi-1*) [59, 60], deficient in the AC gene (*cyaA*), was obtained from the *E. coli* Genetic Stock Centre (Yale University, New Haven, USA; accession No. 7200). The strain was prepared to be chemically competent followed by its transformation with the pTrcHis2-TOPO:AC fusion construct (through heat shock at 42°C for 2 min). The transformed bacteria together with the non-transformed cells were grown at 37°C in Luria-Bertani (LB) media containing kanamycin (15 μ g/ml) up until their cell culture had reached an optical density (OD_{600}) of 0.5. Both groups of cells were streaked on MacConkey agar supplemented with 15 μ g/ml kanamycin and 0.5 mM IPTG (Sigma-Aldrich Corp., Missouri, USA) (for transgene induction) before the streaked plates were incubated for 40 h at 37°C, for visual evaluation of the cultured cells on the media. After incubation, an ability of the induced transformed mutant cells to now ferment lactose would then be considered as an indication of the expressed recombinant protein's ability to generate cAMP from ATP, as a functional AC. As a result, the induced transformed cells would turn deep red or purple (just like wild-type cells) while the mutant control cells would remain yellowish or colorless [59, 60]. (Refer to **Figure 2**).

3.5.8 Phylogenetic analysis of the targeted protein candidates

Since classification of plant ACs has not yet been systematically undertaken and also considering that the relationship of plant ACs with the other currently existing six classes of ACs in animals and microorganisms is still very unclear, we assessed to determine if these plant proteins have any probable definitive groupings or clusters, particularly those ones harboring the AC search motif. In this regard therefore, full-length amino acid sequences of all plant proteins known to harbor the AC search motif were retrieved from the TAIR (<https://www.arabidopsis.org/>), NCBI (<https://www.ncbi.nlm.nih.gov/>) and Uniprot (<https://www.uniprot.org/>) websites, followed by construction of a phylogenetic tree through multiple sequence alignment and tree

