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The GRAS gene family and its roles in pineapple (*Ananas comosus L.*) developmental regulation and cold tolerance

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Abstract

Background Pineapple (*Ananas comosus* L.) is a major tropical fruit crop with considerable economic importance, and its growth and development are significantly impacted by low temperatures. The plant-specific GRAS gene family plays crucial roles in diverse processes, including flower and fruit development, as well as in stress responses. However, the role of the GRAS family in pineapple has not yet been systematically analyzed.

Results In this study, 43 *AcGRAS* genes were identified in the pineapple genome; these genes were distributed unevenly across 19 chromosomes and 6 scaffolds and were designated as *AcGRAS01* to *AcGRAS43* based on their chromosomal locations. Phylogenetic analysis classified these genes into 14 subfamilies: OS19, HAM-1, HAM-2, SCL4/7, LISCL, SHR, PAT1, DLT, LAS, SCR, SCL3, OS43, OS4, and DELLA. Gene structure analysis revealed that 60.5% of the *AcGRAS* genes lacked introns. Expression profiling demonstrated tissue-specific expression, with most *AcGRAS* genes predominantly expressed in specific floral organs, fruit tissues, or during particular developmental stages, suggesting functional diversity in pineapple development. Furthermore, the majority of *AcGRAS* genes were induced by cold stress, but different members seemed to play distinct roles in short-term or long-term cold adaptation in pineapple. Notably, most members of the PAT1 subfamily were preferentially expressed during late petal development and were upregulated under cold stress, suggesting their special roles in petal development and the cold response. In contrast, no consistent expression patterns were observed among genes in other subfamilies, suggesting that various regulatory factors, such as miRNAs, transcription factors, and cis-regulatory elements, may contribute to the diverse functions of *AcGRAS* members, even within the same subfamily.

Conclusions This study provides the first comprehensive analysis of *GRAS* genes in pineapple, offers valuable insights for further functional investigations of *AcGRASs* and provides clues for improving pineapple cold resistance breeding.

Keywords Pineapple, GRAS transcription factor, Gene expression, Genome-wide analysis

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Background

The plant-specific GRAS transcription factor family plays a pivotal role in transcriptional reprogramming associated with various biological processes, including development and stress responses [1]. The name "GRAS" originates from the first three identified genes: *gibberellic acid-insensitive* (GAI), *repressor of* GAI (RGA), and *scarecrow* (SCR) [2]. These proteins are characterized by a conserved GRAS domain at the C-terminus, while the N-terminus exhibits significant variability in sequence and length [3, 4]. As more plant genomes are sequenced, the GRAS gene family has been systematically identified across numerous species, including *Arabidopsis* (*Arabidopsis thaliana*) [5], rice (*Oryza sativa*) [5], muskmelon (*Cucumis melo*) [6], apple (*Malus domestica*) [7], grape (*Vitis vinifera*) [8], tomato (*Solanum lycopersicum*) [9], as well as mosses and ferns [10]. Phylogenetic analyses have grouped GRAS genes into various subfamilies on the basis of structural similarity, reflecting their evolutionary relationships and suggesting functional homology [5, 11–14]. In *Arabidopsis*, the GRAS family has been classified into eight subfamilies: LISCL, PAT1, SCL3, DELLA, SCR, SHR, LS, and HAM [11]. However, Cenci and Rouard [13] expanded this to 17 subfamilies in angiosperms, identifying five new subfamilies: DLT, RAD1, RAM1, SCLA, and SCLB. This finding indicates that GRAS family classification may vary across species and depend on the number of species analyzed [14].

The GRAS gene family has gained increasing attention because of its broad biological functions and wide distribution across the plant kingdom. Differential expression of *GRAS* genes in various plant tissues highlights their diverse roles in plant growth and development. For example, in *Arabidopsis*, *SCR* and *SHR* play important roles in root growth and development [15–17], whereas members of the HAM subfamily, such as *LOM1* or *LOM2*, are crucial for maintaining the shoot apical meristem [18]. In *Solanum lycopersicum*, overexpression of *SlGRAS24* disrupts gibberellin (GA) and auxin signaling, leading to dwarfism, shorter primary roots, fewer lateral roots, and more lateral shoots, suggesting that HAM genes regulate the GA/auxin balance in different meristems [19]. In rice, *OsSCR1* and *OsSCR2* act upstream of *OsMUTE* and *OsFAMA*, playing early roles in stomatal development [20]. GRAS transcription factors are also involved in flower, embryo, seed, and fruit development. In *Arabidopsis*, a quintuple DELLA mutant exhibits early flowering, suggesting that these transcription factors act as inhibitors of flowering [21]. In lily (*Lilium longiflorum*), *LlSCL* is expressed predominantly in anthers during pre-meiosis, indicating a role in microsporogenesis [22]. In tomato, the overexpression of *SlGRAS24* reduces fruit set by 75%, whereas the silencing of *SlFSR* (*SlGRAS38*) significantly extends fruit shelf-life and reduces the activity of enzymes involved in cell wall degradation [23]. Additionally, the *GRAS* gene family is also a key component in signaling during responses to abiotic stresses, enhancing tolerance by regulating stress-related genes [24]. Overexpression of *PeSCL7* from poplar (*Populus euphratica*) in transgenic *Arabidopsis* and poplar improved drought and salt stress tolerance by activating enzymes involved in carbohydrate metabolism and alleviating oxidative stress [25]. In *S. lycopersicum*, overexpression of *SlGRAS4* enhances drought stress tolerance, whereas RNAi lines exhibit hypersensitivity to this stress. Expression profiles suggest that *SlGRAS4* may also play a role in cold stress tolerance [9]. *VaPAT1*, a gene from wild Amur grape (*Vitis amurensis*), is induced by low temperatures, and its ectopic expression in *Arabidopsis* enhances cold tolerance. This gene is also involved in regulating jasmonic acid biosynthesis in response to cold stress in grapevines [26]. Similarly, the overexpression of the *ZjCIGR1* gene from zoysiagrass (*Zoysia japonica Steud.*), which belongs to the PAT1 subfamily of the GRAS protein family, confers cold stress resistance in zoysiagrass [27].

Pineapple (*Ananas comosus* L.), a perennial herbaceous plant from the family Bromeliaceae, is one of the four major tropical and subtropical fruits cultivated globally [28, 29]. Currently, pineapples are cultivated in approximately 90 countries and regions worldwide, with a total cultivation area exceeding 400,000 hectares, primarily located in Asia, the Americas, and Africa. The top 10 pineapple-producing countries, including Thailand, the Philippines, China, Brazil, and India, collectively contribute about 73% of the global production. Pineapple remains one of the most active varieties in the global tropical fruit trade, with an annual trade volume surpassing 2.5 billion USD [28]. Pineapple is favored by consumers for its unique flavor, aroma, and high nutritional value, and its inflorescence is the source of the fruit [30]. However, pineapple cultivation faces a significant challenge due to its sensitivity to low temperatures, especially given its long production cycle of at least 14 months, which often includes exposure to cold stress during winter in subtropical regions and thus restricts year-round production [31, 32]. When exposed to 0 °C or below, ice formation in leaf tissues can cause significant damage, leading to symptoms similar to scalding and rapid tissue necrosis. Extended periods of cold, particularly in prolonged rainy weather with daily temperatures below 8 °C, can result in chilling injuries that severely impact the meristem and young leaves, leading to tissue rot and stunted growth, ultimately causing substantial losses in yield and quality [33]. Therefore, research on the genes related to the regulation of pineapple flower and fruit

development, as well as the cold stress response, can provide important reference information for pineapple breeding and production. As GRAS transcription factors play key roles in these processes, a systematic study of the *GRAS* gene family in pineapple is essential. Using the high-quality pineapple genome [34], we conducted a genome-wide identification and analysis of the *GRAS* gene family in pineapple, including its sequence characteristics and expression profiles. These results offer valuable insights into the potential roles of this important gene family in pineapple development and the cold stress response.

Materials and methods

Identification and sequence analysis of *GRAS* genes in pineapple

Genomic data for pineapple was downloaded from the Phytozome database (version: *Ananas comosus* v3; variety: F153; https://phytozome-next.jgi.doe.gov/info/Acomosus_v3) [35]. GRAS protein sequences from *Arabidopsis* (33) and rice (50) were retrieved from the Plant Transcription Factor Database (http://planttfdb.gao-lab.org/index.php) [36]and used as queries for BLASTP searches. The hidden Markov model (HMM) for the GRAS domain (PF03514) was obtained from the PFAM database (http://pfam.xfam.org) [37] and employed for HMMER (v3.3) searches within pineapple protein sequences, with an E-value cutoff of 0.00001. After removing redundant sequences, the remaining candidates were further analyzed using NCBI CDD (https://www.ncbi.nlm.nih.gov/cdd/) [38] and the SMART tool (http://smart.embl-heidelberg.de/) [39] to confirm the presence of conserved GRAS domains. The identified *GRAS* genes were renamed as *AcGRAS01* to *AcGRAS43* according to their chromosomal distribution. The physical and chemical properties, including protein length, molecular weight (kDa), theoretical pI, grand average of hydropathicity (GRAVY), and instability index of the AcGRAS proteins, were calculated using the ExPASy website (https://web.expasy.org/compute_pi/) [40]. Subcellular localization of the AcGRAS proteins was predicted using Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) [41].

Phylogenetic analysis and classification of AcGRASs

To investigate the evolutionary relationships among the 43 identified *AcGRAS* genes, multiple sequence alignments were performed using ClustalW (http://www.clustal.org/clustal2/) [42] with default parameters. GRAS protein sequences from pineapple (43), *Arabidopsis* (34), and rice (50), as well as the cold resistance gene *ZjCIGR1* from zoysiagrass [27] and *VaPAT1* from wild Amur grape [26] (Additional file 1: Table S1), were used to construct an unrooted phylogenetic tree using IQ-Tree software. The maximum likelihood (ML) method was applied with 5000 bootstrap replicates. The AcGRASs were classified on the basis of their evolutionary relationships with the GRAS members in *Arabidopsis*. The phylogenetic tree was visualized using Evolview (http://www.evolgenius.info/evolview/) [43].

Gene structure, conserved motif, and Cis-regulatory element analyses of AcGRASs

The exon–intron structure of the *AcGRAS* genes was determined via the GFF annotation file of the pineapple genome. Conserved motifs within the AcGRAS proteins were identified using the MEME tool (http://alternate.meme-suite.org) [44] with the following parameters: the number of motifs was set to 8, and the optimal width of each motif was between 6 and 50 residues. The upstream 1500 bp sequence of each *AcGRAS* gene was extracted using TBtools software on the basis of the full-length genomic DNA sequences of the *AcGRAS* genes [45]. Cis-regulatory elements in the promoter regions were predicted using the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [46], and the results were visualized using TBtools.

Three-dimensional (3D) structural modeling of AcGRASs

Homologous protein models for the pineapple AcGRAS proteins were identified using the Protein Data Bank (PDB) database (http://www.rcsb.org/) [47]. The tertiary structures of the AcGRAS proteins were predicted through SWISS-MODEL (https://www.swissmodel.expasy.org/) [48] using default settings. Conserved structural elements were further analyzed with ConSurf (https://consurf.tau.ac.il/) [49]. Visualization and manipulation of the 3D protein models were performed using PyMOL v2.6.0 [50], while protein topology was assessed using Protter (http://wlab.ethz.ch/protter/start/) [51].

