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Establishment and characterization of a new class of adenylate cyclases (class VII ACs) in plants

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A R T I C L E   I N F O

A B S T R A C T

Adenylate cyclase is the key enzyme in the synthesis of cAMP. Now, more and more plant genes which possessing AC function are being identified, but the classification of plant ACs has not yet been systematically studied and the relationship of plant ACs with other existing six classes ACs in animals and microorganisms is still unclear. In this study, we found that 7 of the 15 reported plant ACs with conserved CYTH-like_AC_IV-like domain were clustered into a group with high confidence (Group IV), while the other plant ACs were clustered into other three groups with no common domain. In addition, we also found that the Group IV plant ACs were grouped into an independent and specific class (Class VII), separated from the existing six classes of ACs. The Group IV plant ACs, compared to the existing six classes of ACs, own unique CYTH-like_AC_IV-like conserved domain and EXEXK signature motif, characteristic protein tertiary structures, specific subcellular localization and catalytic conditions. In view of the above, we regarded the Group IV plant ACs as the seventh class of AC (VII AC). This study does the systematic classification of plant ACs which could lay a foundation for further identification and study of the biological functions of the plant-specific VII ACs.

1. Introduction

Cyclic adenosine monophosphate, commonly known as cAMP, is a key second messenger and an important signaling molecule [1, 2]. It was firstly identified by Earl Wilbur Sutherland in 1956 and functioned importantly as a second messenger in liver [3,4]. In addition, Eric Richard Kandel found that cAMP plays a very important role in brain cell repair and could switch short-term memory into long-term memory and relieve the fatigue of brain cells [5,6]. Due to their breakthrough work, Sutherland and Kandel were awarded the Nobel Prize in Physiology or Medicine in 1971 and 2000, respectively. In plants, cAMP has been demonstrated to function as a second messenger involved in pollen tube growth and reorientation in maize, and seed germination, root growth and flowering time regulation in *Arabidopsis thaliana* [1,7,8].

Adenylate cyclase (AC) is the only key enzyme that catalyzes the synthesis of cAMP from ATP by a one-step pathway. Six distinct classes of ACs have been described in various organisms, not including higher plants, according to their common features [9–15]. Class

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I ACs are primarily observed in *E. coli* [16]. Class II ACs are bacterial toxins [17,18]. Class III ACs are frequently detected in eukaryotes and gram-positive prokaryotes [15,19,20]. Class IV ACs have been designated as CyaB and were identified in *Aeromonas hydrophila* and *Yersinia pestis* [11,21]. The other two classes of ACs (V and VI) were primarily observed in specific bacterial groups [10,12].

The discovery of plant AC is severely restricted by its poor sequence conservation and low enzyme activity. However, with the development of biotechnology and the discovery of the important functions of cAMP in plants, the study of plant ACs is becoming more and more active. At present, a total of 26 plant proteins with AC activity have been reported in nine plant species including three monocotyledon plants (*Zea mays*, *Hippeastrum × hybridum*, and *Brachypodium distachyon*), five dicot plants (*Nicotiana benthamiana*, *Arabidopsis thaliana*, *Glycine max*, *Malus domestica*, and *Ziziphus jujuba*) and one basal plant liverwort (*Marchantia polymorpha*) [1,7,8,22–39]. However, the systematical classification study of plant AC is lacking, and the relationship between plant ACs and animal and microbial ACs is still unclear.

In the current study, we made a classification of reported plant ACs and analyzed the relationship between plant ACs and other existing six classes of ACs, basing on our research on ACs of apple and Chinese jujube. The results indicated that the plant ACs containing the CYTH domain were clustered into a new class and independent to the existing six ACs, and regarded as class VII ACs. Then, we elucidated the characteristics of these class VII ACs through protein structure, catalytic activity and subcellular localization analysis. Our results thus could provide a solid foundation for further studies of plant ACs.

2. Material and methods

2.1. Plant materials

Tobacco (*Nicotiana benthamiana*) leaves were used for subcellular localization assays, which was cultivated at 25 °C and 80% humidity under a 16 h (light)/8 h (dark) photoperiod.

Conserved domain and motif finding, transmembrane structure prediction and protein model construction.

