Genome wide identification of bromelain-like cysteine proteases in Puya raimondii

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Short Report

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Abstract

Bromelains are cysteine proteases of the papain family (C1A subfamily). These enzymes are of great commercial value due to their applications in the food, pharmaceutical and biotechnological industries. In plants, they play an important role in multiple physiological processes from germination to senescence, mainly in the defense of plants against biotic and abiotic stresses. In this study, we used available data from the *P. raimondii* genome (Bioproject PRJNA639677) to identify genes encoding bromelain-type proteases. Six bromelain-like nucleotide sequences are postulated in *P. raimondii* draft genome. Hormone, stress and light responsive elements in the *PrBLCP*s sequences were detected. The theoretical molecular weights of the proteins putatives PrBLCPs in *P. raimondii* range from 36643.21-45761.20 Da and theoretical isoelectric point 5.05 to 8.63. Multiple primary sequence alignments and structural model prediction demonstrate that *P. raimondii* putative proteases are very similar to *A. comosus* fruit bromelain (O23791) recognized in the SwissProt/Uniprot database. Phylogenetic relationships between the bromelain-like putative cysteine proteases of *P. raimondii* and the bromelain proteases of *A. comosus* placed the PrBLCPs in two subclades, although with very short phylogenetic distances between them. The results of this study provide valuable information for future genomic studies of bromelain family genes in the genus *Puya*. In addition, it aids in the understanding of the regulatory mechanisms of these proteases and their roles in plant stress tolerance.

Key message

In this work, genes encoding bromelain like proteases were identified in *P. raimondii*. The analysis of putative proteins PrBLCPs demonstrated that they have similarity to fruit bromelain protein from *A. comosus*. These results provide valuable information for future genomic studies of bromelain like genes in the *Puya* genus.

Introduction

Bromelains are cysteine proteases that catalyze the dissociation of peptide bonds from other proteins, resulting in peptides or free amino acids. The name bromelain was originally applied to any protease isolated from Bromeliaceae family plant (Mohamed et al. 2009). Due to their capacity to break down proteins in muscle and hydrolyze collagen and elastin, various cysteine proteases from plants are crucial in food industry operations such as beer processing, milk coagulation, and meat thinning (Arshad et al. 2016). They are also employed to create commercial medications that treat digestive problems, viral and inflammatory illnesses, cutaneous ulcers, cancers, and metastatic diseases (Heinrich et al. 2012; Manzoor et al. 2016). Bromelain has been studied since 1894 and is thought to be a complex combination of proteolytic enzymes with several applications in food, biotechnology and pharmaceuticals (Kwatra 2019). Cysteine proteases play an important role in numerous physiological processes in plants, from germination to senescence. For amino acid recycling, modify or eliminate defective or poorly folded proteins. During seed germination, they destroy storage proteins. They are involved in protein signaling, proenzyme activation, and post-transduction alterations. Senescence and

programmed cell death, as well as modulating responses to biotic and abiotic stress (Buono et al. 2019). Plant proteases with the highest economic value include papain (*Carica papaya*), ficin (*Ficus carica*) and bromelain (*Ananas comosus*).

Bromeliaceae (Poales) comprises 58 genera and 3.140 species (Givnish et al. 2014). Usually epiphytes, caulescent, seismic, and perennial, they are seldom bushy, terrestrial or large. They penetrate harsh habitats due to physiological adaptations and metabolic efficiency, as well as morphological and ecological adaptability. Thus, the family dominated neotropical epiphytes (Eggli and Gouda 2020). Bromelias are ethnobotanical, ornamental, antibacterial, therapeutic, edible, and agricultural, and certain species are used as living fences. Bromeliaceae plants have a greater endopeptidase content than physiologically necessary, hence they have been studied for bromelains, proteolytic enzymes (Albarrán-Mondragón et al. 2022).

Puya raimondii is one of the most researched *Puya* species and has the widest latitudinal spread. The biggest and perhaps the longest-lived bromelia, it grows to 5 m in the vegetative stage and 12 m in the reproductive stage, and blooming individuals survive 60–100 years (Hornung-Leoni et al. 2013). As the Queen of the Andes, it is indigenous to Peru and Bolivia and designated as an endangered species by the International Union for Conservation of Nature (UICN), having 800.000 individuals in Peru and 30.000–35.000 in Bolivia (Lambe 2009). *P. raimondii* provides refuge, food, and nesting sites for numerous birds in its natural ecology. The first divergent members of the subfamily Bromelioideae, the genus *Puya* Molina (Bromeliaceae, Puyoideae), are one of the few long-lived plant lineages with monocarpic specimens and reproductive strategies that can reveal evolution and phylogenetic relationships. They are ideal for researching Andean species radiation due to their ecological, systematic, evolutionary, and biogeographic significance (Jabaily and Sytsma 2013).

A. comosus is a well-studied species in the Bromeliaceae family, from which many cysteine proteases have been identified, the most important of which being stem bromelain, fruit bromelain and comosain (Pang et al. 2020). The stems and fruit crude extracts of *A. comosus* contain a complex combination of tiol proteases and other components that have been poorly described as protease, peroxidase, cellulase, phosphatase, and glucosidase inhibitors. The predominant enzyme in *A. comosus* crude stem extract is stem bromelain (EC 3.4.22.32). It was later shown to include ananain (EC 3.4.22.31) (Rowan et al. 1988) and comosain in minor amounts (Rowan et al. 1990). Fruit bromelain (EC 3.4.22.33), on the other hand, is discovered in larger proportions in the crude extract of *A. comosus* fruit, albeit minor amounts of stem bromelain are also detected. Matagne et al. (2017) identified eight active forms of bromelain from pineapple stem extract: two bromelain acids, three basic bromelains, two ananains, and comosain. Since they all have various substrate specificities and optimal pH, they are classified as unique molecular species in catalytic terms. However, only three bromelain-type enzymes have been confirmed and recognized in the SwissProt/Uniprot database to date: stem bromelain (P14518), fruit bromelain (O23791), and comosain (P80884).

Two pineapple varieties have genome annotations: genome F153 (organized up to the chromosomal level) in the Phytozome database (Ming et al. 2015) and MD2 (organized at the scaffold level) in the NCBI database (Redwan et al. 2016). The new MD2 assembly (MD2v2) by Yow et al. (2021) increased gene count. Yow et al. (2023) found bromelain-family proteases in *A. comosus* using this information. The authors matched four commercial bromelain enzymes to their genes, which they call real bromelains. An orthological and phylogenetic study of all *A. comosus* genome C1A subfamily proteases were also performed.

