



Genome-wide identification and analyses of *ZmAPY* genes reveal their roles involved in maize development and abiotic stress responses

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Abstract

Apyrase is a class of enzyme that catalyzes the hydrolysis of nucleoside triphosphates/diphosphates (NTP/NDP), which widely involved in regulation of plant growth and stress responses. However, apyrase family genes in maize have not been identified, and their characteristics and functions are largely unknown. In this study, we identified 16 apyrases (named as *ZmAPY1-ZmAPY16*) in maize genome, and analyzed their phylogenetic relationships, gene structures, chromosomal distribution, upstream regulatory transcription factors and expression patterns. Analysis of the transcriptome database unveiled tissue-specific and abiotic stress-responsive expression of *ZmAPY* genes in maize. qPCR analysis further confirmed their responsiveness to drought, heat, and cold stresses. Association analyses indicated that variations of *ZmAPY5* and *ZmAPY16* may regulate maize agronomic traits and drought responses. Our findings shed light on the molecular characteristics and evolutionary history of maize apyrase genes, highlighting their roles in various biological processes and stress responses. This study forms a basis for further exploration of apyrase functions in maize.

Keywords Maize · Apyrase · Abiotic stress response · Association analysis · Metabolic

Introduction

Apyrase (APY) enzymes, classified as nucleoside triphosphate (NTP) diphosphohydrolases, belong to the superfamily of guanosine diphosphatase 1 (GDA1)-cluster of differentiation 39 (CD39) nucleoside phosphatase. These enzymes have the capability to remove the terminal phosphate from nucleoside triphosphates (NTPs) and

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nucleoside diphosphates (NDPs) but not from nucleoside monophosphates (NMPs). Based on their subcellular localization, APYs can be broadly categorized into ecto-apyrases and endo-apyrases (Hideaki et al. 1984; Tong et al. 1993; Thomas et al. 1999). ecto-apyrase are located on the cell surface, whereas endo-apyrase are usually located in the endoplasmic reticulum, Golgi and intracellular vesicles (Leal et al. 2005). Some ecto-apyrase possess transmembrane domains at their N- and C-terminals, which are crucial for correct protein folding, membrane targeting, cellular allocation and enzyme activity (Wu et al. 2005; Knowles 2011). The cellular ATP level serves a dual role in providing energy and regulating various cellular processes associated with responses to abiotic stress (Sun et al. 2012). Therefore, the maintenance of cellular ATP homeostasis, regulated in part by apyrases, is essential for preserving normal cell function.

Apyrases are evolutionarily highly conserved (Clark et al. 2014). Their presence in plants was initially identified in potatoes several decades ago (Hideaki et al. 1984). Subsequently, members of the APY family were discovered in the genomes of various plants, including potato (Handa and Guidotti 1996; Riewe et al. 2008), wheat (Liu et al. 2019), soybean (Day et al. 2000), *Arabidopsis* (Yang et al. 2013), cotton (Clark et al. 2010), rice (Chowdhury et al. 2023), and peanut (Sharif et al. 2023). Transcriptome analysis has revealed specific spatio-temporal expression patterns of APY genes and regulation of these genes by biological and abiotic stresses (Liu et al. 2019; Chowdhury et al. 2023; Clark et al. 2024), indicating potential roles in plant growth, development, and stress responses.

Further studies have elucidated the functions of APY genes in plant growth and development. *Arabidopsis* APY genes, *APY1* and *APY2*, are predominantly expressed in rapidly growing tissues or those with high auxin levels. Suppression of their expression affects root and shoot growth (Wu et al. 2007). Additionally, *APY1/APY2* also participate in the phytochrome-mediated signaling pathway that induces differential growth changes in etiolated seedling tissues (Weeraratne et al. 2022). Another member of the APY gene family, *APY7*, acts as a negative regulator of cell growth. *APY7* modulates the growth-inhibiting effects of RALF1 (Rapid ALKalinization Factor), influencing cell wall architecture, composition, and alters the pH of the extracellular matrix (Gupta et al. 2024). Constitutive expression of pea ectoapyrase, psNTP9, in *Arabidopsis* and soybeans leads to a more extensive root system architecture (RSA) (Veerappa et al. 2019). Furthermore, the catalytic activity of GS52 ecto-apyrase is crucial for the early infection process of *B. japonicum*, nodule primordium development initiation, and subsequent nodule organogenesis in soybean (Govindarajulu et al. 2009).