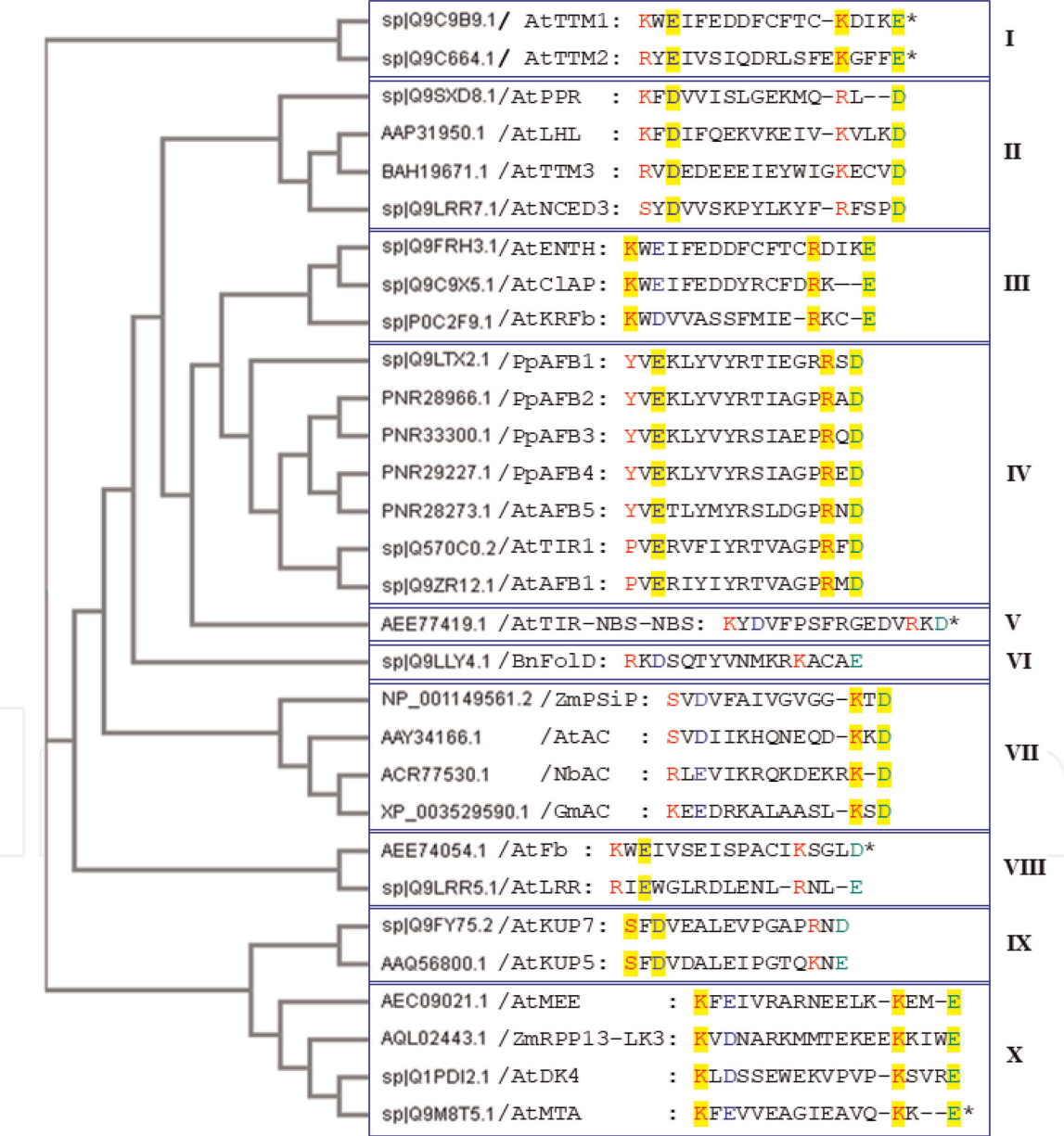


Figure 3. Phylogenetic tree and core motif analysis of all plant proteins currently known to harbor the AC search motif. Specific key amino acid residues in the core AC motif appear to be conserved in a certain pattern per group (yellow highlight), something that possibly could be exploited and used to predict and/or assign groups for any newly identified AC candidate harboring the same AC core motif. Asterisks indicate AC candidates presented herein in this chapter for publication consideration.

generation using Clustal Omega (ClustalO) (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). (Refer to **Figure 3**).

3.5.9 Bioinformatic analysis of the targeted protein candidates

Since in eukaryotes, it is widely accepted that proteins that are co-expressed often have related functions and linked coordinated regulatory systems [61–64], we then sought to explore and gain insights into the probable biological functions of each of our targeted protein candidates. This was achieved by subjecting each protein candidate to correlation expression analysis and stimulus-specific expression analysis so as to obtain its expression partners and then infer function(s). For correlation expression analysis, The Arabidopsis co-expression tool (ACT) (<http://www.arabidopsis.leeds.ac.uk>) [65] was used across all available microarray experiments, using the targeted protein candidate as the driver molecule and leaving the list limit of all other molecules blank to obtain a full correlational list. In this search, the top 50 co-expressed proteins (CEG50) were mostly considered, based on the Pearson correlation coefficient as a measure of similarity between them.

For stimulus-specific expression analysis, the expression profiles of the CEG50 together with the targeted protein candidate (AC-CEG50) were initially screened over all available ATH1:22 K arrays, Affymetrix public microarray data in the Genevestigator V3 (<https://www.genevestigator.com>) using the stimulus and mutation tools [66]. To obtain a greater resolution of the protein expression profiles, the normalized microarray data were subsequently downloaded and analyzed for experiments that were found to induce differential expression of the proteins. The data were downloaded from the following repository sites; GEO (NCBI) (<http://www.ncbi.nlm.nih.gov/geo/>) [67], the NASCArrays (<http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl>) [68], and the TAIR-ATGenExpress (<http://www.ebi.ac.uk/microarray-as/ae/>). The downloaded array data were then analyzed and fold-change (log2) values for each experiment calculated. (See example in **Figure A2**, Appendix A).

3.5.10 Outcomes

Interestingly and overwhelmingly, our research approach managed to confirm AC activity in all of the studied 14 AC candidates as is detailed in **Table 2** below. In the table, it can be seen that 6 of the candidates have already been published by our group while 3 are currently under review, again from our group. Therefore, in order to avoid duplications in this chapter, we only presented results for the last outstanding 5 candidates (**Figure 2**) for publication consideration.