Chromosomal distribution, gene duplication, and collinearity analysis of *AcGRASs*

The chromosomal distribution of all 43 *AcGRAS* genes was determined by mapping them to their respective chromosomes using TBtools, based on physical location data from the pineapple genome annotation file. Whole-genome data for *Arabidopsis*, rice, banana, and grape were downloaded from the Phytozome database (https://phytozome-next.jgi.doe.gov) [35]. Gene duplication events among the 43 AcGRAS genes were identified through TBtools with default settings, and synteny analysis between pineapple and the other four species was also conducted.

Prediction of putative miRNA targets for *AcGRASs*

To predict potential miRNA interactions with AcGRAS genes, pineapple miRNA sequences were retrieved from published literature [52]. The coding sequences (CDS) of AcGRAS genes were extracted and submitted to the psRNATarget online database (https://www.zhaolab.org/psRNATarget/) [53] with default parameters. The interaction networks between *AcGRAS* genes and their predicted miRNA targets were visualized using Cytoscape v3.6software [54].

Transcription factor regulatory network analysis of *AcGRASs*

The Plant Transcriptional Regulatory Map (PTRM) tool (http://plantregmap.gao-lab.org/) [55] was employed to predict transcription factors (TFs) that regulate *AcGRAS* genes. The upstream 2000 bp sequences of *AcGRAS* genes were analyzed with a significance threshold of $P \leq 1e\text{-}7$, using *Arabidopsis* as the reference species. The predicted TFs were visualized as a network using Cytoscape, and word clouds and bar charts were generated using the ggplot2 package in R.

Expression profiling of *AcGRASs* across different tissues and under cold stress based on RNA-seq data

The transcriptomic data from various pineapple floral and fruit tissues were obtained from our previously published work [34, 56]. These samples included four developmental stages of sepal tissues, three stages of petal tissues, six stages of stamen tissues, seven stages of gynoecium tissues, seven stages of ovule tissues, and six developmental stages of fruits. Transcriptomic data from pineapple subjected to cold treatment at 8 °C were generated from our unpublished work, which has been deposited in China National GeneBank DataBase (CNGBdb) with accession number CNP0006260 (https://db.cngb.org/search/project/CNP0006260/). For the cold treatment, pineapple variety Tainong 11 (TN 11) was used, which are provided by the Haixia Institute of Science and Technology, Center for Genomics and Biotechnology, Fujian Agriculture and Forestry University, Fujian, China. The suckers of TN 11 variety was grown in plastic pots containing soil mix under greenhouse conditions (30 °C, 70% humidity, and a 16 h light/8 h dark photoperiod). After three months, healthy TN11 seedlings with well-developed roots were exposed to cold treatment at 8 °C, and leaf samples were collected at 0, 1, 3, 5, 7, 9, 11, 13, 14, and 15 days post-treatment. RNA-seq was performed on samples collected at 0, 3, 7, and 15 days post-treatment with three biological replicates for each group, while the remaining samples were preserved for subsequent qRT-PCR analysis. The transcript abundance of *AcGRAS* genes was calculated as Transcripts Per Million (TPM), and a heatmap based on log2 (TPM + 0.01) values was generated using the heatmap package in R.

RNA extraction and qRT-PCR analysis of selected *AcGRASs*

Since cold stress significantly impacted pineapple growth and development, we further examined the expression pattern of 12 representative *AcGRAS* genes in response to cold stress using qRT-PCR. Total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed with the ThermoScript RT-PCR kit (Thermo Fisher Scientific, Carlsbad, CA, USA). qRT-PCR was conducted using the SYBR Premix Ex Taq II system (TaKaRa Perfect Real Time) on a Bio-Rad Real-Time PCR system (Foster City, CA, USA), with primers listed in Additional file 2: Table S2. The qRT-PCR program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s, and a final step of 95 °C for 15 s. The pineapple *Actin2* gene was used as the internal reference. For each analysis, three technical replicates and three biological replicates were performed, and gene expression levels were calculated using the 2^−ΔΔCT method.

Results

Identification and physicochemical properties of *GRAS* genes in pineapple

In this study, a total of 43 *GRAS* gene family members were identified in pineapple genome (Additional file 3: Table S3) and designated *AcGRAS01* to *AcGRAS43* according to their chromosomal localization. The proteins encoded by the *AcGRAS* genes exhibited a diverse range of lengths, spanning from 110 amino acids (AcGRAS16) to 782 amino acids (AcGRAS14), with an average length of 449.7 amino acids. The predicted isoelectric points (pI) of AcGRAS proteins ranged from 4.07 (AcGRAS16) to 10.86 (AcGRAS13). The minimum molecular weight was determined to be 11,876.43 Da (AcGRAS16), while the maximum molecular weight reached 82,426.32 Da (AcGRAS14). Subcellular localization predictions indicated that all AcGRAS proteins were likely located in the nucleus. Based on the instability index, only AcGRAS16 could be considered stable, while the other 42 were predicted to be unstable. Additionally, the GRAVY index ranged from − 0.87 (AcGRAS29) to 0.146 (AcGRAS13), with most AcGRAS proteins (41 of 43) showing negative values, indicating that AcGRAS proteins were generally hydrophilic. Collectively, AcGRAS proteins displayed considerable variation in their physicochemical properties, implying potential functional diversity.

Classification and phylogenetic relationships of *AcGRASs*

Phylogenetic analysis of the GRAS members from pineapple (AcGRASs), *Arabidopsis* (AtGRASs), and rice (OsGRASs) classified all the GRAS members into 14 subfamilies, including OS19, HAM-1, HAM-2, SCL4/7, LISCL, SHR, PAT1, DLT, LAS, SCR, SCL3, OS43, OS4, and DELLA (Fig. 1, Additional file 1: Table S1). Among these subfamilies, eight AcGRAS members (AcGRAS04, AcGRAS05, AcGRAS06, AcGRAS13, AcGRAS15, AcGRAS22, AcGRAS39, and AcGRAS43) clustered with the reported GRAS cold resistance genes ZjCIGR1 [27] and VaPAT1 [26], belonging to the PAT1 subfamily. Six AcGRAS genes each were classified into the SHR (AcGRAS01, AcGRAS12, AcGRAS21, AcGRAS31, AcGRAS40, and AcGRAS41) and HAM-1 (AcGRAS02, AcGRAS07, AcGRAS14, AcGRAS16, AcGRAS17, and AcGRAS35) subfamilies. Four genes each were found in the SCL3 (AcGRAS08, AcGRAS09, AcGRAS34, AcGRAS42) and SCR (AcGRAS10, AcGRAS20, AcGRAS37, AcGRAS38) subfamilies, and three genes each were found in the DELLA subfamily (AcGRAS03, AcGRAS18, AcGRAS24) and HAM-2 subfamily (AcGRAS23, AcGRAS26, AcGRAS27). The DLT (AcGRAS33, AcGRAS36) and LAS (AcGRAS29, AcGRAS30) subfamilies each contained two gene members, while LISCL (AcGRAS19), OS4 (AcGRAS11),

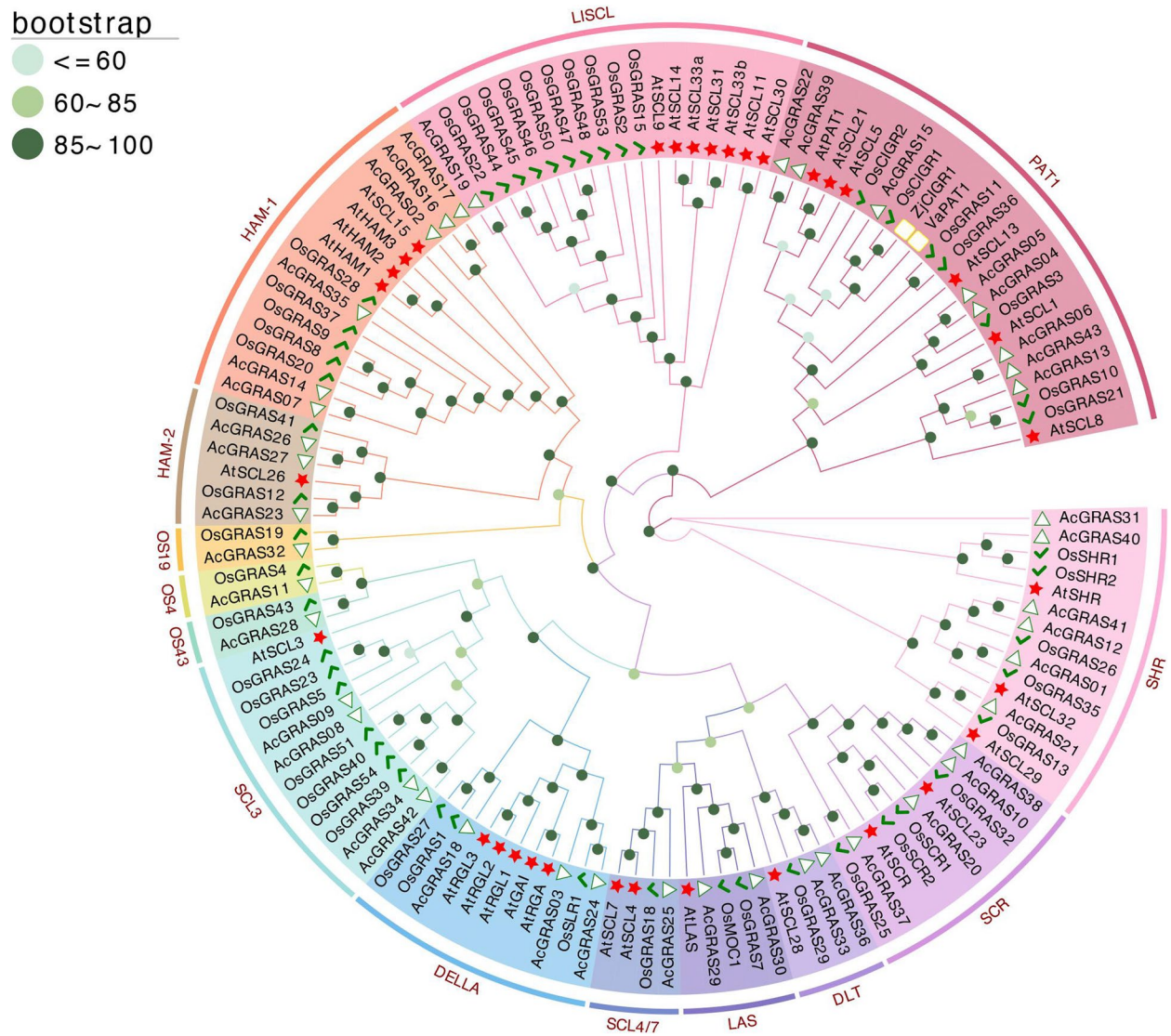

Fig. 1 Unrooted maximum-likelihood phylogenetic tree of GRAS proteins from *Ananas comosus* (Ac), *Arabidopsis thaliana* (At), and *Oryza sativa* (Os). The green triangle, green hook, and red star indicate the GRAS members from pineapple, rice, and Arabidopsis, respectively. The yellow-white squares represent the protein encoded by *ZjCIGR1* from *Zoysia japonica* [27] and the gene *VaPAT1* from *Vitis vinifera* [26]

OS43 (AcGRAS28), OS19 (AcGRAS32), and SCL4/7 (AcGRAS25) subfamilies each contained only one member. Furthermore, most pineapple GRAS proteins clustered closely with rice GRAS proteins, and some subfamilies, such as OS4 and OS19, contained gene members exclusively from rice and pineapple, indicating a close evolutionary relationship. This close clustering suggests that a shared evolutionary process occurred after the divergence of monocots and dicots, contributing to the diversity observed in the GRAS gene family.