In addition to participating in the regulation of plant growth and development, the APY gene also plays a crucial role in stress response (Clark et al. 2021). Overexpression of the pea APY gene *psNTP9* significantly enhances drought resistance and field yield in soybean under both normal watering and drought conditions (Veerappa et al. 2019). *Populus euphratica* APY genes, *PeAPY1* and *PeAPY2*, enhance drought tolerance by modulating stomatal aperture in *Arabidopsis* (Zhang et al. 2021b). Furthermore, overexpression of *PeAPY2* improves cold resistance by modulating vesicular trafficking and

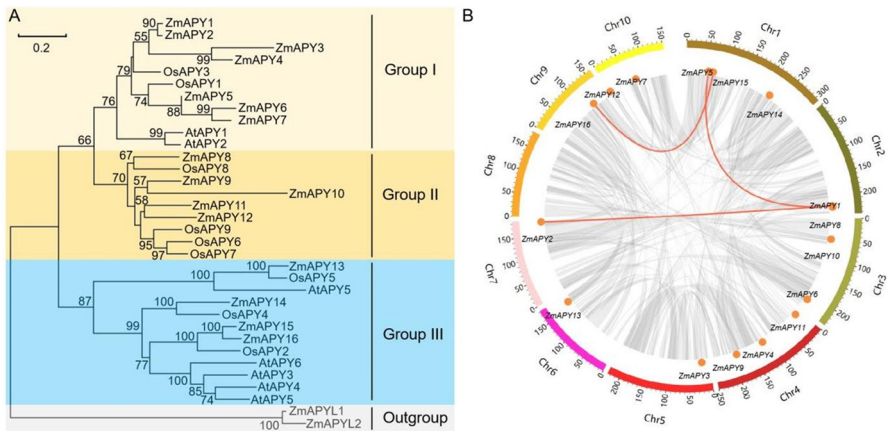


Fig. 1 Phylogenetic analysis, chromosomal distribution and collinearity analysis of *ZmAPY* genes **A** Phylogenetic analysis of APYs from *Z. mays*, *A. thaliana* and *O. sativa*. A total number of 16 *ZmAPY*s from maize, 7 *AtAPY*s from *Arabidopsis* and 9 *OsAPY*s from rice were used to construct the phylogenetic tree. All APY transporter members were classified into four groups. Group I-III and outgroup are distinguished by different colors. **B** Chromosomal distribution and collinearity analysis of *ZmAPY* genes. The chromosomal location of each *ZmAPY* gene was mapped according to the maize genome. The chromosome number is indicated next to each chromosome. The syntenic *ZmAPY* gene pairs are connected by orange lines

extracellular ATP in *Arabidopsis* (Deng et al. 2015). Proteome analysis of soybean roots under waterlogging has demonstrated that *APY* responded to waterlogging stress in soybean (Alam et al. 2010). These findings underscore the multifaceted roles of the *APY* genes in plant stress responses, highlighting its potential as a key player in enhancing plant resilience to various environmental challenges.

Maize, a primary global food crop, faces significant threats from abiotic stresses. Identifying additional abiotic stress resistance genes and further understanding response system in maize remain crucial. While *APY* genes has been reported to be involved in various abiotic stresses in *Arabidopsis*, their presence and biological functions in maize have not been reported. This study aims to characterize *APY* genes and elucidate their roles. Here, 16 *APY* family members were identified in maize genome by phylogenetic analysis (Fig. 1; Table S1). *APY* family expansion patterns and genetic characteristics were analyzed comprehensively (Figs. 1 and 2; Table S2). In addition, potential upstream regulators of *ZmAPY*s were predicted (Fig. 4). Transcriptome analysis revealed tissue-specific and abiotic stress-responsive expression of *ZmAPY* genes in maize (Figs. 3 and 5). Association analysis suggested that variations in *ZmAPY* genes could impact maize agronomic traits and drought responses (Figs. 6 and 7). These findings establish a basis for further investigations into the functions of maize *APY* genes.