Other plants belonging to the Bromeliaceae family which are bromelain like proteases producers include: *Bromelia plumieri* (Monies et al. 1990), *Pseudananas macrodontes* (López et al. 2000; Errasti et al. 2018), B. *balansae* (Pardo et al. 2000; Pardo et al. 2001), B. *hieronymi* (Bruno et al. 2008; Bruno et al. 2011), B. *fastuosa* (Cabral et al. 2006), B. *antiacantha* (Vallés et al. 2007), *Hohenbergia penduliflora* (Pérez et al. 2010)d *pinguin* (Moreno-Hernández et al. 2017). However, no studies of these enzymes in the genus *Puya* have been reported in the literature. The release of the first genome assembly of a *Puya* species, particularly *P. raimondii* (Liu et al. 2021) gives the information needed to begin studying cysteine bromelain-type proteases in the genus.

In this work, genes encoding bromelain-type proteases were discovered in the genome of *P. raimondii* (Bioproject PRJNA639677). The regulatory elements in the cis of the promoters, conserved motifs, evolutionary relationships of *P. raimondii* bromelain-type proteins were explored and probable structures were proposed.

Materials and Methods

Gene identification and sequence characterization of bromelain-like cysteine proteases.

The predicted gene and protein sequences from the recently published draft genome of *P. raimondii* generated by using a combination of PacBio long-read sequencing and Illumina short-read sequencing (Lui et al. 2021) was used as reference to identify the bromelain like proteins in *P. raimondii*. Genomic sequences were retrieved in Fasta format from the National Center for Biotechnology Information (NCBI) database under project PRJNA639677 and the accession number JABWPP000000000. Genes representing bromelain enzymes encoded in the *A. comosus* MD2 v2 genome which encodes proteins likely responsible for at least some of the proteolytic activity reported for commercial bromelain products were selected to compared their annotated genomic sequences against the *P.raimondii* genome by Blastn and the protein sequence by Blastx. The proteins belonging to the C1A family proteases (EC: 3.4.22) were identified among the annotated protein sequences by searching for the C1 peptidase (IPR000668) and/or inhibitor I29 (IPR013201) domains.

To identify regulatory motifs that are represented in the putative promoter sequence among bromelain-like genes, the PLACE (Higo et al. 1999) and plantCARE (Lescot et al. 2002) databases as well as motifs obtained from the literature were used. Only perfect matches to the motif and those with more than four IUPAC letters were considered. The coding regions and the putative amino acid sequences were

compared with bromelain sequences available in NCBI and Phytozome, which confirmed that these sequences indeed correspond to bromelain-like genes. To identify the splice donor sites and splice acceptor sites in the genomic sequence of bromelain like genes from *P. raimondii*, the NetGene2 server was used (Hebsgaard et al. 1996).

Protein sequence analysis

The physicochemical characteristics of predicted bromelain-like proteins, including amino acid sequence length, theoretical isoelectric point (pI) and molecular weight (Mw), were calculated using the ProtParam tool https://web.expasy.org/protparam/. The hidropatic profile was determined using the tool Protscale https://web.expasy.org/protscale/ (Gasteiger et al. 2005). The prediction of functional sites was carried out in tool PROSITE https://prosite.expasy.org/scanprosite/ (de Castro et al. 2006).

To determine the evolutionary relationships between *P. raimondii*, *A. comosus* and related species, were aligned with MUSCLE algorithm the bromelain-like predicted proteins and their orthologs. The phylogeny tree was constructed using the Maximum Likelihood method by MEGA11 software. Node robustness was estimated using the bootstrap method with 100 replications (Tamura et al. 2021). To find conserved motifs, the MEME suite (Bailey et al. 2009) was used to examine bromelain-like protein sequences. Optimal motif size was 10–100 and maximum motif identification was 11.

Protein structural prediction

The SWISS-MODEL server was used for tertiary structure modelling, by using ProMod3 (Waterhouse et al. 2018). Structural information was extracted from the template, and the sequence alignment was used to define insertions and deletions (Biasini et al. 2013). The SWISS-MODEL server was used to build and validate the 3D model, and structural assessment was also performed to validate the model built (Schwede et al. 2003).

Results

Discovery and analysis of genes that encode bromelain-like cysteine proteases in the genome of P. raimondii.

BLAST analysis using *P. raimondii* genomic information (BioProject: PRJNA639677) in the NCBI database identified *PrBLCP* genes to be bromelain-like cysteine proteases. N-terminal regions of bromelain forms discovered by Matagne et al. (2017) were searched. From 15 potential sequences, redundant and non-protein-coding sequences were deleted. Six bromelain-like nucleotide sequences are postulated in *P. raimondii* draft genome, four in accession JABWPP010000050.1 (scaffold 1) and two in accession JABWPP010000047.1 (scaffold 4) (table 1). According to gene structural analysis, all *PrBLCPs* had an intron between 322 and 1276 kb (table S1). The total length of the *PrBLCPs* was 2571 to 3395 bp with CDS between 1023–1230 bp.

Promoter analysis

We examined the cis-regulatory elements that may regulate these genes in *P. raimondii* 1000 bp upstream of the start codon of each sequence. Figure 1 illustrates the discovered components and their location relative to the ATG translation start codon. Also shown are the TATA-box and CAAT-box components closest to the start codon.

This research found hormone, stress, and light-responsive elements in *PrBLCP* sequences (Figs. 1 and 2). We detected hormone response elements for auxin (TGA-element, AuxRR-core, AuxRE), ethylene (ERE), abscisic acid (ABRE, ABRE-like), methyl jamonate (TGACG-motif, JERE), salicylic acid (TCA-element) and gibberellin (P-box). ABRE and ABRE-like components were more common in *PrBLCP1* and *PrBLCP3* than predicted. *PrBLCP4* overexpressed ERE, whereas *PrBLCP3* and *PrBLCP6* overexpressed TGACG-motif. These elements indicate that these genes are involved in plant development and abscisic acid, methyl jamonate, and ethylene-mediated stress responses.

The sequences (table S2) also exhibited overexpression of some stress response elements, including ARE (associated with the development of anaerobiosis) in *PrBLCP1*, *PrBLCP2*, and *PrBLCP3*. The genes *PrBLCP3* and *PrBLCP4* exhibit low temperature responsiveness (LTR), whereas *PrBLCP2* and *PrBLCP4* show drought responsiveness (MBS). Additionally, *PrBLCP2* contains the heat stress response element (STRE). Among these sequences, MYC (involved in drought response) and Unnamed 4 (an unidentified element with no specified function but linked to genes related to abiotic stress response) were present in all of them and were shown to be upregulated in five out of the six genes examined. The coexistence of these components in reaction to stress implies that these cysteine proteases have a significant function in responding to both biotic and abiotic stress in *P. raimondii*.

The promoters of light-responsive genes include many of the cis elements identified in these genes, suggesting that light regulates their expression. These include AE-box, AT1-motif, ATC-motif, Box4, Gap-box, GATA-motif, G-box, GT1-motif, I-box, MRE, Sp1, and TCT-motif. Both *PrBLCP1* and *PrBLCP2* have greater Box4 frequencies than predicted. *PrBLCP5* overexpressed GATA-motif, *PrBLCP3* G-box, and *PrBLCP2* GT1-motif.