Conserved domain was analyzed by National Center for Biotechnology Information (NCBI) CD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The Multiple Em for Motif Elicitation (MEME) suite was used to identify conserved motifs [40]. Transmembrane helices were analyzed by TMHMM Server v.2.0 software (http://www.cbs.dtu.dk/services/TMHMM/). The protein models were generated with the iterative threading assembly refinement (I-TASSER) method by submitting full-length amino acid sequences to the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and selecting the model with the highest *C*-score. Docking of ATP and the catalytic center was performed using Autodock Vina (ver. 1.1.2). The docking images were generated with PyMOL (ver 1.7.4).

2.2. Multiple sequence alignment and phylogenetic tree construction

The amino acid sequences of the 15 previously reported representative plant ACs and the six classes of ACs from microorganisms and animals were downloaded from NCBI. Multiple sequence alignments of the amino acid sequences were performed by the ClustalW program with the parameters of pairwise alignment (gap opening penalty 10 and gap extension penalty 0.1) and multiple alignments (gap opening penalty 10 and gap extension penalty 0.2). The evolutionary history of the ACs was inferred by using the maximum likelihood method based on the poisson correction model with 1000 replicates by MEGA7 [41].

2.3. Protein purification and AC activity detection

The CDSs (complete coding sequences) of *HpAC1* was synthesized by General Biosystems (Anhui) Co., Ltd. And inserted into the pET-28a vector. The specific methods of protein purification and AC activity detection referred to Liu et al. [7,26].

2.4. Subcellular localization observation assay

For observation of the subcellular localization of ZjACs, the *A. tumefaciens* GV3101 strain containing *35S::ZjACs-GFP* was transiently introduced into Tobacco leaves. The images were captured by using the confocal laser-scanning microscope (LSM 710; Carl Zeiss).

2.5. Statistics

Student's *t*-test was used to perform significance analysis by SPSS 16.0 software.

3. Results

3.1. Phylogenetic analysis of the classification of plant ACs

To illuminate the relationships of plant ACs, a phylogenetic tree of typical plant ACs was constructed by using their full-length amino acid sequences. As shown in Fig. 1a, four groups were generated. Seven of the fifteen plant ACs including six ACs from three woody plants (*Ziziphus jujuba*, *Malus domestica* and *Pyrus bretschneideri*) and one AC from a herbaceous plant (*Hippeastrum × hybridum*)
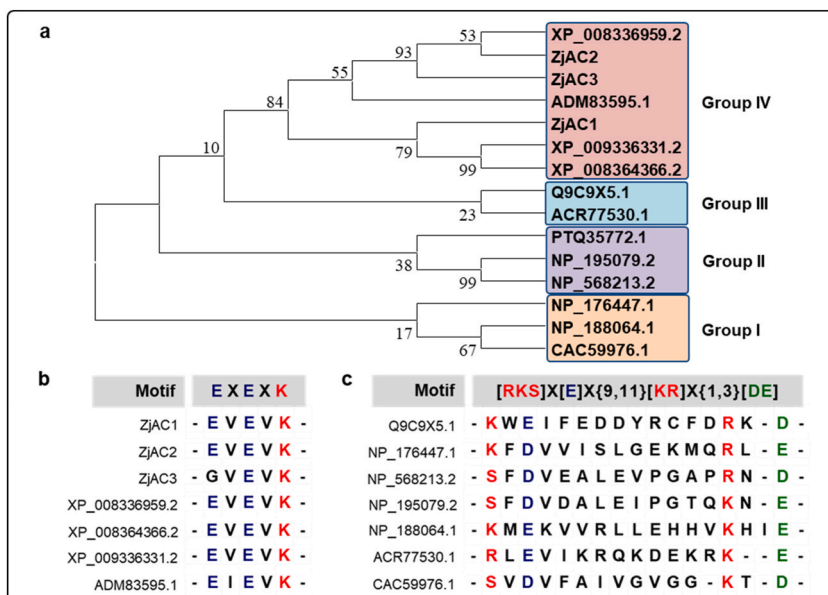
Fig. 1. Phylogenetic tree and core motif analysis of all known plant ACs. a, Phylogenetic analysis of reported plant ACs. The gene names and accession numbers of every group ACs are the following: Group I: *Zea mays* pollen signaling protein with adenylyl cyclase activity (CAC59976.1), *Arabidopsis thaliana* leucine-rich repeat adenylyl cyclase 1 (NP_188064.1), *Arabidopsis thaliana* pentatricopeptide repeat-containing protein (NP_176447.1); Group II: *Arabidopsis thaliana* K$^+$-uptake permease 7 (NP_568213.2), *Arabidopsis thaliana* K$^+$-uptake permease 5 (NP_195079.2), *Marchantia polymorpha* COMBINED AC with PDE (PTQ35772.1); Group III: *Nicotiana benthamiana* adenylyl cyclase (ACR77530.1), *Arabidopsis thaliana* clathrin assembly protein (Q9C9X5.1); Group IV: *Ziziphus jujuba* ZjAC1 (Liu et al., 2022), *Ziziphus jujuba* ZjAC2 (Liu et al., 2022), *Ziziphus jujuba* ZjAC3 (Liu et al., 2022), *Malus domestica* TTM1 (XP_008336959.2), *Malus domestica* TTM2 (XP_008364366.2), *Pyrus* × *bretschneideri* PbrTTM1 (XP_009336331.2), *Hippeastrum* × *hybridum* HpAC1 (AMD83595.1). b, The EXEXK motif of group IV ACs. c, The [RKS]X [E]X{9,11}[KR]X{1,3}[DE] motif of group I ~ III ACs except for MpCAPE.