Gene structure, conserved motif and domain analyses of *AcGRAS*s

The gene structure, conserved motifs, and domains of AcGRASs were analyzed and shown according to their phylogenetic relationships (Fig. 2, Additional file 4: Table S4). Among the 43 AcGRAS proteins, eight conserved motifs (named motif 1 to motif 8) were predicted (Fig. 2B). The results revealed that most conserved motifs in the AcGRASs were situated in the C-terminal domain and were organized in the sequences of Motif 7, Motif 3, Motif 1, Motif 8, Motif 2, Motif 4, Motif 5, and Motif 6, with Motif 3 being the most highly conserved. The SCR and PAT1 subfamilies displayed a relatively stable conserved C-terminal domain with almost no motif deletions. Conversely, members of other subfamilies showed significant deletions of specific motifs, with variations in motif loss among different subfamily members. For example, the DLT subfamily members lost Motif 6, whereas Motif 4 was frequently absent in members of the HAM-1 subfamily. These variations in motif composition might be contributed to the functional diversity observed among different subfamily members. All AcGRAS-encoded proteins contained the conserved GRAS domain (Fig. 2C). Additionally, AcGRAS31 contained the extra Ribosomal_L38 domain, while AcGRAS03 and AcGRAS18, belonging to the DELLA subfamily, contained the extra DELLA domain. The genomic exon–intron structural analysis of the 43 *AcGRAS* genes revealed variability in the number of exons, ranging from 1 to 6 (Fig. 2D). Among them, *AcGRAS31* in the SHR subfamily exhibited the highest number of exons and introns, with 6 exons and 5 introns. *AcGRAS28* and *AcGRAS35* had 4 exons, *AcGRAS20*, *AcGRAS5*, and *AcGRAS4* had 3 exons, and the remaining genes had 1 or 2 exons. Most of these *AcGRAS* genes (26, 60.5%) lacked introns. Moreover, only *AcGRAS31*, *AcGRAS20*, *AcGRAS11*, *AcGRAS5*, and *AcGRAS4* possessed UTRs (non-coding regions), with *AcGRAS11*, *AcGRAS5*, and *AcGRAS4* having two UTRs, while *AcGRAS31* and *AcGRAS20* had only one. These findings suggest that variations in motif compositions and exon–intron structures occurred dynamically

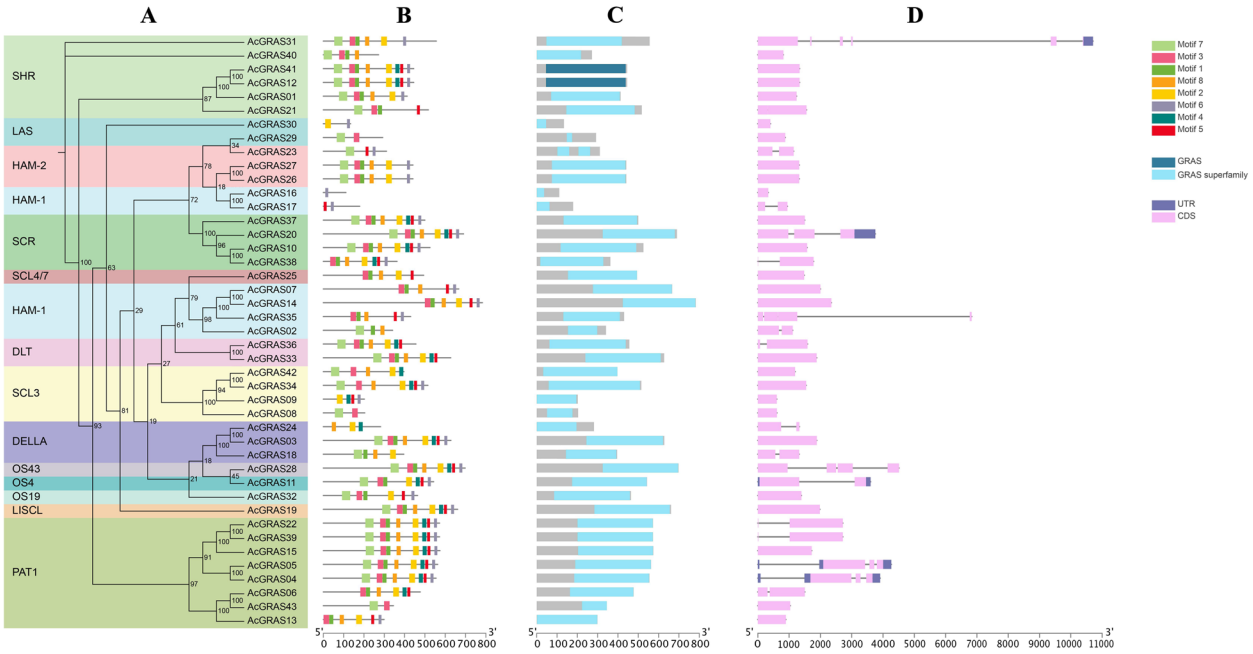

Fig. 2 Phylogenetic relationships, motif compositions, conserved domains and gene structures of AcGRASs. **A** Maximum likelihood phylogenetic tree of AcGRAS proteins; **B** Conserved motif distribution of AcGRAS proteins. A total of eight motifs were predicted, the scale bar indicated 100 aa, and the logo and sequence of the conserved motifs were provided in Additional file 4: Table S4. **C** Conserved domain distribution of AcGRAS proteins; **D** Gene structure of *AcGRAS* genes, including introns (black line), exons (pink rectangle) and untranslated regions (UTRs, purple rectangles). The scale bar represented 1 kb

during the evolutionary development of the *AcGRAS* gene family, and that *AcGRAS* genes with similar features may serve similar functions.

Three-dimensional (3D) structural modeling of AcGRAS proteins

Understanding the 3D structure is essential for elucidating protein function. Homology modeling of all AcGRAS proteins was performed based on the AlphaFoldDB and SWISS-MODEL databases (Additional file 5: Fig.S1). For each subfamily, the structure with the highest GMQE and QMEAN scores (Additional file 6: Table S5) was chosen as the representative model (Fig. 3). The resulting models revealed that the protein structures in each branch could be divided into two main components: an α/β core subdomain and an α-helix domain. Additionally, some proteins, such as AcGRAS35 and AcGRAS36, were found to have an extra α-helix at the N-terminus, connected to the α-helix domain via a random coil. Within the α/β core subdomain, the β-sheets are encased by α-helices, and these β-sheets are conserved [57]. While most AcGRAS proteins contain eight β-sheets, a few, such as AcGRAS09 and AcGRAS30, have fewer. The two proteins in the LAS subfamily contain only two β-sheets, and in AcGRAS43, β-sheets were not detected. Transcription factors typically utilize α-helices to bind directly to the major groove of DNA, whereas β-sheets are crucial for maintaining the structural stability of transcription factors, forming effector domains, and facilitating protein–protein interactions [57]. The variation in the number of β-sheets among AcGRAS proteins may indicate their evolutionary adaptation and functional diversification.

Cis-regulatory element analysis of *AcGRAS* genes

Cis-regulatory elements (CREs) refer to non-coding DNA sequences located in the promoter region of genes, playing a key role in regulating the transcription of associated genes [58]. The CREs in the putative promoter regions of *AcGRAS* genes were predicted and classified into three main types: plant growth and development CREs, phytohormone-responsive CREs, and stress-responsive CREs (Fig. 4, Additional file 7: Table S6). (1) Among the plant growth and development CREs, light-responsive elements were present in nearly

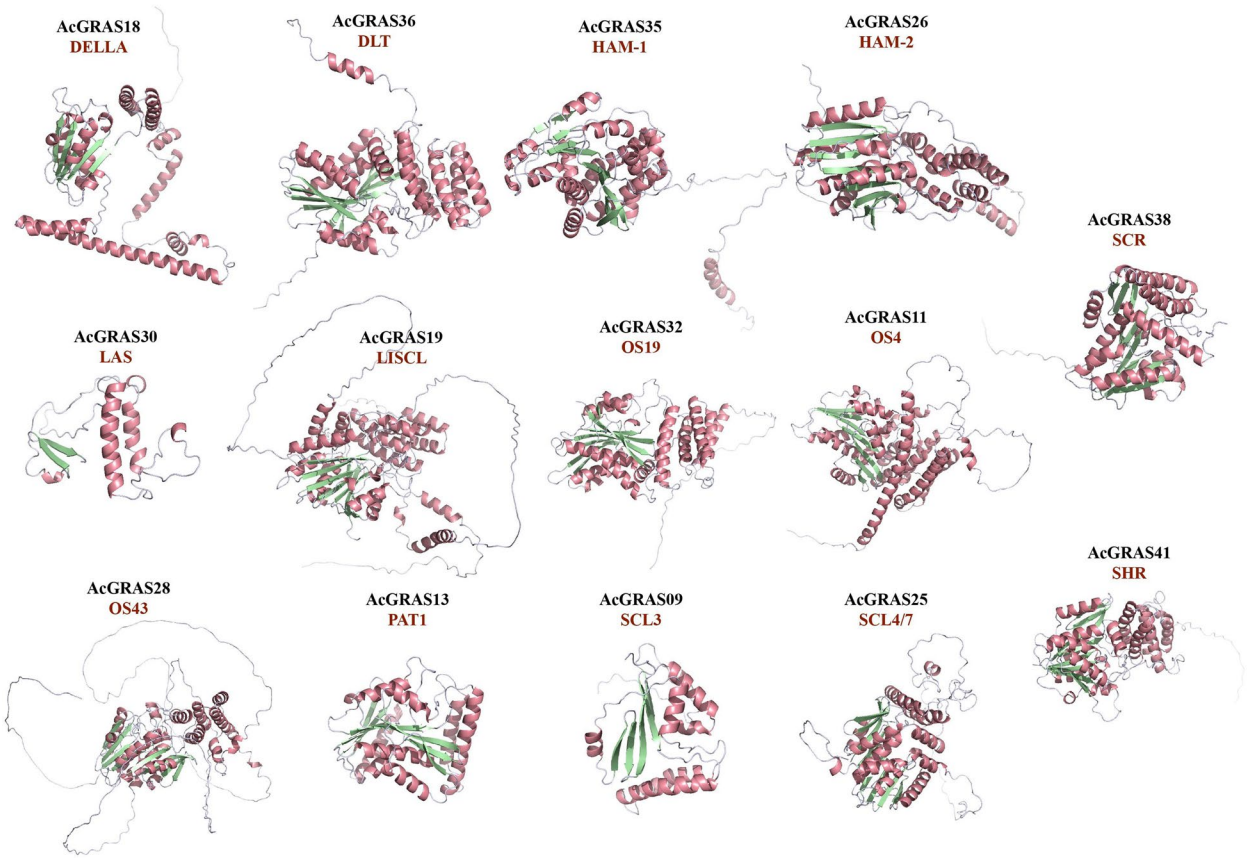

Fig. 3 Predicted 3D structural modeling of AcGRAS proteins. The structure with the highest GMQE and QMEAN scores in each subfamily was selected as the representative model