Protein sequence analysis

The putative PrBLCP in *P. raimondii* has predicted molecular weights of 36643.21–45761.20 Da. PrBLCP1, PrBLCP2, PrBLCP3, PrBLCP4 and PrBLCP5 are acidic proteins with pI values between 5.05–6.06, whereas PrBLCP6 is basic with pI 8.63 (table 2). All have a larger amount of polar amino acids, suggesting hydrophilicity. The six putative proteases identified from genomic sequences in *P. raimondii* have 80–93% identity with proteins described in the F153 genome as cathepsin B-like cysteine proteinases and related to bromelain (EC 3.4.22.33) in the Phytozome database (table S3).

Multiple primary sequence alignments demonstrate that *P. raimondii* putative proteases are very similar to Uniprot *A. comosus* bromelains (Fig. 3). PrBLCPs propeptides have 121–128 amino acids, whereas

PrBLCP6 has 190 amino acids and contains the inhibitory domain I29. In the propeptide, we found the GXNXFXD motif, which is preserved in bromelains. In only PrBLCP5 and PrBLCP6, the first amino acid of this heptapeptide is G instead of A. Next, we find the C1 peptidase domain, which begins with the N-terminal sequence. Compared to the fruit and stem bromelains of *A. comosus*, PrBLCP2 and PrBLCP3 exhibit the fewest N-terminal substitutions (3 for O23791 and 2 for P14518). O23791 and P80884 and all PrBLCPs sequences in our investigation had an amino acids insertion between positions 170–174 (ananain numbering) (Fig. 3).

Functional group identification

The C1A subfamily cysteine proteases have conserved active sites and functional domains. Common active site catalytic dyads include residues C26 and H158 (stem and fruit bromelain locations). The oxanionic cavity is formed by residues Q20 and N179, highly conserved active sites. PROSITE found *P. raimondii* protein active sites (Fig. 4). The inhibitory domain I29 and peptidase domain C1 are conserved in all enzymes (Fig. 5b).

Phylogenetic analysis was used to compare *P. raimondii* bromelain-like proteases to *A. comosus* bromelains (Fig. 5a). This analysis included the three UniProt database bromelain sequences, five *Agave tequilana* sequences with the highest PrBLCPs identity, and genomic annotations F153, MD2v1, and MD2v2, previously grouped in the same orthogroup by Yow et al. (2023).

The PrBLCP1, PrBLCP2, and PrBLCP3 sequences cluster closely with each other and with ACMD2v2_05.02955 (AcC1A31), ACMD2v2_05.02951 (AcC1A28), and ACMD2v2_05.00101 (AcC1A22), all defined as real bromelains by Yow et al. (2023). PrBLCP4, PrBLCP5, and PrBLCP6 were more closely linked to *A. tequilana* proteins than PrBLCP1, PrBLCP2, and PrBLCP3. Only two sequences (ACMD2v2_16.26903 and OAY82768.1) were outside the bromelain group and cannot be functionally defined because they lack the inhibitor domains I29 and peptidase C1. It is possible that there is redundancy in some sequences as they belong to different annotations, however, there is little information on the sequences that code for the bromelains identified as stem bromelain (P14518), fruit bromelain (O23791) and ananain (P80884); for this reason, proteins from the three existing annotations to date were included in this study.

Protein structural prediction

Structural model prediction of PrBLCP1, PrBLCP2, PrBLCP3 (closely related to true bromelains of *A. comosus*) was performed. PrBLCP1 model was based on A0A6P5F3S2.1.A 80.45% identity and 1.0 coverage. For PrBLCP2, the model of A0A6P5F0R8.1.A with 91.36% identity and coverage of 1.0 was used as a template. Both fruit bromelian-like templates are annotated. The structural model of PrBLCP3 utilizes the O23791 template with 84.05% identity and 0.99 coverage. This template matches the UniProt-verified fruit bromelain sequence. In all cases, the PrBLCPs proteases identified in *P. raimondii* show a typical folding of cysteine proteases of the C1A family. The proteins fold into two domains: the L domain with α-helices and the R domain with an antiparallel β-sheet structure (Fig. 6). Oriented to the center of both domains, the oxanionic cavity contains active site residues. The active site amino acids of *P.*

raimondii and *A. comosus* fruit bromelain putative proteins had the same orientation when superimposed (Fig. 7). Residues Q20 and C26 of the three models coincide with numbering in fruit bromelain, while residues H and N differ.: H159 and N181 in PrBLCP1, H160 and N182 in PrBLCP2, and H159 and N180 in PrBLCP3.

Discussion

Several plant species, including cotton, ficus, peanut, and orange, contain genes encoding for papain-like (PLCP) cysteine proteases of the C1A subfamily (Zhang et al. 2019; Zhai et al. 2021; Zhang et al. 2023; Li et al. 2023). Species have different numbers of C1A protease genes. The best-studied genome in the Bromeliaceae family is *A. comosus*, which has 71 protease genes, four of which have been related to recognized activities and named as true bromelains (Yow et al. 2023). In our study, we found six *PrBLCP* genes in *P. raimondii*. The intron-exon structure, motif and conserved domains of all genes were comparable (tables S1 and S6). These traits have been seen in C1A genes across other species exhibiting analogous gene architectures and functional domains (Zhai et al. 2021; Li et al. 2023). At the nucleotide level, all sequences found in *P. raimondi* had more than 80% similarity with the genome of *A. comosus*, suggesting that these proteins are highly conserved (table S5).

Overexpression of ARE, LTR, MBS, STRE, MYC, Unnamed 4, AE-box, AT1-motif, ATC-motif, Box4, Gap-box, GATA-motif, G-box, GT1-motif, I-box, MRE, Sp1, and TCT-motif in the promoters of *PrBLCP*s genes in *P. raimondii* suggested a role for these proteases in stress responsiveness. Different variables affect gene expression via cis-regulatory regions. These areas have enhancers, repressors, and transcription factor binding sites for spatiotemporal gene expression (Marand et al. 2023).

The role of cysteine proteases in plant stress response has received a lot of attention. Response elements to hormones, light, drought, low temperature, and wounding have been discovered in *A. comosus* genes related to stress response such as *HSF* (Wang et al. 2021), plant-pathogen interaction genes such (Zhou et al. 2023) and genes regulating developmental processes such as *AP2/ERF* (Zhang et al. 2021). It has been suggested that under stressful circumstances, multiple regulatory mechanisms interact. DRE (dehydration-responsive element) cis-elements bind DREB proteins, causing dehydration stress response proteins and transcription factors to be activated. *DREB1* (cold-inducible) gene promoters, on the other hand, overexpress hormone-responsive motifs (auxin, GA, ethylene, ABA, Me-JA) (Srivasta et al. 2010). ABRE (abscisic acid-responsive) and light-responsive elements, on the other hand, are overexpressed in *DREB2* (drought-inducible) promoters. W-box and WUN motif elements are associated with wound response and have been discovered in the promoters of *DREB* genes (Ain-Ali et al. 2021). Light-responsive elements, such as Box 4 and G-box, have been found in the promoters of genes that are drought, salinity, cold, or heat-responsive (Shariatipour and Heidari 2020). The findings of this research suggest that the expression of *PrBLCP*s genes may be associated with the capacity of *Puya* species to adapt to arid conditions, such as the steep slopes and rocky outcrops that serve as their natural environment.