4. Impact of our study

It is worth mentioning that our breakthrough in the identification of ACs in *A. thaliana* had an impetus effect of prompting us and other plant scientists across the globe to also search for similar proteins in Arabidopsis and other plants, using either our own motif search approach or other different methods such as omics analysis and homologous cloning. Through the motif search approach, 6 additional ACs were identified in *A. thaliana* (AtKUP7, AtKUP5, AtNCED3, AtTIR1, AtAFB1 and AtAFB5) by two groups [75–79], 4 in the basal plant *Physcomitrella patens*

Protein name	Gene ID	Annotated or confirmed function(s)	Publication status as AC
AtTTM 1	At1g73980	Leaf senescence [70]	Unpublished
AtTTM 2	At1g26190	Pathogen resistance [71]	Unpublished
AtTTM 3	At2g11890	Root development [72]	Under Review
AtLRR	At3g14460	Pathogen defense [73]	Published [51]
AtENTH	At1g25240	Endocytosis, pollen germination and pollen tube growth	Under Review
AtPPR	At1g62590	Chloroplast biogenesis and restoration of cytoplasmic male sterility	Published [50]
AtCIAP	At1g68110	Endocytosis and plant defense	Published [69]
AtMEE	At2g34780	Embryogenesis and response to abiotic stress	Published [52]
AtMTA	At3g02930	Regulation of microtubule assembly and growth	Unpublished
AtAC	At3g21465	Response to biotic stress	Published [53]
AtTIR-NBS-LRR	At3g04220	Disease resistance	Unpublished
AtLHL	At3g18035	Regulation of developmental and reproductive processes, and response to abiotic stress	Published [74]
AtFb	At3g28223	Regulation of the cell cycle	Unpublished
AtKRFB	At4g39756	Regulation of the cell cycle	Under Review

Table 2.
Current research status of the 14 *Arabidopsis* proteins harboring the AC search motif: [RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE] [33].

(PpAFB1, PpAFB2, PpAFB3 and PpAFB4) by one of the two mentioned groups [78, 79], 1 in *Brassica napus* (BnFolD) by another totally new group [80], and 1 (GmAC) by our group in soybean (*Glycine max*) [81]. AtKUP7 and AtKUP5 are permeases responsible for K⁺ ion flux [75, 76] while AtNCED3 participates in the biosynthesis of the stress-related hormone, abscisic acid (ABA) [77]. AtTIR1, AtAFB1, AtAFB5, PpAFB1, PpAFB2, PpAFB3 and PpAFB4 are all auxin receptors responsible for the auxin-mediated regulation of root growth [78, 79]. BnFolD regulates the metabolism of folate [80] while GmAC is involved in early plant development and stress response [81].

Using the other methods, other scientists identified a total of 13 additional ACs of which 1 was identified through the omics analysis method and 12 through homologous cloning. ZmRPP13-LK3, which participates in the ABA-mediated resistance of *Z. mays* plants to heat stress, was identified through omics analysis [82] while NbAC from *Nicotiana benthamiana* [83]; HpAC1 from *Hippeastrum hybridum* [84]; MpAC from *Marchantia polymorpha* [85]; AtDK4 from *A. thaliana* [12]; BdTTM3 and BdGUCD1 from *Brachypodium distachyon* [86, 87]; MdTTM1 and MdTTM2 from *Malus domestica* [88]; and ZjAC1, ZjAC2 and ZjAC3 from *Ziziphus jujuba* [89] were identified through homologous cloning. NbAC plays a role in tabtoxinine-β-lactam-induced cell death during the development of wildfire disease [83] while HpAC1 is involved in responses to infection by the plant fungal pathogen, *Phoma narcissi*, and also to injuries through mechanical damage [84]. MpAC is involved in male organ and cell development [85] while AtDK4 is responsible for the nitric oxide (NO) dependent pollen tube guidance and fertilization [12]. BdTTM3 is

responsible for responses to mechanical wounding [86] while BdGUCD1 is involved in jasmonic acid (JA) mediated responses to *Fusarium pseudograminearum* infection [87]. MdTTM1 and MdTTM2 currently do not have any known function(s) [88] while ZjAC1, ZjAC2 and ZjAC3 are involved in the significant acceleration of seed germination, root growth, and flowering respectively [89]. Notably, ZmPSiP, responsible for the polarized growth and re-orientation of pollen tubes in *Z. mays* and identified through the homologous cloning method [11], is so far the only plant AC to have been identified before our own ACs in *Arabidopsis*. Intriguingly, of the 13 additional plant ACs discovered through either the omics analysis or homologous cloning method, 4 (ZmPSiP, NbAC, AtDK4 and ZmRPP13-LK3) coincidentally harbor the same rationally designed AC search motif, commonly found in the AC protein molecules studied by our group.