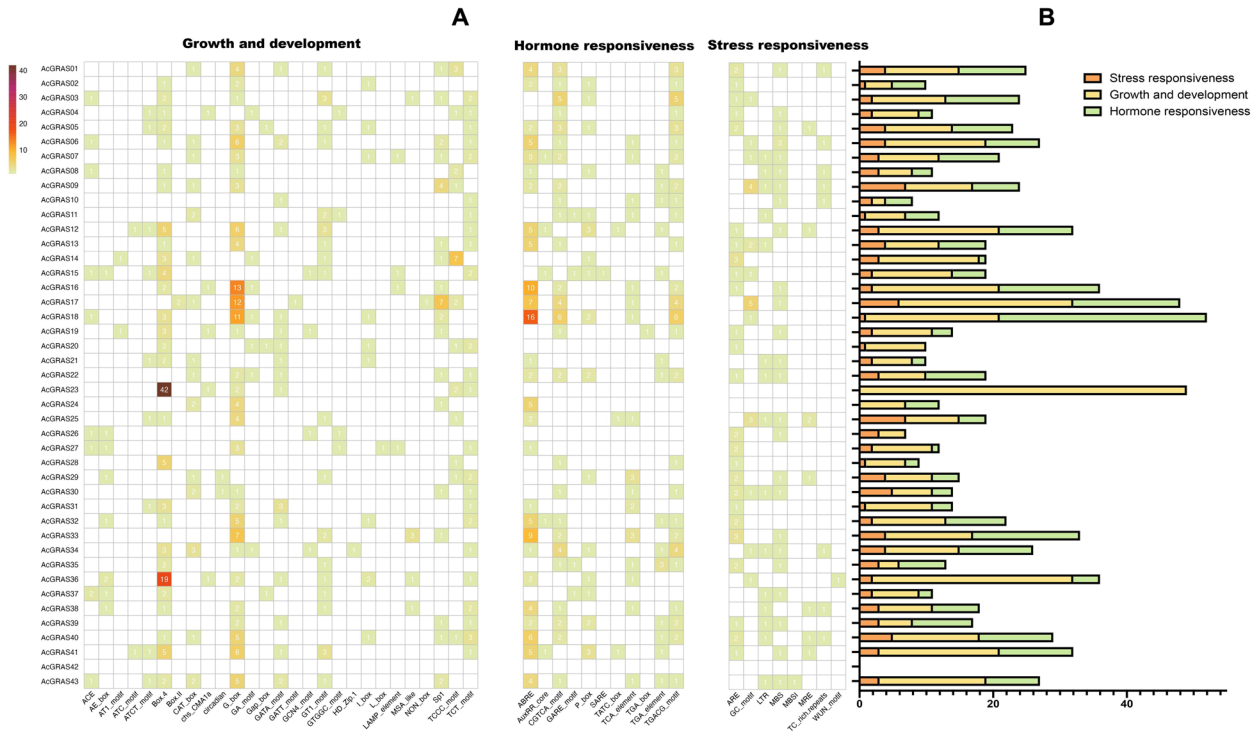

Fig. 4 Cis-regulatory elements in the putative promoter regions of the *AcGRAS* genes. **A** Heatmap of the number of cis-regulatory elements, the different color presented the number of cis-elements. **B** The sum of cis-regulatory elements in each category is shown in the histogram

all *AcGRAS* genes, indicating widespread regulation by light. CREs involved in meristem expression were found in the putative promoters of approximately one-third of the *AcGRAS* members, with multiple occurrences in the promoters of *AcGRAS24*, *AcGRAS17*, *AcGRAS30*, *AcGRAS11*, *AcGRAS43*, and *AcGRAS34*. Other plant growth and development CREs were less common, appearing only in the promoters of specific *AcGRAS* members. For example, CREs related to cell cycle regulation were found only in *AcGRAS03*, *AcGRAS33*, *AcGRAS36*, and *AcGRAS38*, while endosperm expression-related CREs were present only in *AcGRAS26*, *AcGRAS19*, *AcGRAS15*, and *AcGRAS34*. These growth and development-related CREs showed no consistent distribution pattern within subfamilies, suggesting that even within the same subfamily, *AcGRAS* members may play distinct roles in pineapple growth and development. (2) Various phytohormone-responsive CREs were predicted in the promoters of *AcGRAS* genes, indicating that hormones played a significant role in their regulation. Among these, abscisic acid (ABA) response-related CREs were the most abundant (114), which were present in 27 out of 43 *AcGRAS* members including 6 from the PAT1 subfamily (*AcGRAS05*, *AcGRAS06*, *AcGRAS13*, *AcGRAS22*, *AcGRAS39*, and *AcGRAS43*), 6 from the SHR subfamily (*AcGRAS01*, *AcGRAS12*, *AcGRAS21*, *AcGRAS31*, *AcGRAS40*, and *AcGRAS41*), and 4 from the HAM-1 subfamily (*AcGRAS02*, *AcGRAS07*, *AcGRAS16*, and *AcGRAS17*). MeJA response-related CREs were the second most abundant (104), appearing in the promoters of 26 *AcGRAS* members, including 7 from the PAT1 subfamily (*AcGRAS04*, *AcGRAS05*, *AcGRAS06*, *AcGRAS13*, *AcGRAS22*, *AcGRAS39*, and *AcGRAS43*) and 5 from the HAM-1 subfamily (*AcGRAS02*, *AcGRAS07*, *AcGRAS16*, *AcGRAS17*, and *AcGRAS35*). These CREs were especially abundant in two DELLA subfamily members, *AcGRAS03* and *AcGRAS18*, with 10 and 12 CREs, respectively. Gibberellin-responsive CREs were identified in 18 *AcGRAS* members, including 4 from the PAT1 subfamily (*AcGRAS05*, *AcGRAS15*, *AcGRAS22*, and *AcGRAS39*), 3 from the HAM-1 subfamily (*AcGRAS02*, *AcGRAS14*, and *AcGRAS35*), and 2 from the DELLA subfamily (*AcGRAS03* and *AcGRAS18*). Additionally, auxin- and salicylic acid-responsive CREs were also identified but were found only in few specific *AcGRAS* members. (3) Diverse stress-responsive CREs were also identified involved in low-temperature responsiveness, defense and stress responses, and MYB binding sites involved in drought inducibility. These elements presented varied distributions and combinations in the promoter region across *AcGRAS* members. For example, low-temperature responsive CREs were present in the promoters of 18

AcGRAS members, including 4 from the PAT1 subfamily (*AcGRAS13*, *AcGRAS22*, *AcGRAS39*, and *AcGRAS43*), 3 from the SCL3 subfamily (*AcGRAS08*, *AcGRAS09*, and *AcGRAS34*), and 1 from the DELLA subfamily (*AcGRAS07*). The variation in CRE distribution and combinations in the promoters of different *AcGRAS* members contributed to their diverse roles in pineapple growth, development, and stress responses.

Chromosomal location and collinearity analysis

The 43 AcGRAS genes were unevenly distributed across 19 chromosomes and 6 scaffolds (Fig. 5). Within the pineapple genome (LG01-LG25), no genes were found on LG02, LG06, LG10, LG14, LG16, or LG18. There are 5 GRAS genes each on LG01 and LG15, 3 GRAS genes each on LG04, LG11, and LG17, 2 genes each on LG03, LG08, LG19, and LG24, and 1 gene each on the remaining chromosomes. Most genes were distributed in regions with high gene density and high recombination frequency, such as near the chromosome ends. The expansion of gene families is generally driven by various gene duplication patterns, which are considered to be a key force in species evolution. Gene duplication events were analyzed using the MCScanX method, revealing only three

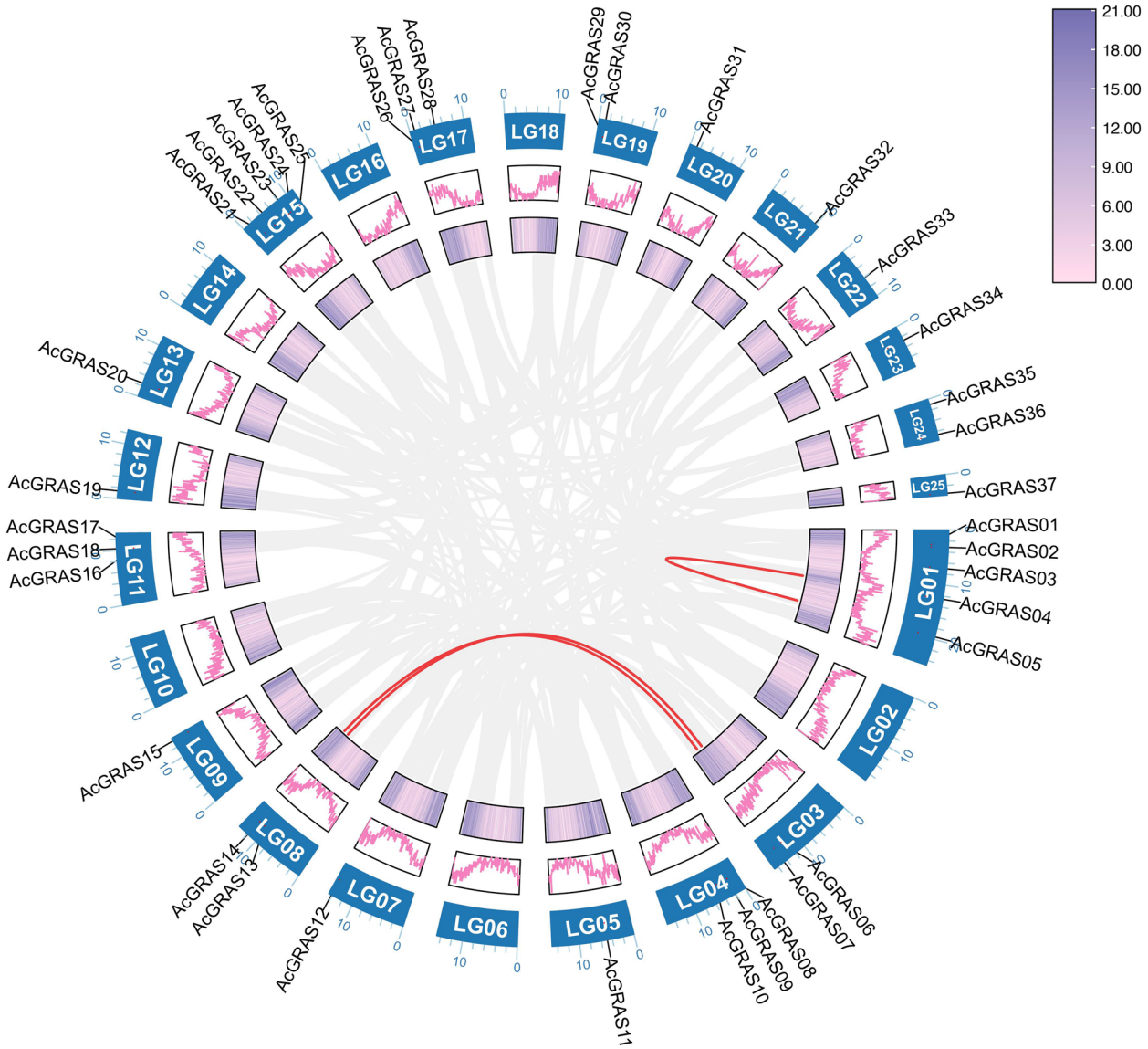

Fig. 5 Distribution and collinearity of *AcGRAS* genes in the pineapple genome. The background gray lines represent all the syntenic blocks in the pineapple genome, and the red lines represent duplicate Ac*GRAS* gene pairs. Chromosome numbers are shown at the bottom of each chromosome. The two rings in the middle represent the gene density of each chromosome

segmental duplicated gene pairs: *AcGRAS04*/*AcGRAS05*, *AcGRAS14*/*AcGRAS07*, and *AcGRAS13*/*AcGRAS06*. Combining with the phylogenetic analysis results (Fig. 1), it was found that the genes within each duplicated gene pair clustered together in the same subfamilyCombining with the phylogenetic analysis results , it was that the genes within each duplicated gene pair clustered together in the same subfamily. Specifically, one pair (AcGRAS07 and AcGRAS14) clustered within the HAM-1 subfamily, while the other two pair (AcGRAS04 and AcGRAS05, AcGRAS06 and AcGRAS13) both clustered within the PAT1 subfamily. These findings suggest that gene duplication might have played an important role in the development of the AcGRAS gene family in the pineapple genome.