Although not all mechanisms are understood, bromelains are known to participate in the hypersensitivity response and a type of programmed cell death associated with this response (Salguero-Linares and Coll 2019). They found that *Candidatus liberibacter* infection produced *CsPLCP*s in *Citrus sinensis* (Li et al. 2023). Zhao et al. (2023) demonstrated that cytokinins, ethylene, and methyl jasmonate all promoted *PLCP* gene expression in grapes, which in turn-controlled resistance to *Phytophthora capsici* via hormonal signaling pathways. In fig, light positively regulates *PLCP* gene expression and herbivory control is linked to high *PLCP* gene expression and ficin accumulation in the inflorescence, receptacle, and latex (Zhai et al. 2021). *PrBLCP*s expression is linked to pathogen and herbivore defense genes in *P. raimondii*. These findings aid species management and phytosanitary control, enabling conservation measures to conserve natural populations.

MEROS classifies papain-like cysteine proteases (subfamily C1A) as inactive proenzymes with an inhibitory domain I29 and a peptidase domain C1. In these enzymes, the inhibitory domain I29 acts as a propeptide of the inactive zymogen, preventing substrate access to the active site. It also helps enzyme folding and transport (Wiederanders 2003). These domains and catalytic amino acids are conserved in this subfamily. All PrBLCPs found in this work had a propeptide with the inhibitory domain I29 and peptidase domain C1. PrBLCPs, like other cysteine proteases, include the heptapeptide GXNXFXD, which is highly conserved in the propeptide and helps these enzymes fold correctly, according to Ramli et al. (2018) the D residue of this motif participates in the correct folding of these enzymes.

The core structure of *P. raimondii* PrBLCPs is quite like fruit bromelain, as shown by multiple alignment and functional domain analysis. Physicochemical properties suggest they are more like fruit bromelain than stem and ananain (table S7). Purified stem bromelain has 212 amino acids, glycosylation at residue N117 and a molecular mass of 23.40-35.73 kDa and pI 9.55 (Ritonja et al. 1989). Fruit bromelain contains 351 amino acids, 25–31.00 kDa molecular mass, no glycosylation and pI 4.6 (Yamada et al. 1976). Ananain, on the other hand, is a non-glycosylated 216-residue sequence with a theoretical mass of 23,464 kDa (Lee et al. 1997). Another distinguishing characteristic found in PrBLCP sequences is an amino acid insertion (GTKYW) between positions 170–174 (ananain numbering), which is comparable to ananain. This area is found in fruit bromelain as well as other cysteine proteases including chymopapin and actinidin, but not in stem bromelain or papain (Lee et al. 1997). Ananain crystal structure investigations revealed that this insert creates the -chain beginning and operates by stabilizing the D167-S168-S169 loop structure (Yongqing et al. 2019). Although these proteases are quite similar, with substantial similarity in their main sequences, they vary in substrate selectivity, inhibitory characteristics and immune response (Azarkan et al. 2020).

The C1 peptidase domain is a monomer of 150 to 200 amino acids that constitute two domains structurally, the L domain with α helices and the R domain with β antiparallel sheet structure. Residues C26 and H158, positioned in the gap between the R and L domains, include the unique catalytic dyad of C1A subfamily enzymes. The R domain residue N179 and the L domain residue Q20 help to generate the oxanionic cavity and make hydrogen bonds with the protonated side chain of residues H158 to orient the imidazole ring (Azarkan et al. 2020). These active sites were found in PrBLCP2, PrBLCP3, PrBLCP4, and

PrBLCP5, however, PROSITE did not detect C26 as an active site in PrBLCP1 (Fig. 4). This might be explained by the replacement of G24 by S24, which results in two consecutive serine residues (S24-S25) upstream of C26. The SH group of C works as a nucleophile by giving a pair of electrons in the catalytic process, but two OH groups so near to the S groups might substitute the SH function and prevent PROSITE from recognizing residue C as an active site (Fig. 3). The potential folding of PrBLCP1 shows residue C26 orientated toward the oxanionic cavity, as in other bromelain-like, therefore enzymatic experiments are required to determine whether C mediates proteolytic action. The estimated structural model for PrBLCP1, PrBLCP2, and PrBLCP3 shows a typical bromelain folding that is comparable to O23791 (fruit bromelain) (Figs. 6 and 7).

Phylogenetic relations between *P. raimondii* putative bromelain-like cysteine proteases with *A. comosus* bromelains placed the PrBLCPs in two subclades, however the distances are minor, possibly due to recent evolutionary changes (Fig. 5). PrBLCP1, PrBLCP2, and PrBLCP3 grouped around the actual *A. comosus* bromelains discovered by Yow et al. (2023) (ACMD2v2v2_05.00101, 05.00102, 05.02951, and.02955). No difference in stem or fruit expression of these genomic sequences codes for commercial bromelains with recognized activities. PrBLCP4, PrBLCP5, and PrBLCP6 were connected to the most ancestral *A. comosus* bromelain orthogroup members (ACMD2v2_16.26904, 16.26905 and 1626906). The distances between the two subclades in the phylogram imply two major bromelain clusters, presumably from one orthologous gene family. These findings support the recent and fast divergence of these species and their high gene family overlap (Liu et al. 2021). The great conservation of these genes and the structural properties of the proteases they express imply that bromelains are crucial to *Puya* species evolution and adaption to severe conditions.

The C1A protease family members have crucial roles in responding to abiotic stressors, such as regulating cell death programs and protecting against herbivory and pathogen infections. Currently, there is a lack of characterization research on these enzymes specifically in the species *Puya*. The findings of this study, together with its application to other species within the same genus, might serve as a valuable addition to the understanding of the regulatory mechanisms of these proteases in plants. Furthermore, the use of cysteine proteases in fields such as agriculture, biotechnology, and health may have implications for the preservation of plants belonging to the Bromeliaceae family amidst the ongoing climate change.

Declarations

Author contribution

ICC conducted the bioinformatics analyses, interpreted the data and wrote the manuscript; MHT conceived the idea, edited and revised the manuscript; DRC performed in silico searches of the genomic sequences and contributed to the bioinformatics analyses; JDL model and analyze structural prediction and edit the manuscript; MFR designed the study, interpreted the bioinformatic analysis and edited the manuscript.