All in all, a total of 30 plant proteins are currently known to harbor the rationally designed AC search motif among which 22 have been experimentally confirmed as functional ACs while 8 are still yet to be confirmed [11, 12, 33, 50–53, 69, 73–83]. Apparently, when this whole lot of 30 plant proteins harboring the rationally designed AC search motif was clustered together phylogenetically, using ClustalO, a total of 10 distinct groups emerged (**Figure 3**). Interestingly and when closely analyzed, the AC search motif in each of those 10 generated groups, tended to have a certain pattern, whereby its key catalytic residues are somewhat uniformly conserved per group, proposing that the motif could perhaps be utilized to predict and/or assign a group to any newly identified plant ACs harboring the very same motif. This is quite tempting and persuading considering that currently, plant ACs have not yet been systematically classified as compared to animal and microorganismal ACs that have six established and well-known classes [90]. Thus, in future, the AC search motif could possibly be of vital essence to both the identification and classification of more/new plant ACs.

5. Brief discussion and self-reflection

It is evident that the advent of high-throughput technologies in the twenty-first century ultimately revolutionized science, allowing the uncovering of most of the previously obscured areas such as the identification of ACs in plants in this case. New technologies such as omics analysis, homologous cloning and motif search approach proved very useful and key in the identification of ACs in plants. For instance, of the total (31) currently confirmed ACs in plants, the omics analysis method identified 1 AC, the homologous cloning method identified 12 while the motif search approach identified 18. More so, this whole AC identification spectrum also covered a wide range of plant species (eleven to be specific), which among them are seven herbaceous plants (*A. thaliana*, *N. benthamiana*, *Z. mays*, *H. hybridum*, *B. distachyon*, *B. napus* and *G. max*), two woody plants (*M. domestica* and *Z. jujuba*) and two basal plants (*M. polymorpha* and *P. patens*). In addition to this, there are also six dicots (*A. thaliana*, *N. benthamiana*, *M. domestica*, *G. max*, *B. napus* and *Z. jujuba*) and three monocots (*Z. mays*, *H. hybridum* and *B. distachyon*). Ideally, the wide-spread distribution of ACs in plants noted here, clearly shows the importance and significance of this group of proteins in plants as key enzymes for essential cellular processes such as growth, development and survival.

Symbolically, the fact that our own research method (motif search approach) has overall identified up to 18 plant ACs, i.e., 12 in *A. thaliana* and 6 in other plants, strongly demonstrates its strength among other methods currently known and/or

used. In addition, this also lends lots of confidence into this method for searching and identifying ACs in plants, and perhaps even in going further to classify such newly identified ACs into specific and/or particular groups. Thus, all in all, the motif search method can be confidently widened further to cover as many species as possible across the whole spectrum of the plant kingdom.

6. Conclusion

The report given here has three very key/important messages. Firstly, it proves the strength and usefulness of the method (motif search approach) we used in our research group to search and identify ACs in *A. thaliana* and therefore, the method can too be applied, confidently so, to other plants in the plant kingdom to bring back valid results. Secondly, it proposes the possibility of using the AC search motif to determine and classify newly identified ACs into specific and recognizable groups. Lastly, it proved the relevance and usefulness of *A. thaliana* as a model organism for plant research, particularly when it comes to the designing of experiments and modeling of concepts that are relevant and applicable to the rest of the plant kingdom.

7. Future prospects and recommendations

Considering that ACs and their functions have also now been identified in a number of commercially important plants such as maize, tobacco, soybean, apple, cabbage and jujube, more work underlying the mechanisms of action of these important proteins should be pursued and interrogated. Such an approach will then help elucidate or reveal the exact functions or roles of these enzymes and/or their cAMP-linked signaling systems in plants.