Collinearity analysis among different species is an effective method to explore their evolutionary relationships. Here, we conducted a comparative collinearity analysis between pineapple and four representative species: two dicots (*Arabidopsis thaliana* and *Vitis vinifera*) and two monocots (*Oryza sativa* and *Musa nana*) (Fig. 6, Additional file 8: Table S7). A total of 35 *AcGRAS* genes show collinearity with the rice genome, followed by bananas (31), grapes (21), and *Arabidopsis* (9) (Additional file 8: Table S7). These findings suggest that the collinearity between the pineapple and monocot genomes is greater than that between the pineapple and dicot genomes. The 34 *AcGRAS* genes with collinearity to rice were primarily located on chromosomes 3, 8, 15, and 17, while those with collinearity to *Arabidopsis* were distributed mainly on chromosomes 1 and 12. Some homologous genes exhibited one-to-many or many-to-one relationships. Notably, two *AcGRAS* genes (*AcGRAS01* and *AcGRAS19*) displayed collinearity across all four selected species, indicating that these GRAS family genes may have played a significant role in evolutionary processes.

Prediction of putative miRNAs directing *AcGRASs*

MicroRNAs (miRNAs) played a crucial role in gene expression regulation by targeting mRNA degradation [59]. Previous studies indicated that many GRAS members were regulated by miRNAs, particularly miRNA171 [60, 61]. To investigate the potential regulatory relationships between miRNAs and *AcGRAS* genes in pineapple, a total of 385 miRNA-gene target pairs were found (Fig. 7). Specifically, 38 out of 43 *AcGRAS*

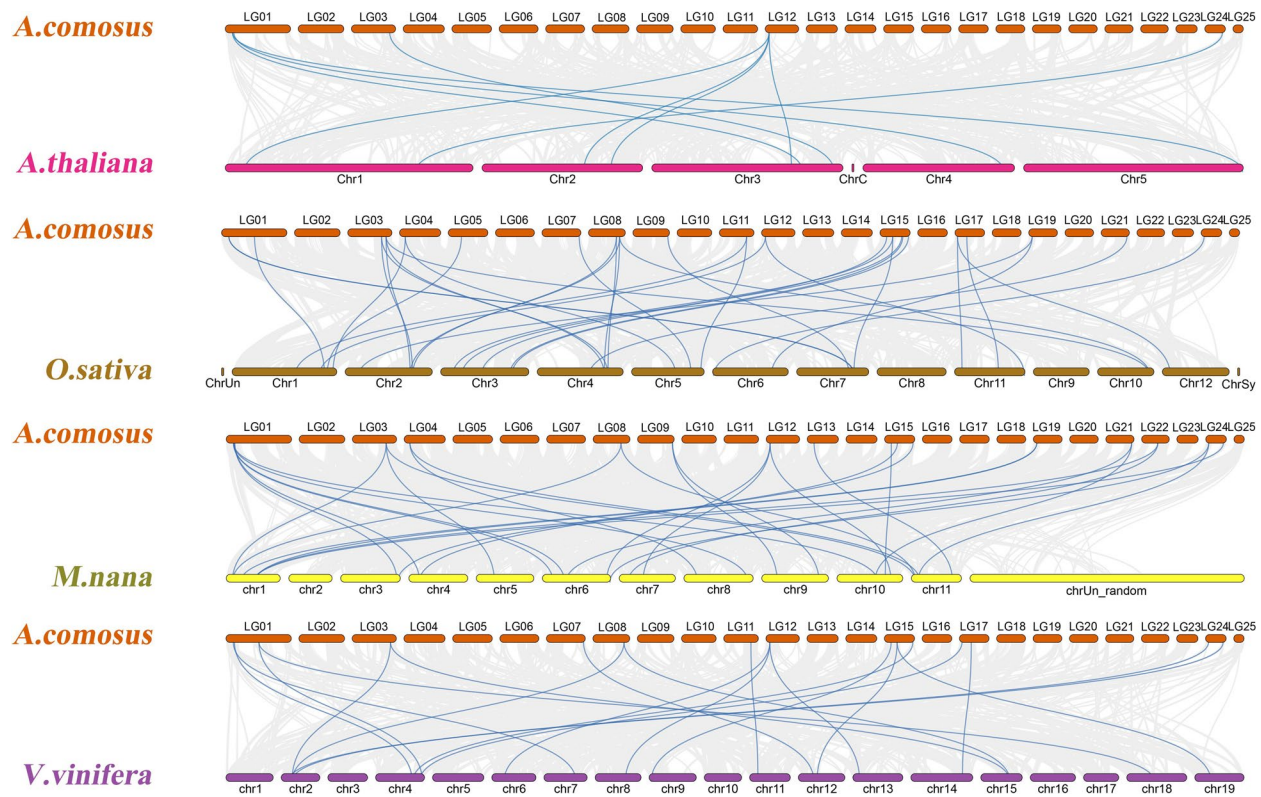

Fig. 6 Synteny analysis of *AcGRAS* genes and four representative plant species. Grey lines in the background indicate collinear blocks in pineapple and other plant genomes, whereas the colored lines highlight syntenic *GRAS* gene pairs. Species names are prefixed with 'A. thaliana', 'O. sativa', 'V.vinifera' and 'M.nana', denote *Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera* and *Musa nana*, respectively

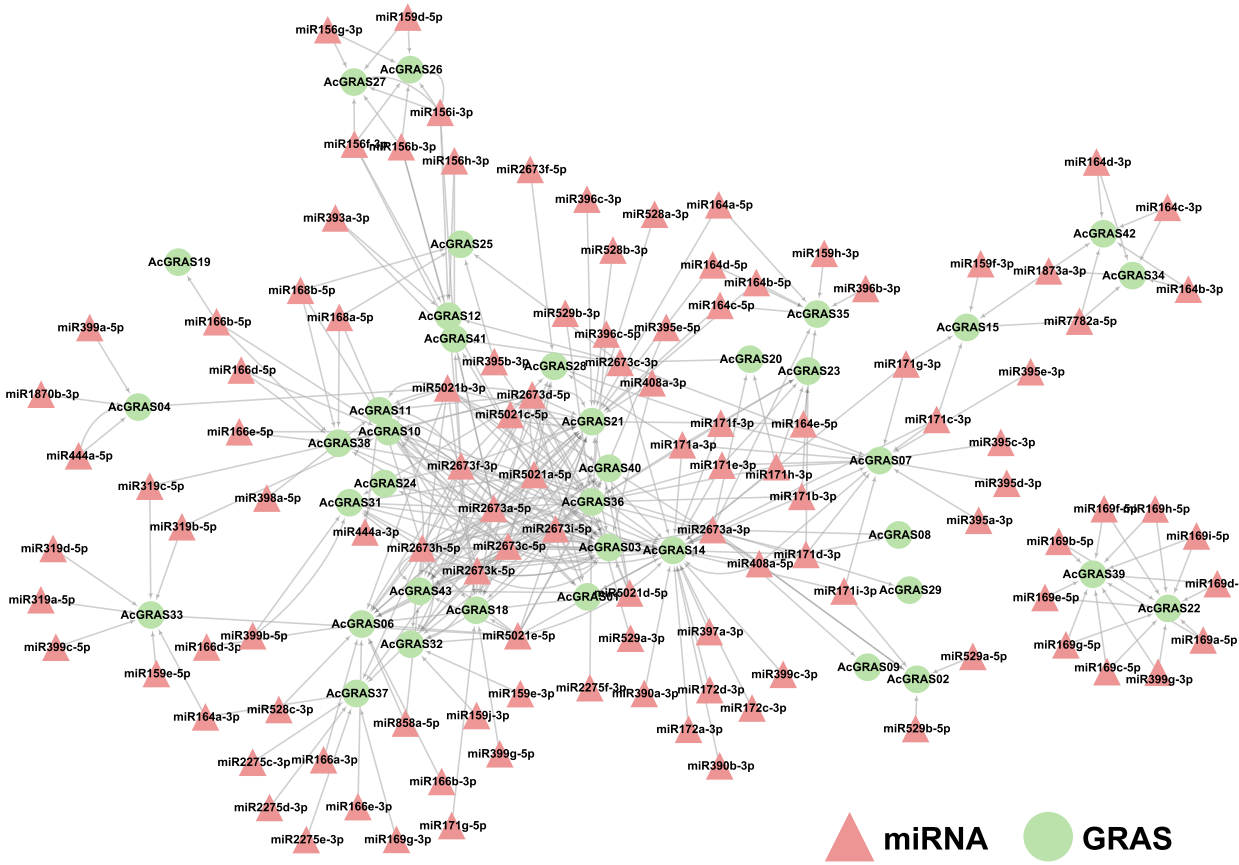

Fig. 7 Predicted miRNAs targeting *AcGRAS* genes. The network diagram shows the predicted miRNA targets for *AcGRAS* genes. Red triangular nodes represent the predicted miRNAs, and green circular nodes represent the targeted *AcGRAS* genes

genes were found to be targeted by miRNAs (excluding *AcGRAS5/13/16/17/30*). *AcGRAS14* (HAM-1) was the one targeted by most miRNAs (36), followed by *AcGRAS03* (DELLA, 30) and *AcGRAS21* (SHR, 30). Among the 36 miRNAs targeting *AcGRAS14*, the miRNA2673 family had the most members (12). The miRNA2673 family also had the highest number of target mRNA interactions, with 158 pairs, and the gene most frequently targeted by this family was *AcGRAS03* (27 times). Among all miRNAs, *miR2673a-5p* targeted the most *AcGRAS* genes (17), while its counterpart from the same precursor, *miR2673a-3p*, targeted only 10 *AcGRAS* genes, with only four genes overlapping between these two miRNAs. These findings suggest that even miRNAs derived from the same precursor can have significantly different functions depending on their processing. The interaction network results of the predicted miRNA targets for *AcGRAS* genes revealed that even members within the same subfamily were regulated by different types and numbers of miRNAs. For example, *AcGRAS14* from the HAM-1 subfamily was primarily regulated by different members of the miRNA2673 family, while *AcGRAS35*, also from the HAM-1 subfamily, was mainly targeted by various members of the miRNA164 family. Similarly, *AcGRAS07* from the same subfamily was predominantly regulated by members of the miRNA171 family. Previous studies have indicated that several GRAS members are regulated by miRNAs, especially miRNA171 [60, 61]. In pineapple, the miRNA171 family was predicted to target eight *AcGRAS* gene members from the DLT, HAM-1, HAM-2, OS43, PAT1, and SCR subfamilies (Additional file 9: Table S8). Among these, the members from the HAM-1 and HAM-2 subfamilies were targeted by the most miRNA171 family members. For instance, *AcGRAS14* (HAM-1 subfamily) was predicted to be targeted by nine different miRNA171 family members, including miR171a-3p, miR171g-3p, miR171b-3p, miR171c-3p, miR171d-3p, miR171e-3p, miR171f-3p, miR171h-3p, and miR171i-3p. Additionally, *AcGRAS07* (HAM-1 subfamily) and *AcGRAS23* (HAM-1 subfamily) were predicted to be targeted by seven and six different miRNA171 family members, respectively. These findings suggested that the miRNA171 family may also have

played a specific role in regulating the HAM subfamily members in pineapple.