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Ethical approval

Not applicable

Competing interests

The authors declare no conflict of interest

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Tables

Table 1 Bromelain-like nucleotide sequences identified in the partial genome of *P. raimondii*

Sequence ID	Accesion number	Position on the Scaffold	Total length (bp)	Promoter region (bp)	CDS (bp)
PrBLCP1	JABWPP010000050.1	33204980-33207550	2571	1109	1077
PrBLCP2	JABWPP010000050.1	33218547-33221500	2954	1134	1083
PrBLCP3	JABWPP010000050.1	33233566-33236550	2985	1088	1071
PrBLCP4	JABWPP010000050.1	100521000-100523700	2701	1099	1023
PrBLCP5	JABWPP010000047.1	15277875-15281269	3395	1054	1041
PrBLCP6	JABWPP010000047.1	15283330-15286588	3259	1026	1230

Table 2 Physico-chemical characteristics of bromelain-like cysteine proteases in *P. raimondii*

Protein ID	Sequence length (aa)	MW (Da)	pI	Grand average of hydropathicity (GRAVY)
PrBLCP1	358	39918.05	5.05	-0.275
PrBLCP2	360	40024.99	5.15	-0.202
PrBLCP3	356	39638.40	5.85	-0.303
PrBLCP4	340	36586.16	5.52	-0.249
PrBLCP5	346	38733.72	6.05	-0.314
PrBLCP6	409	45761.20	8.63	-0.235

Figures

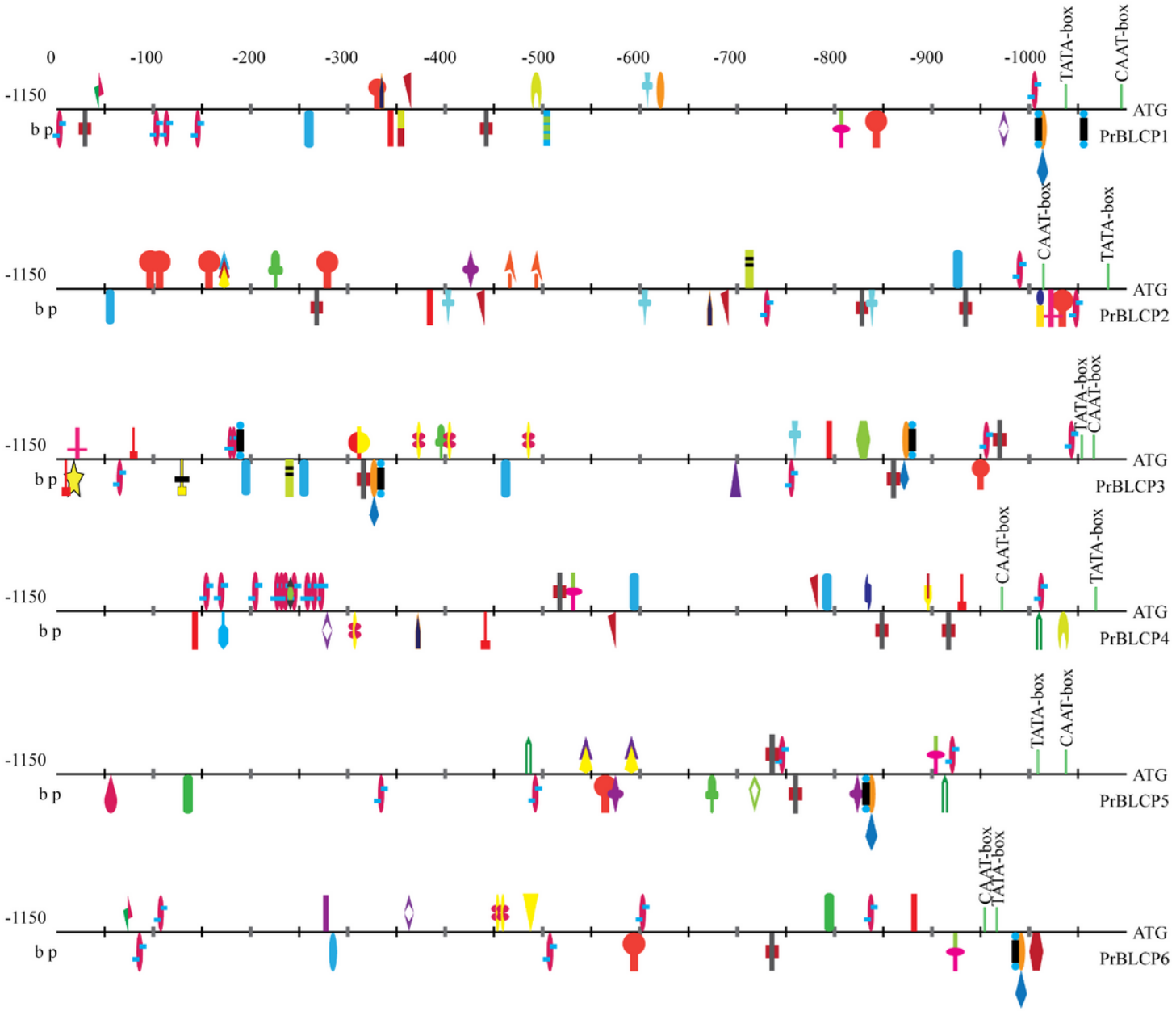

Figure 1

Location of cis-regulatory elements in the PrBLCP promoter regions in the partial genome of *P. raimondii*

Symbol	Cis-element	Function	Symbol	Cis-element	Function
	AAGAA-motif	Developmental related element		JERE	MeJA-responsiveness
	ABRE	Abscisic acid responsiveness		LTR	Low-temperature responsiveness
	ABRE-like	Abscisic acid responsiveness		MBS	MYB binding site involved in drought-inducibility
	AE-box	Part of a light responsive element		MRE	MYB binding site involved in light responsiveness
	ARE	Element essential for the anaerobic induction		MYB	Drought related
	AT1-motif	Part of a light responsive element		MYB recognition site	Drought related
	ATC-motif	Part of a light responsive element		MYC	Drought related
	ATCT-motif	Part of a light responsive element		Myc	Drought related
	AuxRE	Part of an auxin-responsive element		O2-site	Zein metabolism regulation
	AuxRR-core	Element involved in auxin responsiveness		P-box	Gibberellin-responsive element
	Box 4	Part of a light responsive element		RY-element	Seed-specific regulation, ABA responsiveness
	CAT-box	Meristem expression		Sp1	Light responsive element
	chs-CMA1a	Part of a light responsive element		STRE	Stress response element (heat stress)
	Circadian	Element involved in circadian control		TCA-element	Element involved in salicylic acid responsiveness
	CTAG-motif	Involved in growth and development		TC-rich repeats	Defense and stress responsiveness
	DRE core	Dehydration responsive element		TCT-motif	Part of a light responsive element
	ERE	Ethylene responsive element		TGACG-motif	MeJA-responsiveness
	Gap-box	Part of a light responsive element		TGA-element	Auxin-responsive element
	GATA-motif	Part of a light responsive element		Unnamed 4	Present in abiotic stress response genes
	G-box	Light responsive element		W box	Biotic and abiotic stress response (wounding related)
	GT1-motif	Light responsive element		WRE3	Wounding responsiveness element
	HD-Zip 1	Differentiation of the palisade mesophyll cells		WUN-motif	Wound responsive element
	I-box	Part of a light responsive element			