Author contributions

OR conceived the idea; PC, AS-M, TD, EB, KS, GM, NM, MT and DK did the experiments. OR wrote the manuscript and PC edited it. All authors read and approved the manuscript.

Funding

The project was funded by the National Research Foundation (NRF) of South Africa (Grant Numbers: CSUR78843 & CSUR93635) and the Organization for Women in Science for the Developing World (OWSD).

Conflict of interest

The authors declare no conflict of interest.

Appendix A: Illustration of some of the results obtained during our process of searching and identifying ACs in *A. thaliana*

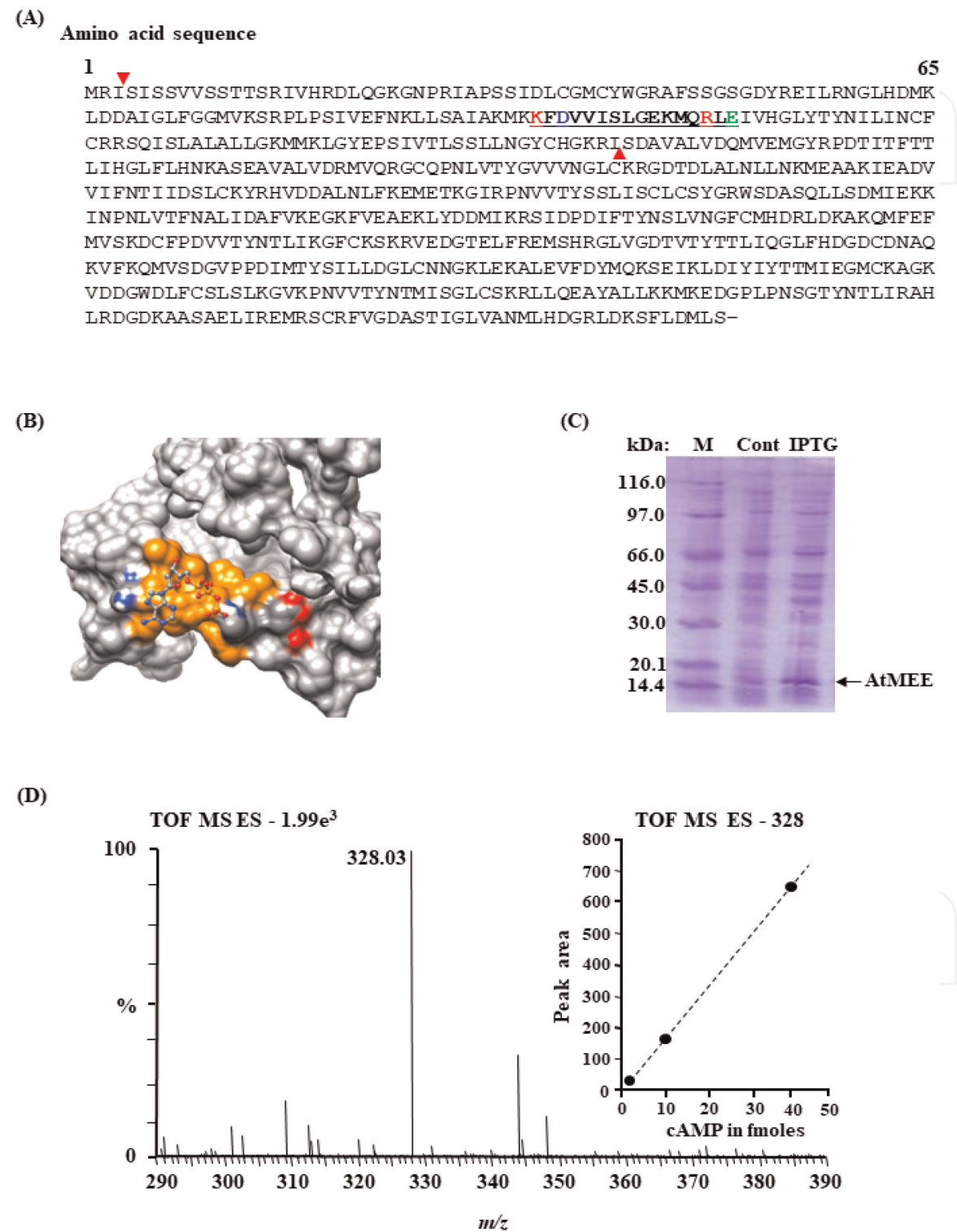


Figure A1.
(A) Amino acid sequence of AtPPR showing presence of the AC search motif in the cloned and recombinantly expressed protein fragment (inverted red triangles) [50]. (B) 3D structure of AtLRR showing the AC search motif (gold) in a solvent-exposed position that is amenable for an unimpeded ATP binding and ultimately catalysis [51]. (C) Expression profile of the recombinant AtMEE protein [52]. (D) Mass spectrometric analysis of the in vitro AC activity of AtAC [53].