Transcription factor regulatory network of *AcGRASs*

To gain a more comprehensive understanding of the factors influencing *AcGRAS* gene expression, the potential transcription factor (TF) regulatory network for all 43 *GRAS* genes in pineapple was analyzed using the PTRM online database (http://plantregmap.gao-lab.org/). The analysis revealed that, except for *AcGRAS42*, the promoter regions of the other 42 *GRAS* genes were enriched with 21 types of TFs (Fig. 8, Additional file 10: Table S9). Among all TFs, ERF was the most abundant (2,241), followed by BBR-BPC (1,545) and MIKC-MADS (392). *AcGRAS17* was found to be the most transcriptionally regulated gene (894), followed by *AcGRAS24* (503) and *AcGRAS29* (485), with *AcGRAS17* and *AcGRAS24* being predominantly regulated by ERF TFs (Additional file 10: Table S9). The proportion of ERF among the TFs regulating different *AcGRAS* gene members ranged from 89.52% to 100%, indicating a distinct preference for ERF binding motifs in the promoter regions of most *AcGRAS* genes. Diverse TFs involved in plant growth and development, including MIKC-MADS, LBD, bHLH, and AP2, were identified. Some stress-related TFs, such as bZIP [62] and NAC [63], have also been identified. Detailedly, the NAC TFs targeted members from the PAT1 (*AcGRAS06*) and DELLA (*AcGRAS03*) subfamilies, while various bZIP TFs primarily targeted members from the HAM-1 (*AcGRAS07*, *AcGRAS16*, *AcGRAS17*), SHR (*AcGRAS01*, *AcGRAS12*, *AcGRAS41*), and DELLA (*AcGRAS18*) subfamilies.

Expression patterns of *AcGRAS* in different tissues of pineapple

To explore the potential functions of *AcGRAS* genes, the expression profiles of the 43 *AcGRAS* genes in various pineapple tissues, including floral organs and fruit at different developmental stages, were analyzed using RNA-seq data (Fig. 9). After filtering out genes with low expression levels, 29 *AcGRAS* genes remained. Most of these genes exhibited preferential expression in specific tissues, suggesting that members of the *GRAS* gene family tend to have roles in particular tissues or at specific developmental stages. Hierarchical clustering grouped the *AcGRAS* genes into five clusters (blocks A-E), revealing diverse expression profiles even among genes within the same subfamilies. The *AcGRAS* genes in different blocks displayed distinct temporal and spatial expression patterns: (A) The *AcGRAS* genes in block

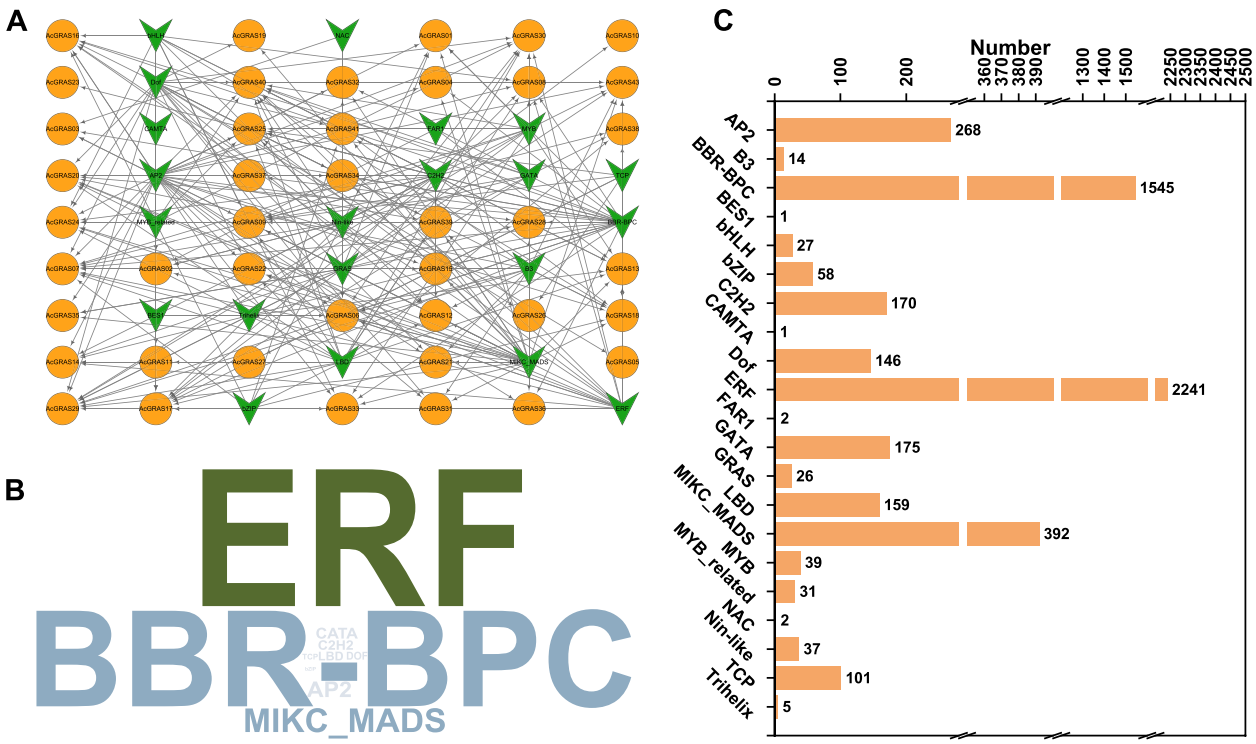

Fig. 8 Putative TF regulatory network analysis of *AcGRAS* genes. **A** Network diagram illustration of the predicted TFs that target *AcGRAS* genes. Green arrow-shaped nodes represent TFs, and orange circular nodes represent *AcGRAS* genes. **B** Word cloud of TFs, where the font size is positively correlated with the number of corresponding TFs. **C** Statistical results of the number of TFs

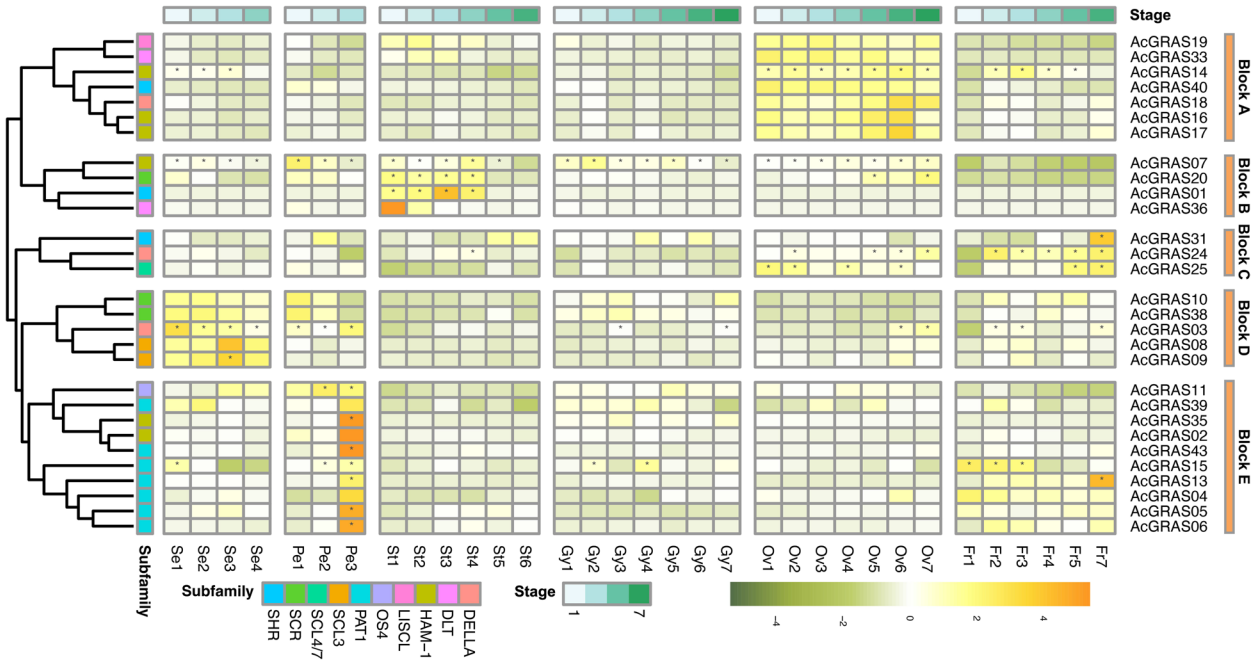

Fig. 9 Hierarchical clustering of the expression profiles of *AcGRASs* in floral tissues and fruits at different developmental stages. Se, sepal; Gy, gynoecium; Ov, ovule; Pe, petal; St, stamen; Fr, fruit; numbers represent developmental stages as described in Wang et al. (2020) [56]; the heatmap was created based on the $\log_2$(TPM + 0.01) value of *AcGRASs* and normalized by row. The TPM value higher than 50 was shown as abundant genes and marked with "*". Differences in gene expression changes are shown in color as the scale, orange for high expression and dark green for low expression

A were preferentially expressed during ovule development, including one member each from the LISCL (*AcGRAS19*), DLT (*AcGRAS33*), SHR (*AcGRAS40*), and DELLA (*AcGRAS18*) subfamilies, as well as three members from the HAM-1 subfamily (*AcGRAS16*, *AcGRAS17*, and *AcGRAS14*). Among these, *AcGRAS14* was also highly expressed during the early stages of sepal development and the middle stages of fruit development. (B) *AcGRAS* genes in block B were highly expressed during the early developmental stages of stamens, including one member each from the HAM-1 (*AcGRAS07*), SCR (*AcGRAS20*), SHR (*AcGRAS01*), and DLT (*AcGRAS36*) subfamilies. (C) The *AcGRAS* genes in block C tended to have higher expression levels at the late developmental stages of fruit, including one member each from the SHR (*AcGRAS31*), DELLA (*AcGRAS24*) and SCL4/7 (*AcGRAS25*) subfamilies. Besides, *AcGRAS24* and *AcGRAS25* were also showing high expression at certain stages of ovule development. (D) The *AcGRAS* genes in block D exhibited preferential expression during sepal development, including one member from the DELLA (*AcGRAS03*) subfamily and two members each from the SCR (*AcGRAS10*, *AcGRAS38*) and SCL3 (*AcGRAS08*, *AcGRAS09*) subfamilies. Among these genes, *AcGRAS03* tended to decrease in expression during sepal development but was highly expressed at the later stages of gynoecium, ovule, petal, and fruit development. (E) The *AcGRAS* genes in block E were preferentially expressed during petal development, with an ascending trend, including one member from the OS4 (*AcGRAS11*), subfamilies, two members from the HAM-1 (*AcGRAS35*, *AcGRAS02*), and seven members from the PAT1 subfamily (*AcGRAS43, AcGRAS15, AcGRAS13, AcGRAS04, AcGRAS05, AcGRAS06, AcGRAS39*). Among these genes, *AcGRAS13* was also highly expressed at the late developmental stages of fruit, while *AcGRAS15* was also higher expressed at the early development stages of fruit. In summary, different *AcGRAS* genes displayed diverse expression profiles within or across subfamilies, with most showing tissue- or developmental stage-specific expression patterns. For example, *AcGRAS35*, *AcGRAS43*, *AcGRAS02*, *AcGRAS05* and *AcGRAS06* in block C were specifically preferentially expressed only at the late developmental stage of petals. However, a few genes, such as *AcGRAS07*, exhibited relatively high expression levels across all floral tissues.