were clustered into Group IV, which contain the same CYTH-like_AC_IV-like domain and EXEXK signature motif (Figs. 3a and 1b). The other three groups of plant ACs did not contain the common domain and AC conserved domain, except for MpCAPE, which has the AcyC conserved domain of class III ACs. However, the plant ACs that lack the AC conserved domain were also observed to contain the AC core motif [RKS]X [E]X{9,11}[KR]X{1,3}[DE] (Fig. 1c). Thus, only the Group IV ACs is common and functionally conserved AC in plant.

3.2. Establishment of a novel class of AC-the class VII ACs specific in plants

Six distinct nonhomologous classes of ACs have been identified in microorganisms and animals [9,12,15] and named by using Roman numerals, following the general scheme introduced by Danchin [9]. To illustrate the relationship between the Group IV plant ACs and the existing six classes of ACs, a phylogenetic tree was constructed by aligning multiple sequences of the AC conserved domain. We observed that the AC proteins from microorganisms, animals and humans were classified into six classes (Class I to VI) (Fig. 2), which was consistent with the findings described in previous reports [9,12,15]. The MpCAPE (PTQ35772.1), from the basal higher plant liverwort, was grouped into class III ACs (Fig. 2), which was also in keeping with the previous report [29]. Interestingly, the Group IV plant ACs were grouped into an independent additional class, separated from class IV ACs (Fig. 2), although they shared the same CYTH superfamily domain. Thus, we designated them as a new class of ACs, namely Class VII ACs.

3.3. The conserved domain and motif of class VII ACs

To clarify the common characteristics of class VII ACs, we compared the conserved domains and motifs between class VII ACs and class IV ACs because both of them belong to the CYTH superfamily. As shown in Fig. 3, the class IV ACs from bacteria contain a CyaB conserved domain, and the class VII ACs from plants contain a CYTH-like_AC_IV-like conserved domain. The motifs differed significantly between the two classes. We found that the class IV ACs contain four main motifs, but six signature motifs were identified in class VII ACs, and all the motifs of class VII ACs were different from class IV ACs (Fig. 3). The motif difference originating from the protein primary structure may have caused the separation of class VII ACs and class IV ACs in the phylogenetic tree.