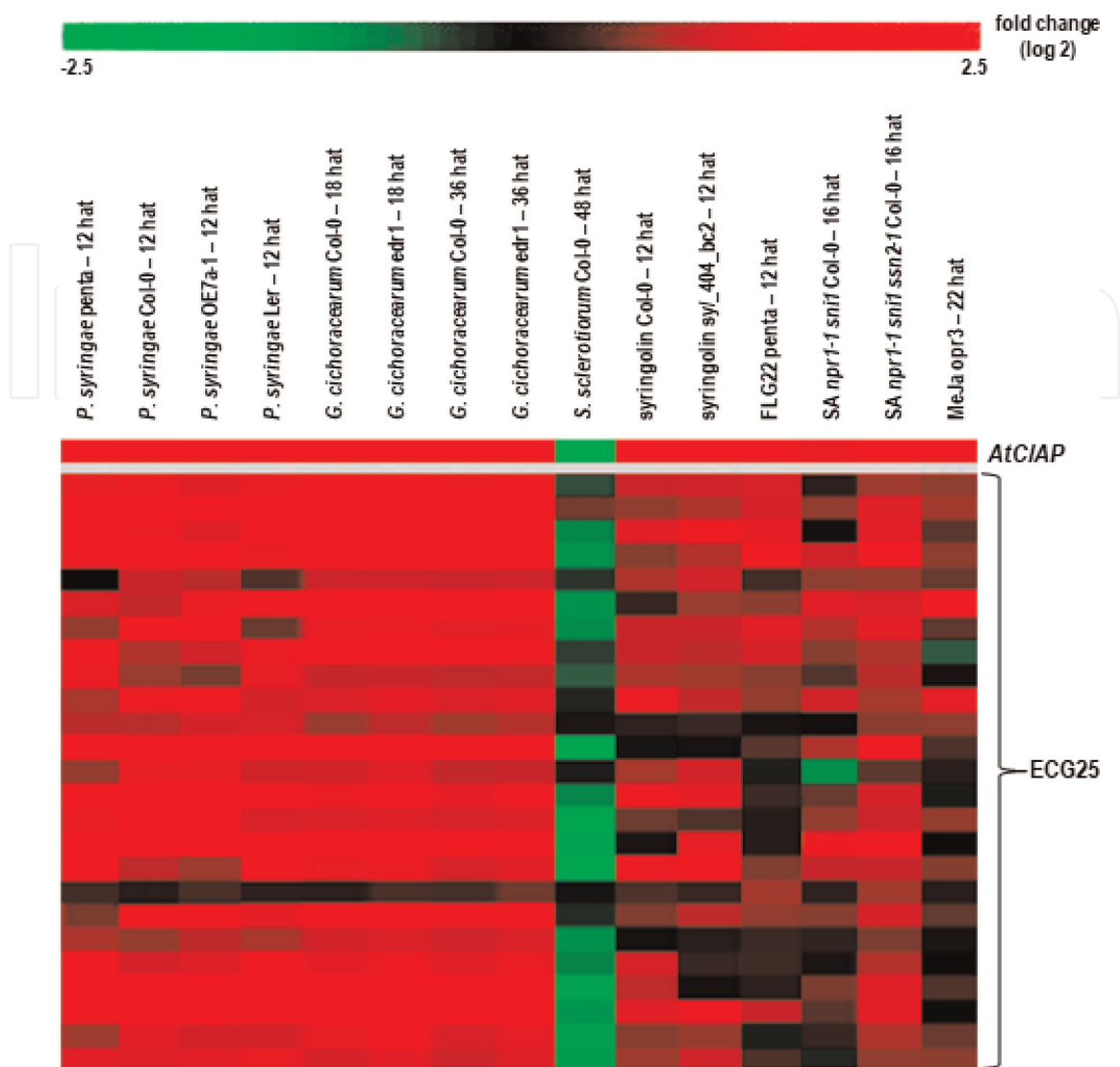


Figure A2.
Heat map of AtCIAP showing its correlation and differential expression patterns as an AC [69].