Low temperature has the most significant impact on pineapple production among various stressors, and many *GRAS* genes have been reported to play roles in plant cold stress responses [64]. To elucidate the response of *AcGRAS* genes to low-temperature stress, we analyzed their expression profiles in pineapple leaves at different

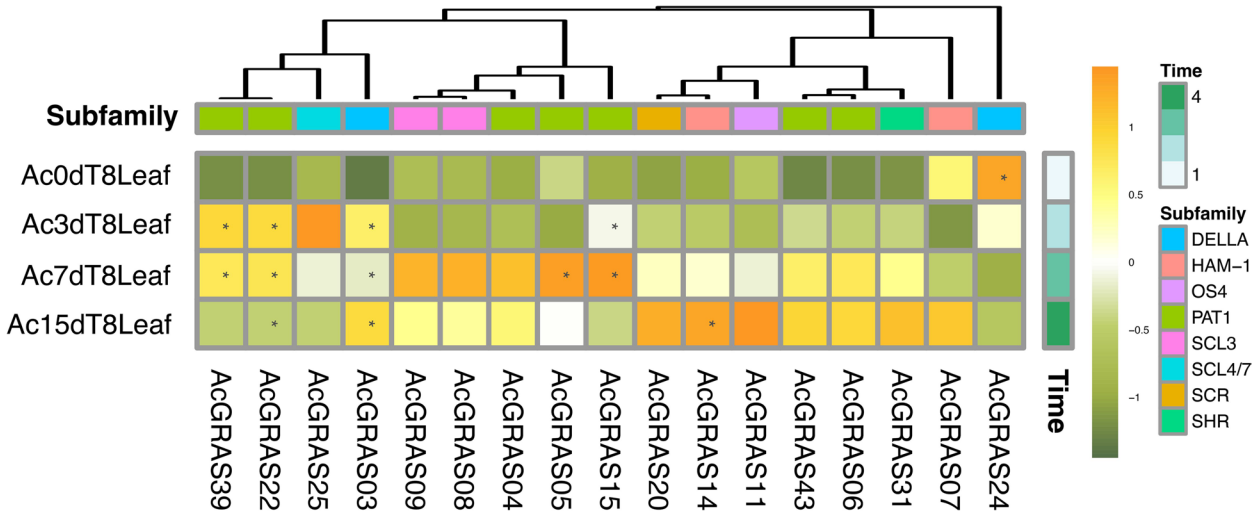

Fig. 10 Hierarchical clustering of the expression profiles of the *AcGRASs* under cold treatment at 8 °C (0 d, 3 d, 7 d, and 15 d). The heatmap was created based on the $\log_2$(TPM + 0.01) value of *AcGRASs* and normalized by row. The TPM value higher than 50 was shown as abundant genes and marked with "*". Differences in gene expression changes are shown in color as the scale, orange for high expression and dark green for low expression

time points under long-term cold treatment at 8 °C (0 d, 3 d, 7 d, and 15 d) via RNA-seq data (Fig. 10). After filtering out low-expression genes, our results indicated that, except for *AcGRAS24*, which was downregulated by cold stress, all other *AcGRAS* members were upregulated in response to low temperature. A few genes, including *AcGRAS15*, *AcGRAS25*, *AcGRAS22*, and *AcGRAS39*, exhibited peak expression during the mid-phase of cold treatment, while other members, such as *AcGRAS03*, *AcGRAS04*, *AcGRAS05*, *AcGRAS07*, *AcGRAS11*, *AcGRAS14*, *AcGRAS19*, and *AcGRAS43*, showed increased expression in the mid-to-late stages of cold treatment.

To validate the cold response trends of *AcGRAS* genes, we further analyzed the expression of 12 representative *AcGRAS* genes at additional time points (0 d, 1 d, 5 d, 7 d, 9 d, 11 d, 13 d, and 14 d) under the same batch of cold treatments as the transcriptome data using qRT-PCR (Fig. 11). These genes included seven members from the PAT1 subfamily, two from the DELLA subfamily, two from the HAM-1 subfamily, and one from the LISCL subfamily. Under cold stress conditions, the expression of *AcGRAS03*, *AcGRAS14*, and *AcGRAS24* was significantly downregulated, initially decreasing and then showing some recovery with extended treatment time. Other *GRAS* genes were upregulated to varying degrees in response to cold stress. Specifically, *AcGRAS04*, *AcGRAS05*, and *AcGRAS43* exhibited decreased expression early in the cold treatment but were significantly upregulated after 11 days, suggesting their involvement in the long-term adaptation of pineapple to cold environments. In contrast, *AcGRAS07*, *AcGRAS22*, and *AcGRAS39* showed an initial increase in expression followed by a decrease, with peak expression occurring in the mid-treatment period. While expression levels of most *AcGRAS* genes varied throughout the cold treatment, *AcGRAS15* was consistently upregulated at different time points, indicating its potential ongoing role in pineapple's cold stress response. With a few exceptions, the results of the qRT-PCR analysis were largely consistent with the transcriptome data in revealing the response of *AcGRAS* genes to cold stress. Different *AcGRAS* members played roles in the short-term response or long-term adaptation of pineapple to low temperatures.

Discussion

Pineapple is an important tropical fruit crop, and the normal development of its flowers and fruits is crucial for fruit quality formation [56]. The growth and development of pineapple are significantly affected by low temperatures, with winter cold limiting year-round production [32]. Identifying gene resources involved in regulating flower and fruit development, as well as cold response, is of great significance for molecular breeding in pineapple. Members of the plant-specific GRAS gene family play essential roles in plant growth, fruit maturation, and stress responses including cold stress [1]. In this study, we identified all GRAS gene family members in the pineapple genome and systematically analyzed their structural characteristics, phylogenetic relationships, regulatory elements, and expression patterns. This research provides

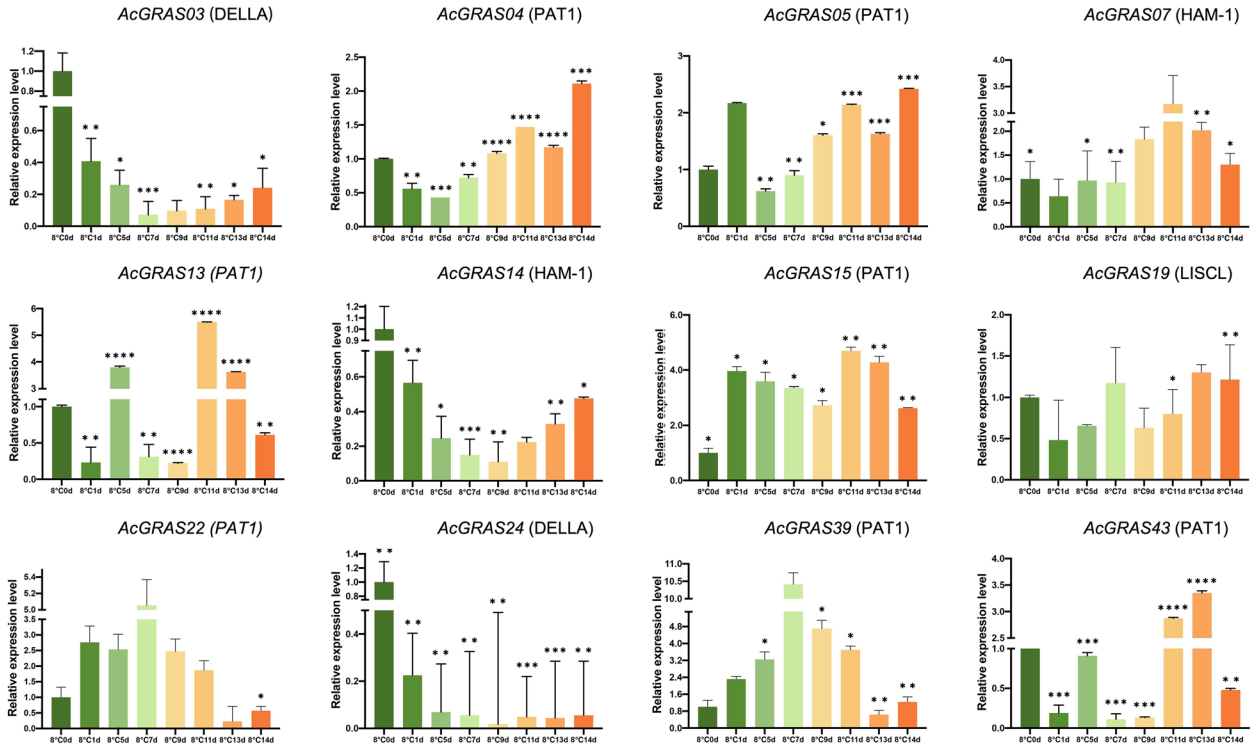

Fig. 11 qRT-PCR analysis of 12 representative *AcGRAS* genes (*AcGRAS03, AcGRAS14, AcGRAS07, AcGRAS24, AcGRAS19, AcGRAS39, AcGRAS22, AcGRAS15, AcGRAS13, AcGRAS05, AcGRAS43, and AcGRAS04*) under cold (8 °C) stress in pineapple. All the experiments were conducted independently at least three times. Error bars indicate the standard deviation across three replicates. Asterisks denote significant differences in transcript levels relative to the blank control without treatment (0 d) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

a reference for future functional studies of GRAS genes and molecular breeding in pineapple.

A total of 43 GRAS genes were identified in the whole pineapple genome, which were unevenly distributed across 19 chromosomes and 6 scaffolds, and named *AcGRAS01* to *AcGRAS43* based on their chromosomal locations (Additional file 3: Table S3, Additional file 11: Fig S2). The number of GRAS genes in pineapple was comparable to species like *Arabidopsis thaliana* (34), *Cucumis melo* (37), small gourd (*Lagenaria siceraria*) (37), physcomitrella moss (*Physcomitrium patens*) (42), cocoa (*Theobroma cacao*) (44), and wintersweet (*Prunus mume*) (46), but fewer than in *Oryza sativa* (50), *Populus trichocarpa* (106), *Malus domestica* (127), and winter rape (*Brassica napus*) (87). It seemed that the number of GRAS genes did not appear to correlate with genome size, for example, pineapple possesses a larger genome (526 Mb) [34] with fewer *GRAS* gene family members (43) compared to *Brachypodium distachyon* (271 Mb, 63*GRASs*) [65, 66]. Previous studies have shown that gene duplication events likely drive the expansion of the GRAS gene family [67] In pineapple, we identified only three segmental duplicated gene pairs: *AcGRAS04/AcGRAS05*, *AcGRAS14/AcGRAS07*, and *AcGRAS13/AcGRAS06* (Fig. 5). Phylogenetic analysis revealed that each duplicate gene pair, two genes are clustered in the same subfamily, and 3 gene pairs are distributed in 3 subfamilies (Fig. 1). Expression analysis showed that *AcGRAS04/AcGRAS05* and *AcGRAS13/AcGRAS06* were predominantly expressed during late petal development (Fig. 9) and were induced by cold stress during the later stages of treatment (Figs. 10 and 11), suggesting possible functional redundancy. Differently, while *AcGRAS14/AcGRAS07* were both highly expressed during ovule development, *AcGRAS14* was predominantly expressed during mid-fruit development, whereas *AcGRAS07* showed abundant expression in other flower organs (Fig. 9), with differing expression trends under cold stress (Figs. 10 and 11), indicating possible functional divergence. Gene duplication might have played role in the development of the *AcGRAS* gene family in pineapple, and the limited duplication events may explain the relatively smaller size of the GRAS gene family in this species.