Figure 2

Cis-regulatory elements identified in the PrBLCP promoter regions, symbols and function

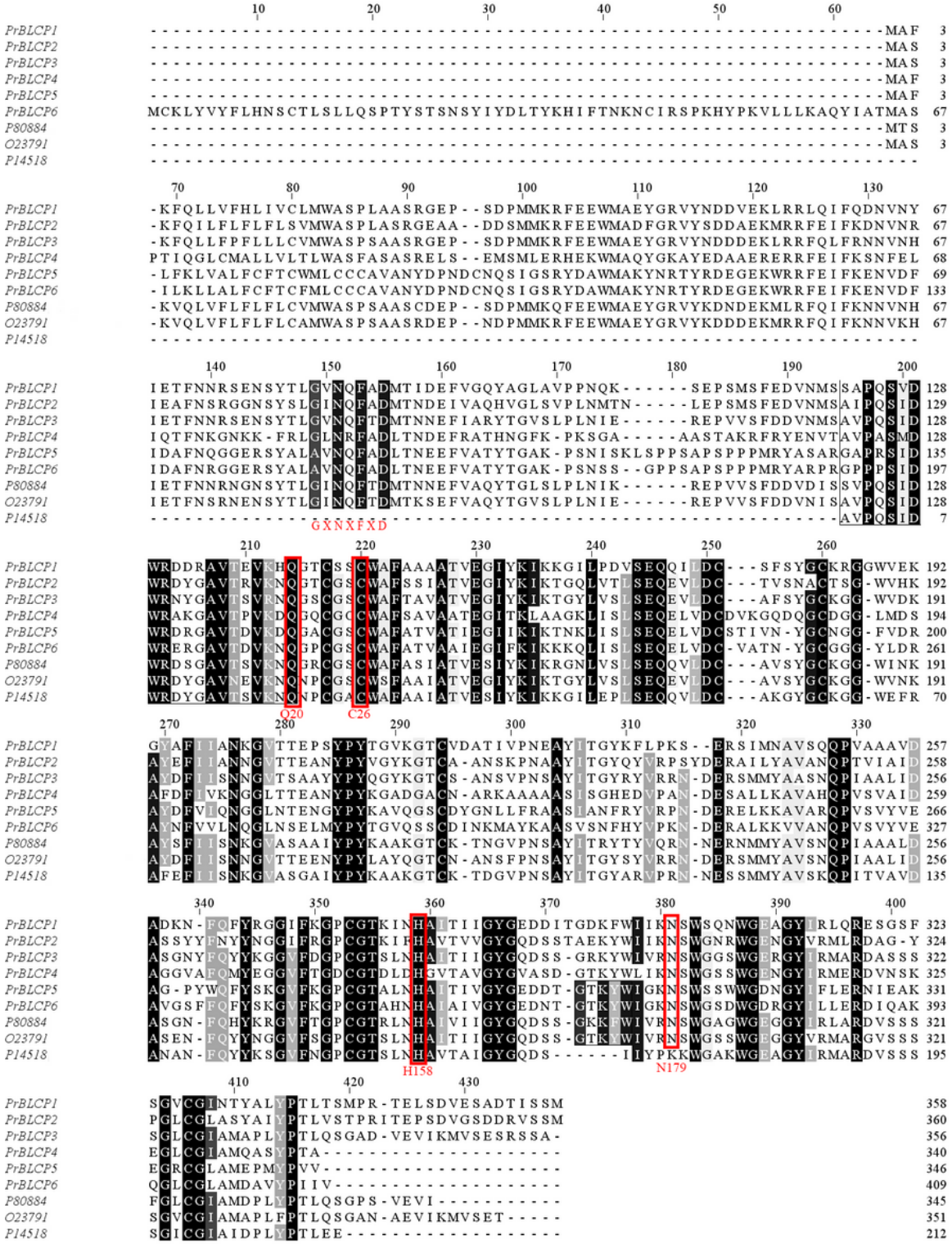

Figure 3

Multiple alignment of PrBLCP in *P. raimondii* and *A. comosus* bromelains: stem bromelain (P14518), fruit bromelain (O23791) and ananain (P80884). The heptapeptide of the propeptide and the amino acids from the active sites (numbering of the fruit bromelain) are highlighted in red