3.4. The characteristic tertiary structure of class VII ACs

To further identify the characteristics of class VII ACs, protein models were generated with the iterative threading assembly
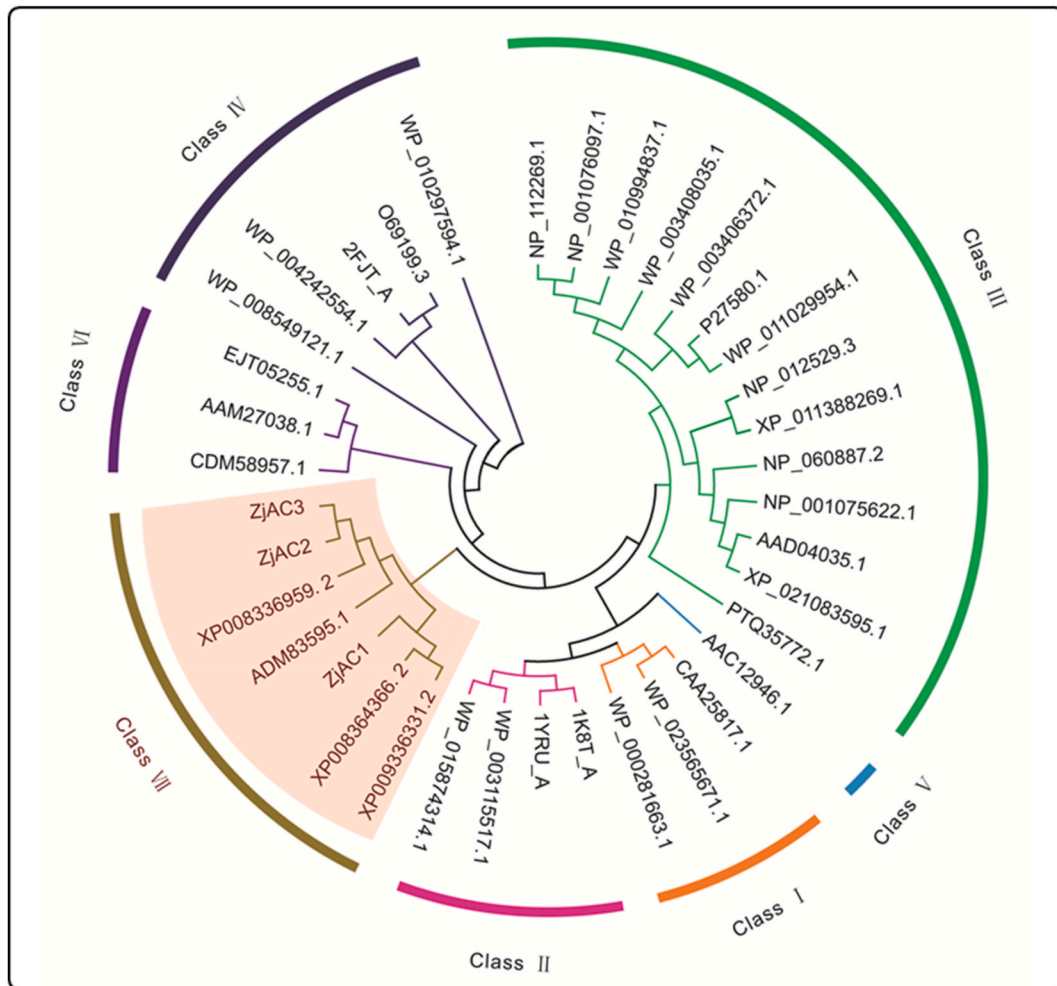
Fig. 2. Evolutionary relationship of the plant ACs with six other classes of ACs. The pink box shows a novel class of ACs from plants. The gene names and accession numbers of every class ACs as follow: Class I ACs: *Escherichia coli* adenylyl cyclase (CAA25817.1), *Escherichia coli* class I adenylyl cyclase (WP_023565671.1), *Escherichia coli* class I adenylyl cyclase (WP_000281663.1); Class II ACs: *Bacillus anthracis* calmodulin-sensitive adenylyl cyclase (1K8T_A), *Bordetella pertussis* bifunctional haemolysin-adenylyl cyclase (1 YRT_A), *Pseudomonas* type III secretion system adenylyl cyclase effector ExoY (WP_003115517.1), *Candidatus Hamiltonella defensa* CyaA/EF/ExoY family adenylyl cyclase toxin (WP_015874314.1); Class III ACs: *Rattus norvegicus* adenylyl cyclase type 2 (NP_112269.1), *Oryctolagus cuniculus* adenylyl cyclase type 5 (NP_001076097.1), *Nostocaceae* adenylyl/guanylate cyclase domain-containing protein (WP_010994837.1), *Mycobacterium tuberculosis complex* adenylyl cyclase (WP_003408035.1), *Mycobacterium tuberculosis complex* adenylyl cyclase (WP_003406372.1), *Glutamicibacter nicotianae* adenylyl cyclase (P27580.1), *Streptomyces* adenylyl/guanylate cyclase domain-containing protein (WP_011029954.1), *Saccharomyces cerevisiae S288C* adenylyl cyclase (NP_012529.3), *Ustilago maydis 521* adenylyl cyclase (XP_011388269.1), *Homo sapiens* adenylyl cyclase type 10 isoform 1 (NP_060887.2), *Oryctolagus cuniculus* adenylyl cyclase type 10 (NP_001075622.1), *Rattus norvegicus* soluble adenylyl cyclase (AAD04035.1), *Mesocricetus auratus* adenylyl cyclase type 10 (XP_021083595.1), *Marchantia polymorpha* Mp*CAPE* (PTQ35772.1); Class IV ACs: *Pectobacterium carotovorum* class IV adenylyl cyclase (WP_010297594.1), *Aeromonas hydrophila* adenylyl cyclase CyaB (O69199.3), *Pectobacterium carotovorum* class IV adenylyl cyclase (WP_010297594.1), *Proteus* class IV adenylyl cyclase (WP_004242554.1), *Pseudovibrio* class IV adenylyl cyclase (WP_008549121.1); Class V ACs: *Prevotella ruminicola* adenylyl cyclase (AAC12946.1); Class VI ACs: *Rhizobium* sp. *CCGE 510* adenylyl cyclase protein (EJT05255.1), *Rhizobium etli* adenylyl cyclase CyaC (AAM27038.1), *Rhizobium favelukesii* adenylyl cyclase protein (CDM58957.1); Class VII ACs: *Ziziphus jujuba* ZjAC1 (Liu et al., 2022), *Ziziphus jujuba* ZjAC2 (Liu et al., 2022), *Ziziphus jujuba* ZjAC3 (Liu et al., 2022), *Malus domestica* MdTTM1 (XP_008336959.2), *Malus domestica* MdTTM2 (XP_008364366.2), *Pyrus × bretschneideri* PbrTTM1 (XP_009336331.2), *Hippeastrum × hybridum* HpAC1 (AMD83595.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