Appendix B: Cloning of the Arabidopsis proteins harboring the AC search motif

AC molecule	Gene ID	Primer set
AtTTM 1*	At1g73980	Fwd: 5'-TCACCCAGAATAACTTTTGAAGTTAGTGTT-3'
		Rev.: 5'-AGATGCGGATGCGGAGAATGATAAGACAGA-3'
AtTTM 2*	At1g26190	Fwd: 5'-GGTCAAGACAGCAATGGAATTGAGTTTCAT-3'
		Rev.: 5'-GTCATAGTCCGTTAACCGTGGATCATCAAA-3'
AtTTM 3	At2g11890	Fwd: 5'-GAAGTCGAAGTCAAGCTCCGTCCTAACC-3'
		Rev.: 5'-AGGAAGTTTTCCTGACCGGAAAACAGCAAA-3'
AtLRR	At3g14460	Fwd: 5'-CCAGGGGTTGGAAAGACTACCTTGACAGAG-3'
		Rev.: 5'-TACTAATTCCTCCCTATCGAAAACATGACC-3'

AC molecule	Gene ID	Primer set
AtENTH	At1g25240	Fwd: 5'-GAATTTGGGGTCTCAAACGCGCACGACATT-3'
		Rev.: 5'-GAAGGTAATCAAATCTGGTAACGTGTGAGT-3'
AtPPR	At1g62590	Fwd: 5'-CGGAGCCAAATCGAGAAGATGAGGATCTCG-3'
		Rev.: 5'-CATTTCCACCATTGATCAACCAAAGCTAC-3'
AtCIAP	At1g68110	Fwd: 5'-GAATTCTGCAAAGGTTTCGGTGCTCGAAC-3'
		Rev.: 5'-GAATGTAATCAAATCTGGCATTGTATAAGT-3'
AtMEE	At2g34780	Fwd: 5'-GCCCCGAAGGATCCAATGTCGGAGTTGGAG-3'
		Rev.: 5'-GCGCCGGAATTCCGAGACTAATTGCGCTTC-3'
AtMTA*	At3g02930	Fwd: 5'-ACTGATAAGAGGTCCCCCAAAGCTCCAACC-3'
		Rev.: 5'-CAAAGCCTTCAACCGTATTAAGTCTGACGA-3'
AtAC	At3g21465	Fwd: 5'-GCTGCCAAAAGAGGAGACACAGAGTCGTTA-3'
		Rev.: 5'-GCTAAGAAGAGCTTCATTCTTGTTTAACTC-3'
AtTIR-NBS-LRR*	At3g04220	Fwd: 5'-GATTCTTCTTTTTTACTCGAAACTGTTGCT-3'
		Rev.: 5'-TGGATCCACTTTGTAGAAAATGACTATCAC-3'
AtLHL	At3g18035	Fwd: 5'-GGAAGGCCTAGGAGAGTTGTTGACCCTAGC-3'
		Rev.: 5'-GAACAGAGCTTCTTGCATTGCCTCTGCTTC-3'
AtFb*	At3g28223	Fwd: 5'-AACAACATATCGTGATCACCTTGTGTATCC-3'
		Rev.: 5'-AAGCTTGTCGGTTGGAGGCGAGGAAGGGAT-3'
AtKRFB	At4g39756	Fwd: 5'-GGAATTCCCATGGCTACTGGTACGGAATCT-3'
		Rev.: 5'-GACTCGAGCGTAGCAGCCAATGCGGGAGAG-3'

Each primer set was designed in such a way that the two annealing regions kept the AC search motif in between so that each resultant gene fragment would be containing the motif. Asterisks represent candidates presented herein in this chapter for publication.

Table B1.
Primers used for amplification of the gene fragments of the Arabidopsis protein candidates harboring the AC search motif [RK][YFW][DE][VIL][FV]X(8)[KR]X(1,3)[DE] [33].

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