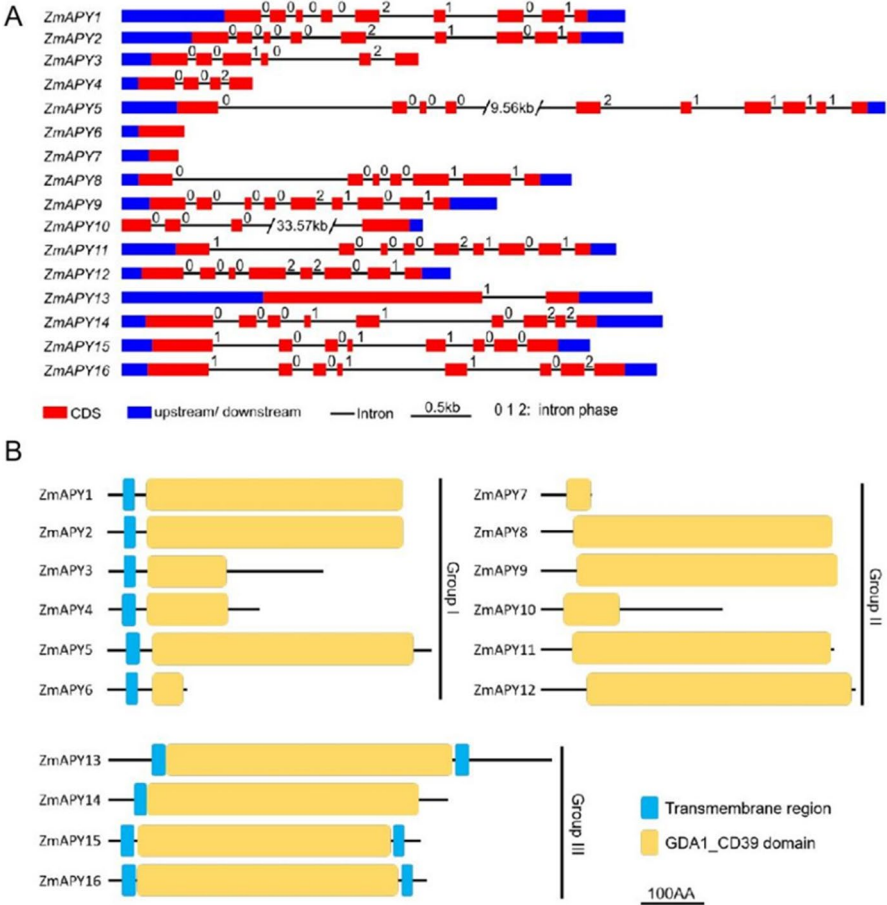


Fig. 2 Exon–intron structure and conserved motifs of *ZmAPY* gene family **A** Exon–intron structures of *ZmAPY* genes. Red boxes represent exons (CDS), black lines represent introns, and blue boxes represent 5' and 3'UTR regions. 0, 1 and 2: Intron phase, defined as the position of the intron within a codon, as introns are located either between codons (phase 0) or within codons (phase 1 and 2). **B** Predicted conserved protein domains of *ZmAPY* proteins. The black lines represent the length of each protein sequence, and the conserved protein kinase domains are depicted with colored boxes. blue boxes represent transmembrane region and yellow boxes represent GDA1_CD39 domain

Materials and methods

Plant materials and growth condition

The maize inbred line B73 was used in this study. Maize seeds are germinated on moist filter paper in a plant growth chamber at 28°C for 2–3 days. Uniformly germinated seeds are then transplanted into soil-filled pots (10 cm × 10 cm × 9 cm) and grown in the chamber until the three-leaf stage for subsequent stress treatments. For drought stress, water is withheld from three-leaf stage maize seedlings for 10