refinement method. We found that class VII ACs comprise an eight-strand β-barrel that constructs a topologically closed tunnel inside, which locates two or three Glu binding Mn^{2+} (Fig. 4a), demonstrating a potential solvent exposed catalysis center. To explore the feasibility of catalysis activity, ATP molecular docking was conducted, which suggested that ATP could dock at the catalysis center with a good free energy and binding pose. For example, in ZjAC3 (Fig. 4a), both sides of the tunnel were exposed to solvent, and it comprises Mn^{2+}-binding amino acid residues (Glu 41, Glu 194 and Glu 196) and phosphate oxygen-binding residues (Arg 91, Lys 104,
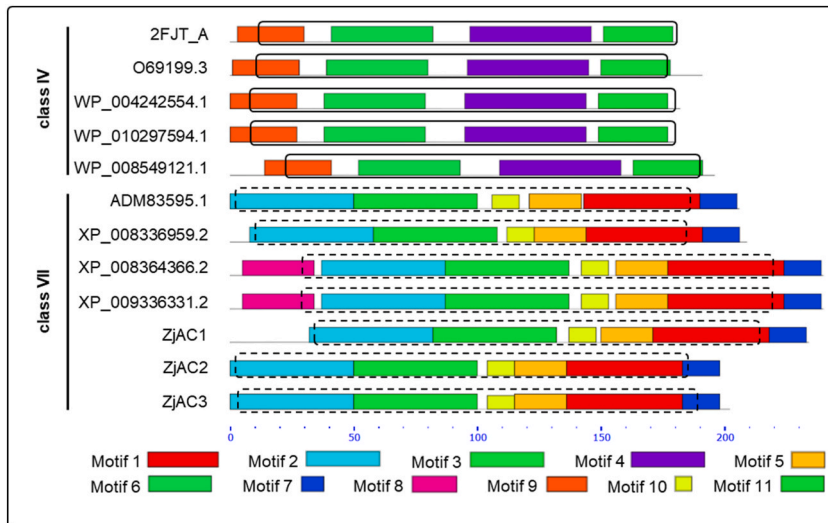
Fig. 3. Conserved domain and motif analysis of class VII and class IV ACs. The solid-line box represents the location of the CyaB conserved domain of class IV ACs, and the dashed-line box represents the location of the CYTH-like_AC_IV-like conserved domain of class VII ACs. b, c, Representative protein model of class VII ACs (ZjAC3) and class IV ACs (YpAC). d, *ZjAC1*, *ZjAC2* and *ZjAC3* are all localized in the nuclei and cytoplasm of the cell.
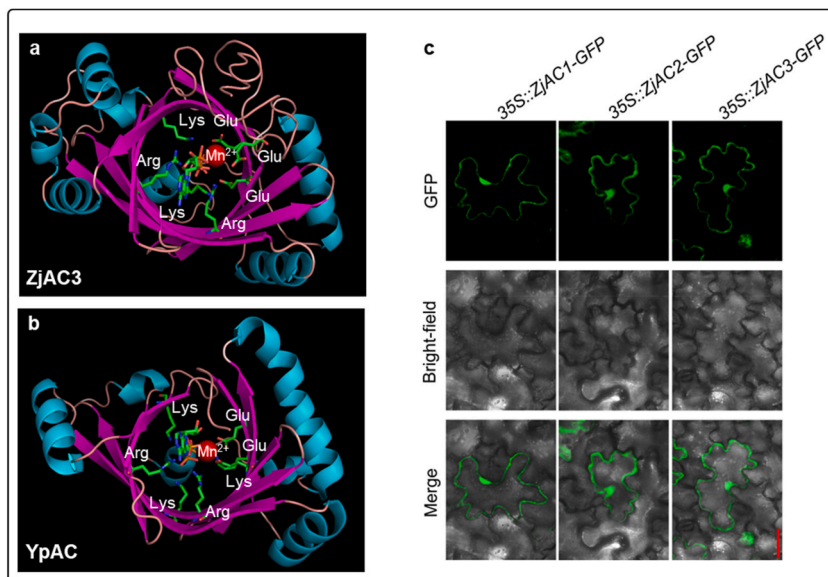