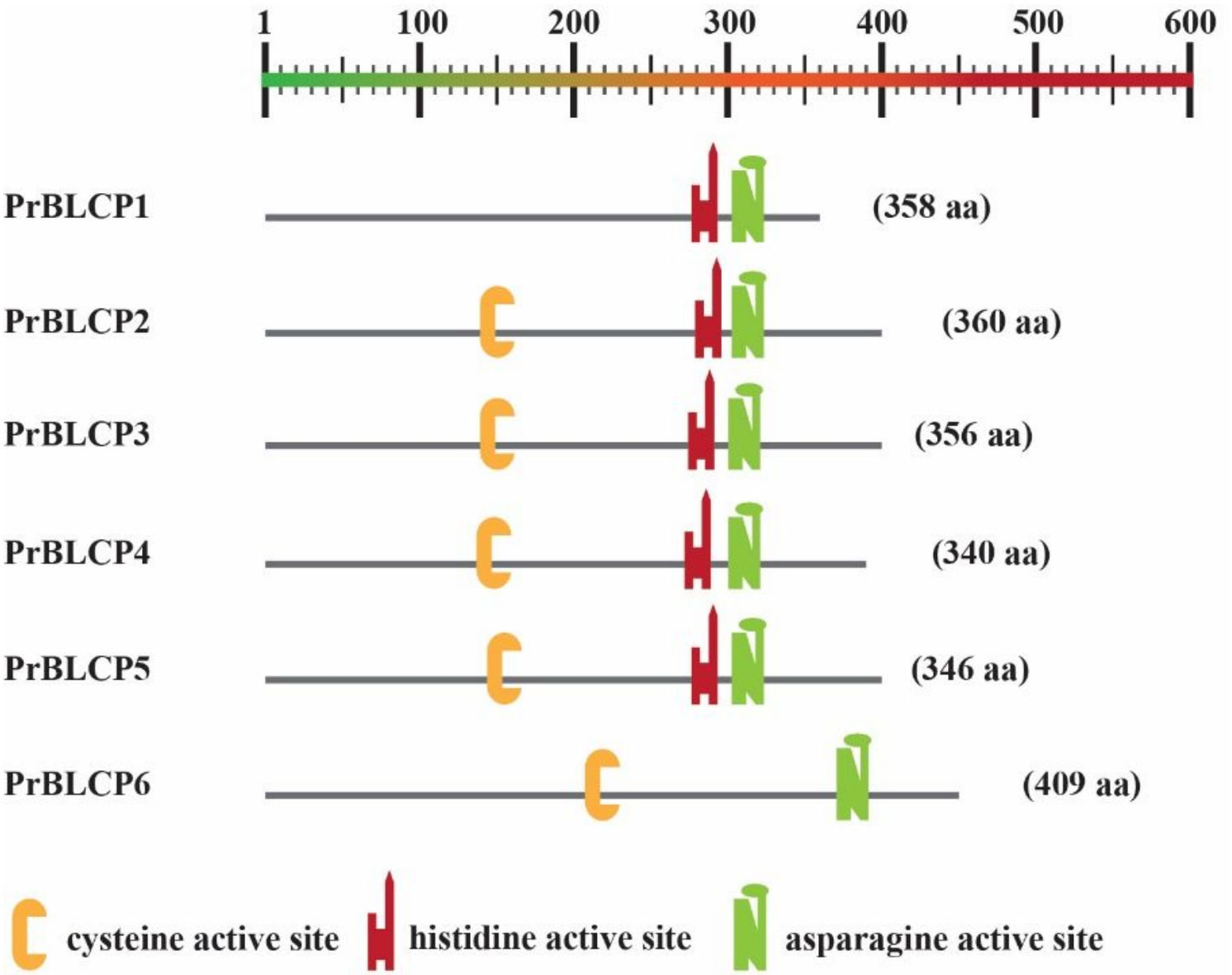

Figure 4

Prediction of active sites in of PrBLCP in *P. raimondii* in tool PROSITE

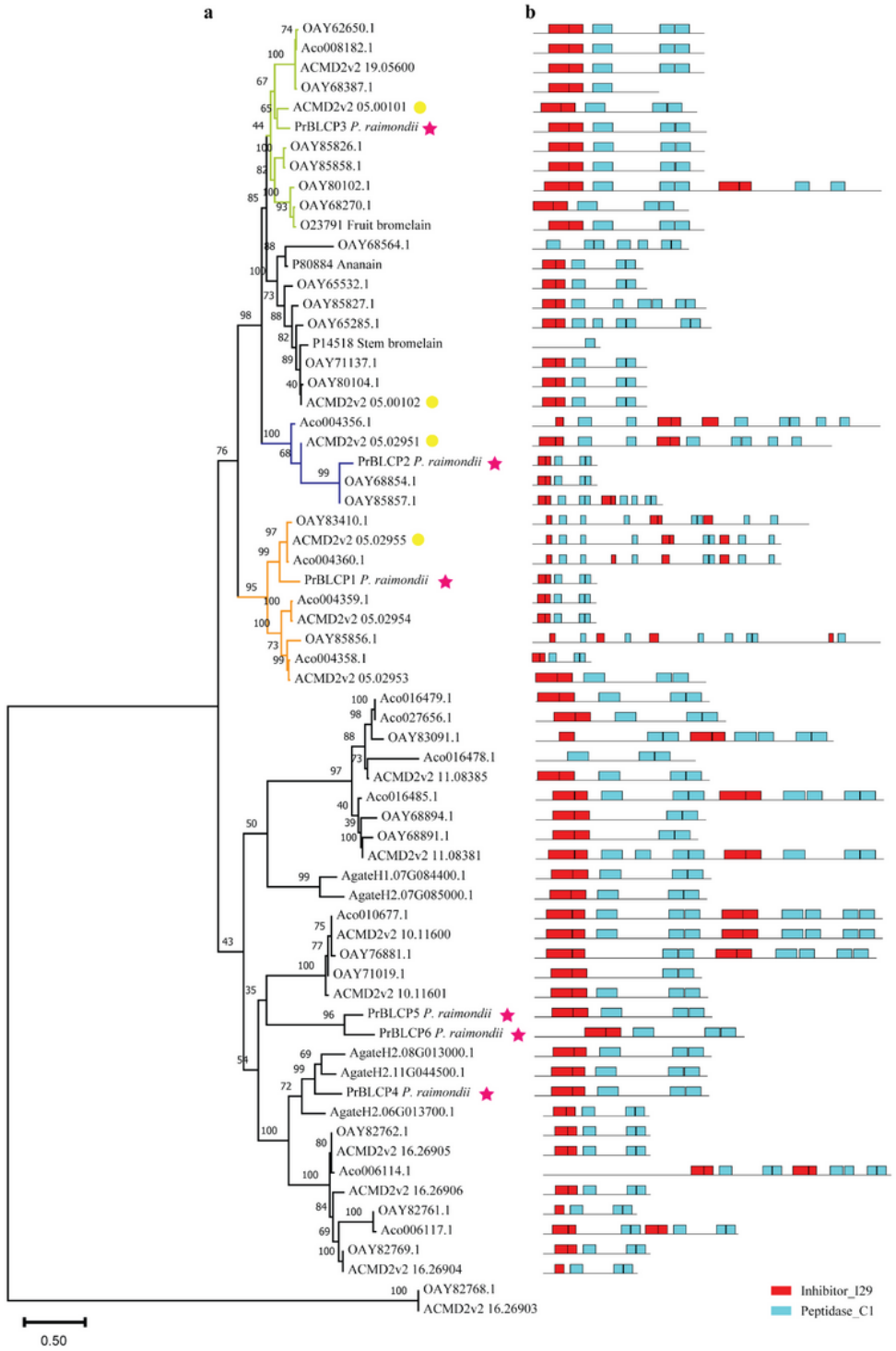

Figure 5

(a) Phylogenetic tree represents the relationship between the PrBLCP proteases identified in *P. raimondii*, those of *A. comosus* grouped within the bromelain subfamily by Yow et al. (2023) and those previously annotated in the UniProt database (stem bromelain (P14518), fruit bromelain (O23791) and ananain (P80884)). Yellow circles indicate sequences identified as true bromelains by Yow et al. (2023). (b) MEME analysis of PrBLCP, inhibitory domain I29 and peptidase domain C1