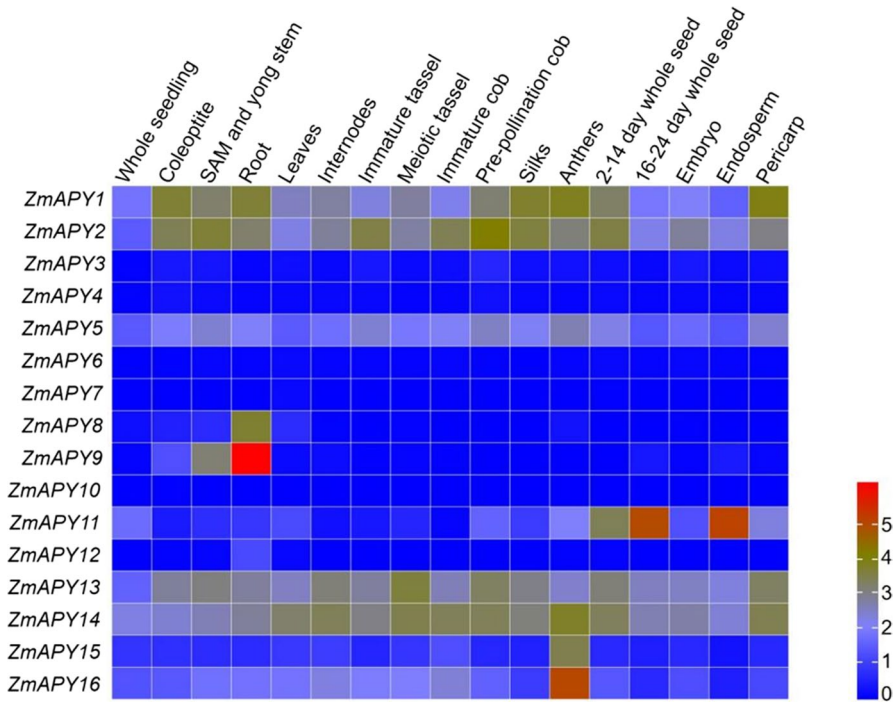


Fig. 3 Expression analysis of *ZmAPY* genes in different tissues The genes were labeled on the left and the tissues were displayed at the top of each column. The gene expression values are reported as square root transformations of the fragments per kilo bases per million mapped reads (FPKM). Different colors in map represent FPKM values, as shown in the bar to the right

days, while the control group is watered normally. Leaf samples are collected for RNA extraction to analyze *ZmAPY* genes expression levels. For heat and cold treatments, three-leaf stage maize seedlings were placed in growth chambers at 50°C and 4°C for 4 h and 24 h, respectively, while the control group continues growing at 28°C. Leaf samples are then collected for RNA extraction to evaluate *ZmAPY* genes expression level.

Identification and Phylogenetic analysis of the *ZmAPY* genes in maize

Gene ID of all *APY* genes in *Arabidopsis thaliana* and *Oryza sativa* was collected previous study (Chowdhury et al. 2023), protein sequences of these APYs were downloaded from Ensemble Plants (<http://plants.ensembl.org/>) and aligned for constructing HMM model. Protein sequences for maize (*Zea mays*) were also collected from Ensemble Plants, and *ZmAPY* genes were identified using HMMsearch with default parameters. Proteins identified by HMMsearch that lacked conserved domains were removed after submission to SMART (<https://smart.embl.de/>). Subsequently, MEGA X was used for multiple sequence alignment and phylogenetic

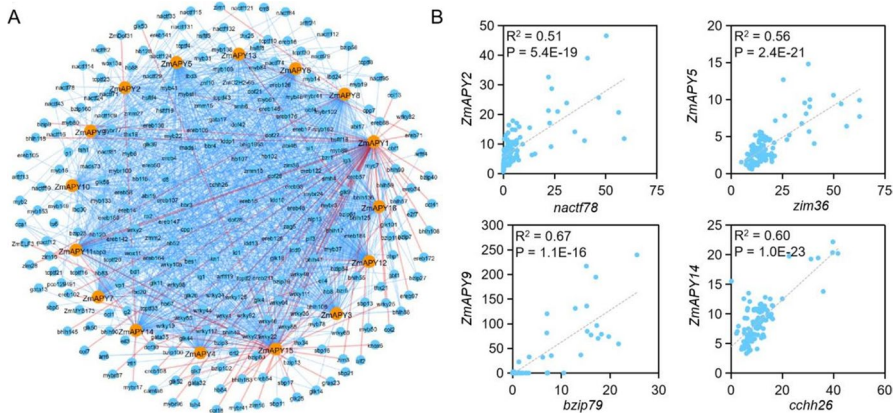


Fig. 4 Prediction of upstream regulators of *APY* genes **A** Upstream regulators of *ZmAPYs* were predicted by ChIP-seq data of 104 transcription factors and planttfdb software. Orange dots represent *ZmAPYs* and blue dots represent potential upstream regulators of *ZmAPY* genes. The red line connects the transcription factors and *ZmAPY* genes that may have regulatory relationship analyzed by ChIP-seq data, while the blue line connects the transcription factors and *ZmAPY* genes that may have regulatory relationship predicted by planttfdb software. **B** Correlation analysis between the expression levels of potential upstream regulators *nactf78*, *zim36*, *bzip79*, *cchh26* and *ZmAPYs*. The *P* values were calculated by test of correlation coefficient