Fig. 4. Distinct characteristics of class VII ACs. a and b, Representative protein model of class VII ACs (ZjAC3) and class IV ACs (YpAC). c, *ZjAC1*, *ZjAC2* and *ZjAC3* are all localized in the nuclei and cytoplasm of the cell.

Arg 168 and Lys 225). Almost all residues binding Mn^{2+} were from the same β-strand in both bacterial class IV ACs and plant class VII ACs, but the residues from plants were not as far inside as those from bacteria, especially compared to YpAC (Fig. 4b). There are flexible residues near the tunnel in class VII ACs, which may affect the catalytic activity for blocking ATP (Figure S1).

3.5. The specific subcellular localization of class VII ACs

The analysis of transmembrane helices indicated that class VII ACs has no transmembrane structure. We further investigated the localizations of *ZjAC1~3* genes, which belong to class VII ACs. The CDSs of *ZjAC1~3* were constructed with a *C*-terminal green fluorescent protein (GFP) tag driven by a *35S* promoter and transiently introduced into tobacco leaves. As shown in Fig. 4c, the GFP signal was observed in the nuclei and cytoplasm, which indicated that the *ZjAC1~3* localized in the nuclei and cytoplasm and might perform their functions there, not like most reported ACs, which are localized in membranes [42].
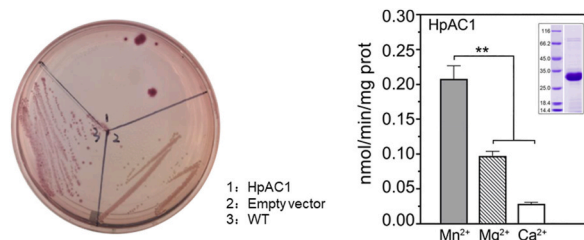
Fig. 5. Functional verification of HpAC1. a, Complementation of the SP850 *E. coli* cyaA mutation by HpAC1. Colonies of host cells transformed with a recombinant vector showed a purple colour, while cells harboring an empty vector remained colourless. b, Enzymatic activity of HpAC1 under different divalent cations of Mn^{2+}, Mg^{2+}, and Ca^{2+}. Reaction mixtures (1 mL) containing 50 µM Tris-HCl, pH 7.5, each divalent cation (1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ or 0.5 mM $MnCl_2$) and HpAC1 enzyme as specified were incubated for 30 min at 37 °C. SDS protein gel stained with Coomassie blue showing the HpAC1 protein band in bacterial extracts collected at the indicated times after IPTG induction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. The distinct catalytic conditions of class VII ACs