Phylogenetic analysis indicated that GRAS members from pineapple, *Arabidopsis*, and rice clustered into 14 subfamilies (Fig. 1), with most subfamilies containing GRAS members from all three species. However, some subfamilies, such as OS4 and OS19, contained genes only from rice and pineapple, suggesting distinct development

processes of GRAS gene family in these species. In most subfamilies, pineapple GRAS proteins clustered closely with rice GRAS proteins, indicating a close evolutionary relationship. Comparative collinearity analysis between pineapple and four representative species also showed higher collinearity between the pineapple genome and monocots (Fig. 6). The classification of GRAS genes in rice and *Arabidopsis* was consistent with previous reports [68], suggesting the reliability of our phylogenetic tree. However, the number of subfamilies differed from other studies; for example, 13 subfamilies were identified in the phylogenetic analysis of grapevine and *Arabidopsis* GRASs [8], while 10 subfamilies were identified in the phylogenetic analysis of GRASs from orchids (*Dendrobium chrysotoxum*), *A. thaliana*, and *O. sativa* [69]. These variations may reflect differences in the species included in the analysis. As more species undergo GRAS gene family analysis, species-specific subfamilies are being discovered [1], underscoring the importance of studying GRAS gene evolution in a broader range of species to gain a comprehensive understanding of their evolutionary history.

Gene exon–intron structural analysis of the 43 *AcGRAS* genes revealed variability in the number of exons, ranging from 1 to 6 (Fig. 2D). Most *AcGRAS* genes (26, or 60.5%) lacked introns, a phenomenon also reported in other GRAS family studies [70]. The origin of plant GRAS genes is thought to stem from horizontal gene transfer from ancient prokaryotic soil bacteria, followed by duplication events in flowering plants, which may explain the prevalence of intronless genes [71]. Over time, some GRAS genes developed different exon–intron structures, potentially acquiring new functions to adapt to specific environments. We found that most conserved motifs in AcGRAS proteins were located in the C-terminal domain and arranged in the sequences of Motif 7, Motif 3, Motif 1, Motif 8, Motif 2, Motif 4, Motif 5, and Motif 6, with Motif 3 being the most highly conserved. The SCR and PAT1 subfamilies displayed relatively stable C-terminal domains with almost no motif loss, whereas other subfamilies exhibited significant motif deletions (Fig. 2B), contributing to the functional diversity observed among subfamily members. Protein 3D structure prediction showed relatively higher similarity among proteins within the same subfamily, while there were distinct differences in proteins from different subfamilies (Fig. 3, Additional file 5: Fig. S1). This structural diversity might contribute to the functional diversity of GRAS family members.

Research has demonstrated that GRAS family members play critical roles in various aspects of plant growth and development, including root and shoot development, lateral organ formation, flower, embryo, and seed development, as well as fruit development and maturation [1]. Expression analysis of *AcGRAS* genes during pineapple floral organs and fruit development revealed that, after filtering out low-expression genes, most *AcGRAS* members were predominantly expressed in specific tissues or at certain developmental stages (Fig. 9). For instance, genes in Block A, which include members from the HAM-1, DLT, DELLA, and SHR subfamilies, were highly expressed during ovule development but showed relatively low expression in other flower organs and during fruit development. Previous studies have also shown that GRAS genes from different subfamilies are involved in flower and fruit development regulation [9, 21]. For example, GRAS proteins from the SHR subfamily have been shown to play a key role in ovule polarity establishment in *Arabidopsis* [72]. Genes from different subfamilies clustered into distinct blocks based on their expression patterns (Fig. 9). Nearly all members of the PAT1 subfamily clustered in Block E and were predominantly expressed during late petal development, suggesting a specialized role in this process. However, the expression patterns of other subfamily members across different tissues were less consistent. Although members of the same subfamily shared some similarities in gene and protein structure, the composition and distribution of CREs related to growth and development varied significantly among subfamily members (Fig. 4, Additional file 7: Table S6). Most *AcGRAS* genes were also predicted to be targeted by miRNAs (Fig. 7, Additional file 9: Table S8), indicating that the expression of these members might be regulated by miRNAs. Consistent with previous reports, we found that *AcGRAS* members from the HAM-1 and HAM-2 subfamilies were predominantly targeted by the miRNA171 family, reflecting conserved complementarity between HAM subfamily genes and miRNA171 across species [1]. However, even within the same subfamily, the miRNAs targeting different GRAS gene members varied considerably in type and number. Additionally, the TF predictions showed that various growth and development-related TFs were predicted to regulate different *AcGRAS* gene members (Fig. 8, Additional file 10: Table S9). Collectively, the diversity of these regulatory factors likely contributed to the functional diversification of GRAS gene members during the growth and development in pineapple.

Many GRAS family members have been reported to play roles in stress responses across various plants [1]. The growth and development of pineapple is particularly sensitive to cold stress. Our results showed that, except for a few *AcGRAS* genes downregulated under cold stress,

such as *AcGRAS03* and *AcGRAS24* from the DELLA subfamily, the expression of most *AcGRAS* members were upregulated in response to cold. Some genes, such as *AcGRAS22* and *AcGRAS39* from the PAT1 subfamily, were predominantly expressed during early to mid-cold treatments, while most genes, including *AcGRAS04*, *AcGRAS05*, *AcGRAS13*, and *AcGRAS43*, were highly expressed during the later stages of prolonged cold exposure. These findings suggest that different *AcGRAS* members may play distinct roles in short-term or long-term cold adaptation in pineapple. Additionally, the expression of *AcGRAS15* from the PAT1 subfamily was upregulated at all time points during cold stress, indicating its continuous involvement in cold stress adaptation. These findings highlighted the critical role of the PAT1 subfamily in pineapple's response to cold stress. In wild Amur grape, the PAT1 subfamily member *VaPAT1* has been shown to regulate jasmonic acid biosynthesis in response to cold stress [26], and in zoysiagrass, overexpression of the PAT1 subfamily gene *ZjCIGR1* has been found to confer cold stress resistance [27]. CRE prediction analysis revealed that many stress-related CREs were present in the promoter region of most PAT1 subfamily members, including ABA-responsive, MeJA-responsive, and low-temperature-responsive elements (Fig. 4). These candidate genes involved in pineapple cold stress response could serve as potential targets for future research and molecular breeding efforts.

Conclusion

In summary, this study provides the first comprehensive analysis of the GRAS gene family in pineapple, identifying 43 *AcGRAS* genes and revealing their diverse roles in development and stress responses. The findings highlight the functional diversity of *AcGRAS* genes, particularly their tissue-specific expression and significant involvement in cold stress adaptation, with specific emphasis on the unique roles of the PAT1 subfamily in petal development and cold response. These insights not only deepen our understanding of the molecular mechanisms underlying pineapple development and stress tolerance but also offer potential applications in breeding programs aimed at enhancing cold resistance in this economically important crop. This research also serves as a valuable resource for future functional studies of *AcGRAS* genes in pineapple.

Abbreviations

GAI	Gibberellic acid-insensitive
RGA	Repressor of GAI
SCR	Scarecrow
CREs	Cis-regulatory elements
GMQE	Global Model Quality Estimation
QMEAN	Quality Model Energy Assessment
GRAVY	Grand Average of hydropathicity
miRNAs	MicroRNAs
pI	Isoelectric point
RNAi	RNA interference
TF	Transcription Factor
TPM	Transcripts Per Million
UTR	Untranslated Region
CDS	Coding Sequence
ABA	Abscisic Acid
MeJA	Methyl Jasmonate

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05913-9.

Additional file 1: Table S1. Table S1.1 shows the gene IDs and GRAS subfamily information for pineapple, Arabidopsis, rice, ZjCIGR1 and VaPAT1, and Table S1.2 shows the protein sequences used to construct the maximum likelihood (ML) phylogenetic tree.

Additional file 2: Table S2. The primer sequences for qRT–PCR used in this study.

Additional file 3: Table S3. Physicochemical properties of AcGRAS gene family members in pineapple.

Additional file 4: Table S4. Putative motifs identified from AcGRAS proteins via MEME. The sequence logos were generated via WebLogo.

Additional file 5: Fig. S1. Homology modeling of all AcGRAS proteins. All AcGRAS proteins were homologously modeled via the AlphaFoldDB and SWISS-MODEL databases and classified by subfamily.

Additional file 6: Table S5. GMQE and QMEAN scores of AcGRAS protein 3D structure modeling.

Additional file 7: Table S6. Related information on the cis-regulatory elements in the promoter regions of AcGRASs.

Additional file 8: Table S7. Information on collinearity analysis and interpecies collinear gene repetition events.

Additional file 9: Table S8. Prediction of putative miRNAs directing AcGRASs.

Additional file 10: Table S9. Potential transcription factor (TF) prediction information for all 43 GRAS genes in pineapple.

Additional file 11: Fig. S2. Chromosomal localization of all AcGRAS genes. The colors on the chromosomes indicate gene density, with red indicating relatively high density and blue indicating relatively low density.

Acknowledgements

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Authors' contributions

P.Z., Y.Q. and R.L. designed the experiments and revised the manuscript; J.L., J.W. and X.C. performed the gene family analysis; D.Z. conducted the cold treatment and collected the samples; Q.Y., S.X. and X.X. collected the different pineapple tissues; S.C. generated the expression data from RNA-seq; L.D., L.L., and C.L. performed the qRT–PCR and constructed the vectors. P.Z., J.L. and R.L. wrote the original manuscript; P.Z., Y.Q. and X.W. reviewed and edited the manuscript.

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Data availability
The entire Ananas comosus L. genome sequence information was obtained from the Phytozome website (version: Ananas comosus v3; https://phytozome-next.jgi.doe.gov/info/Acomosus_v3). The original RNA-seq data of pineapple floral organs used in this study were obtained from the European Nucleotide Archive (ENA) under accession number PRJEB38680. Data on the different stages of pineapple fruit development were obtained from https://de.iplantcollaborative.org/de/?type=data&folder=/iplant/home/cmwai/coge_data/Pineapple_tissue_RNAseq. Transcriptomic data from pineapple subjected to cold treatment at 8 ℃ were generated from our unpublished work, which has been deposited in China National GeneBank DataBase (CNGBdb) with accession number CNP0006260 (https://db.cngb.org/search/project/CNP0006260/). The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethics approval and consent to participate
The experimental research and method on pineapple species comply with relevant institutional, national, and international guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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