tree construction of the identified 18 protein sequences with those from rice and *Arabidopsis thaliana*'s *APY* proteins. The tree was built using Neighbor-Joining (NJ) method, pair-wise deletion, and bootstrap value set to 1000. As a result, 16 genes were identified as *ZmAPY* genes based on the evolutionary tree and named as *ZmAPY1* -*ZmAPY16*, respectively. The Synteny analysis of *ZmAPY* genes was employed by MCScanX by using default parameter.

Prediction of physicochemical properties of *ZmAPY* proteins

To obtain data such as the number of amino acids, molecular weight, isoelectric point, and average hydrophobicity index of the *ZmAPY* proteins, a physicochemical property analysis of *ZmAPY* protein sequences was employed by using an online tool ProtParam on ExPASy (<https://web.expasy.org/protparam/>). Gene structure analysis was performed by GSDS2.0 (<https://gsds.gao-lab.org/>). The subcellular localization of *ZmAPY* proteins were predicted by WoLF PSORT (<https://wolfpsort.hgc.jp/>) and CELLO (<http://cello.life.nctu.edu.tw/>).

Expression analysis of *ZmAPY* genes in different tissues

The expression data for the *ZmAPY* genes were obtained from various tissues, including seedling, roots, leaves, seeds, shoot apical meristems, internodes, tassel, cob, coleoptile, pericarp and anthers. These data were sourced from the qTeller

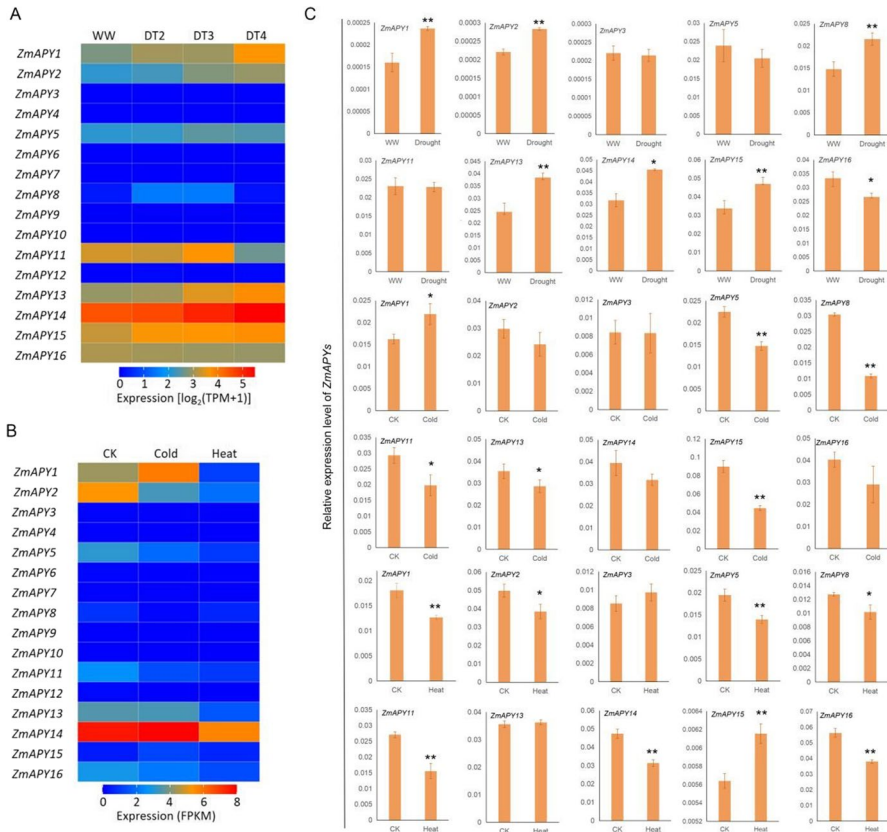


Fig. 5 Expression analysis of *ZmAPYs* under drought, cold, heat stresses **A** Expression analysis of *ZmAPY* genes under three different drought degree stresses. **B** Expression analysis of *ZmAPY* genes under cold and heat stresses. The genes were labeled on the left and the abiotic stresses were displayed at the top of each column. The FPKM / TPM values of the *ZmAPY* genes were used to draw a heat map by using HEMI. **C** qRT-PCR in analyzing the expression of *ZmAPYs* under drought, cold and heat stress. Statistical significance was determined by Student's *t* test: ** $P < 0.05$ and *** $P < 0.01$

(<https://qteller.maizegdb.org/>). The FPKM values of the *ZmAPY* genes were used to draw a heat map by using HEMI (Deng et al. 2014).