The class VII AC enzymes showed the highest catalytic activity under Mn^{2+}, rather than Mg^{2+} or Ca^{2+}. For example, ZjAC1 and ZjAC2 had no activity under Mg^{2+} and very weak activity under Ca^{2+} [7]. Similarly, the catalytic efficiencies of MdTTM1, MdTTM2 and HpAC1 under Mn^{2+} were also considerably higher than those under Mg^{2+} and Ca^{2+} (Fig. 5) [34]. To date, according to the most recent literatures, all the other classes of ACs have the best catalytic activity under the Mg^{2+} except a few members of class III ACs [29, 43–46].

4. Discussion

Although more and more *ACs* were identified in plants, the classification of plant ACs is lacking. In this study, a phylogenetic tree of reported plant ACs was constructed by using 6 woody plant ACs identified by our team [7,34] and other previously reported plant ACs [1,22,23,26,27,29–31]. Based on the phylogenetic analysis, we found that the 6 woody plant ACs together with the previously reported HpAC1 were classified into a new group, which contain the same CYTH superfamily domain, but Groups I ~ III of plant ACs that have a *C*-terminal AC catalytic domain similar to those of class III ACs [29] or contain the core motif [RKS]X [E]X{9,11}[KR]X{1,3}[DE] derived from functionally assigned residues in the catalytic center of plant guanylyl cyclases (GCs) [31,47,48].

The classification of ACs in microorganisms, animals and humans has long been studied, but not in plants. A classification system for purine nucleotide cyclases was proposed by Danchin in 1993, primarily based on structural features (e.g., similarities of amino acid sequences), which define three distinct groups [9]. To date, ACs have been subdivided into six classes according to their sequence homology within their catalytic domains [9,12,15]. We observed that the Group IV plant ACs could form a new cluster based on the amino acid sequence similarity of the ACs conserved domain, which differs from the existing six classes of ACs with distinct motifs compared to the existing six classes of ACs including class IV ACs, although the Group IV plant ACs also contains the CYTH conserved domain and the EXEXK signature motif like class IV ACs mostly found in bacterial.

The catalytic process of the Group IV plant ACs depends on metal ions notably different from the existing six classes of ACs. Most ACs have been reported to prefer Mg^{2+} as an ion cofactor [49,50]. However, the Group IV plant ACs shows universally higher activity under the condition of Mn^{2+} ions than that under Mg^{2+} and Ca^{2+}. This property may be related to the differences in the ion molecular weight and the AC amino acid binding sites, which need to be further verified. Under the condition of *in vitro* catalysis, all ZjACs, belonging to the Group IV plant ACs, showed certain AC activity with the assistance of divalent ions, especially Mn^{2+}. It is possible that the larger atomic radius of Mn^{2+} may be able to compensate for the structural deficiency and coordinate with the phosphoric acid of ATP and the acidic amino acids, thereby producing a higher AC activity.

Therefore, the Group IV plant ACs containing CYTH superfamily domain could be considered as a new class of ACs, VII ACs, in terms of its distinct conserved domain and motif, characteristic tertiary structure as well as specific subcellular localization and catalytic conditions. The members of VII ACs will be extended further as the genome sequencing of plants becomes increasingly common.

Author contribution statement

Zhiguo Liu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ye Yuan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lixin Wang: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Haonan Cao, Chenyu Wang: Performed the experiments, Analyzed and interpreted the data.

Xuan Zhao, Lili Wang: Analyzed and interpreted the data.

Mengjun Liu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18612.

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