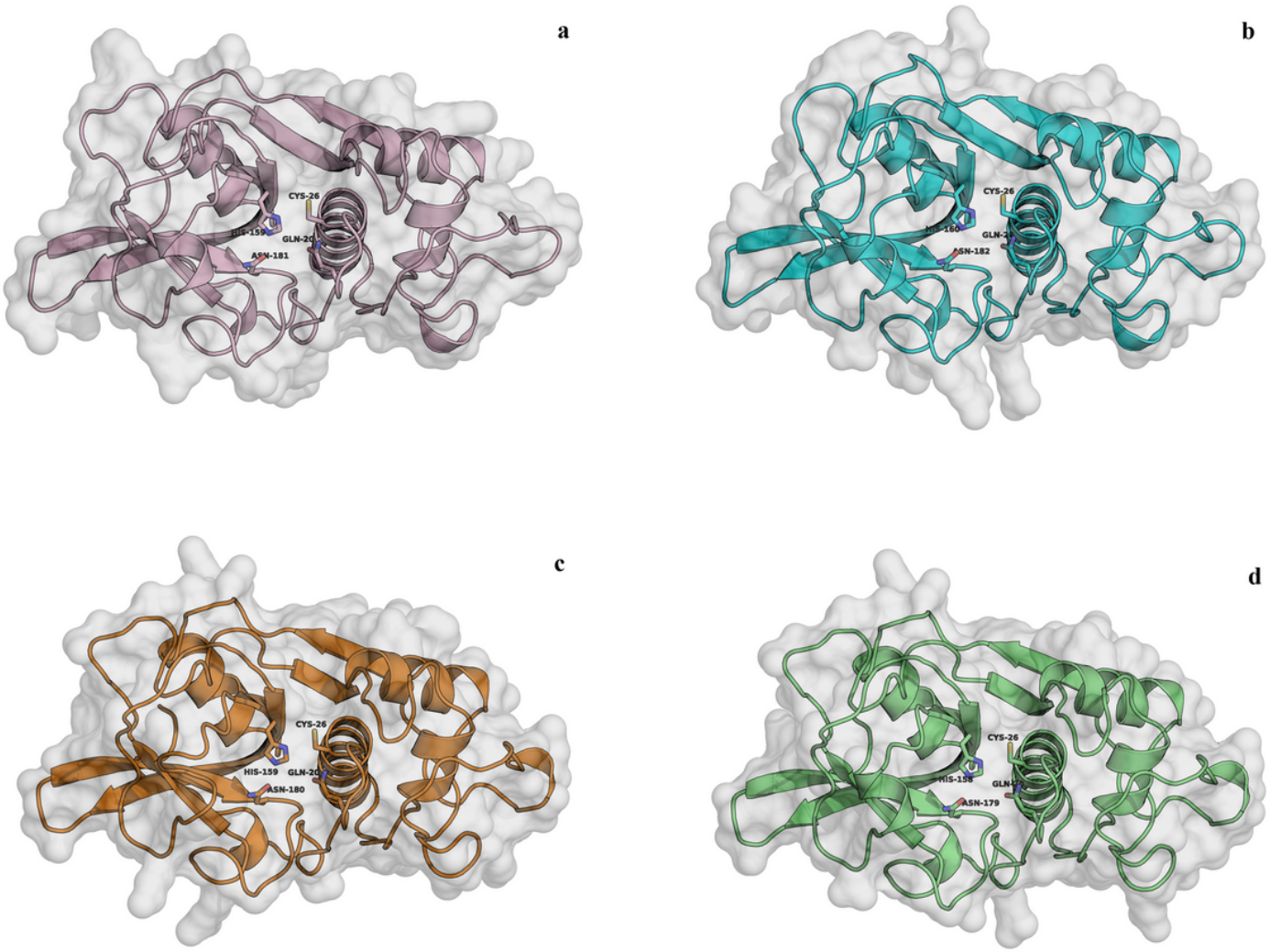

Figure 6

Protein structural prediction of PrBLCP1 (a), PrBLCP2 (b), PrBLCP3 (c) and O23791 fruit bromelain (d), modeled from Swiss Model and viewed by PyMOL. In sticks representation of amino acids of the active site Q20, C25, H158, N179 (numbering of the fruit bromelain)

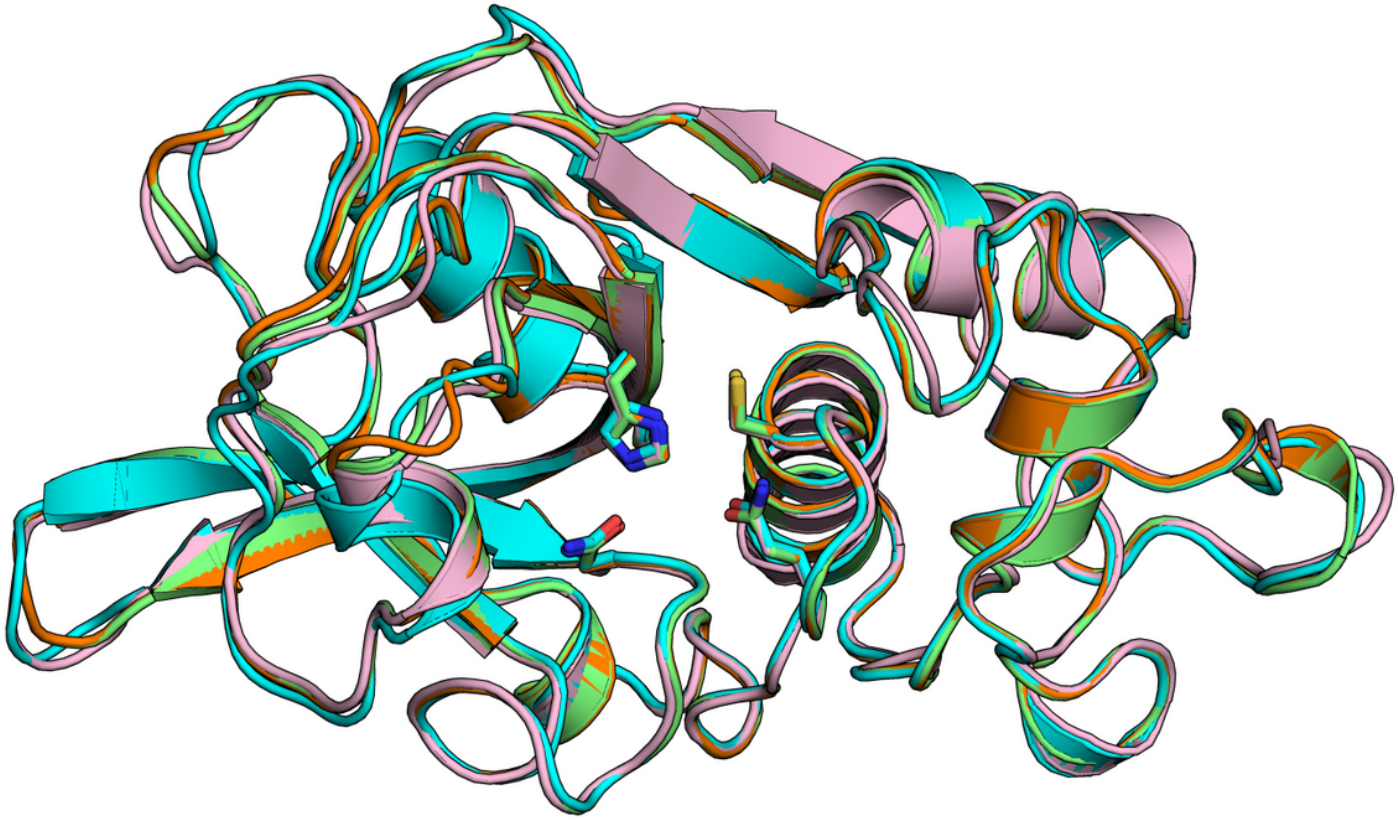

Figure 7

Structural model overlapping of PrBLCP1 (pink), PrBLCP2 (light blue), PrBLCP3(orange) and O23791 fruit bromelain (green), modeled from Swiss Model and viewed by PyMOL. In sticks representation of amino acids of the active site

Supplementary Files

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