Upstream transcription factor prediction of *ZmAPY* genes

The 2kb upstream sequences of each *ZmAPY* gene were obtained from the maize genome for analysis. The PlantTFdb (<https://plantfdb.gao-lab.org/>) and jbrowse on maizeGDB (<https://jbrowse.maizegdb.org/>) were used to analyze the retrieved sequences and identify. All predicted upstream transcription factors were visualized by using Gephi (<https://gephi.org/>). Then expression correlation coefficients between potential upstream transcription factors and *ZmAPY* genes were calculated by using the expression profiles collected from qTeller.

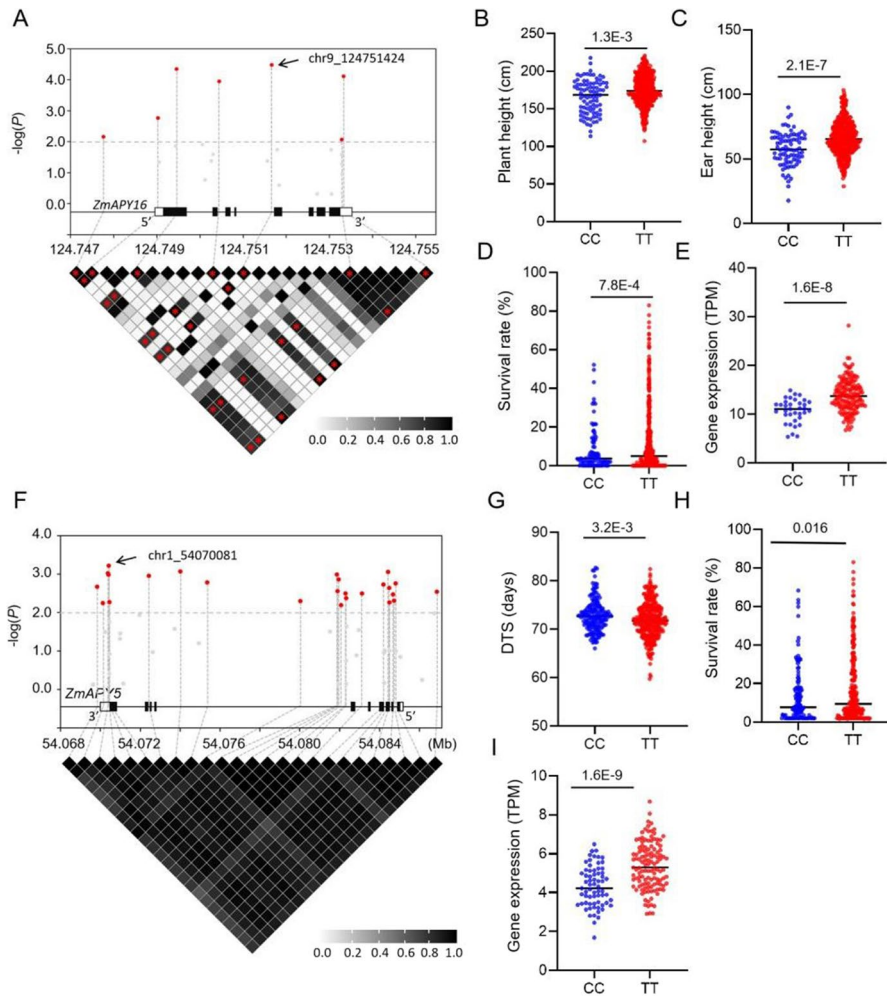


Fig. 6 *ZmAPYs* was associated with agronomic traits and drought resistance of maize **A**. Association of SNPs from *ZmAPY16* with plant height. chr9_124751424 is most significantly associated with plant height compared with other SNPs. SNPs significant associated with plant height ($p < 0.01$) were marked in red. **B-E** Genetic variation of *ZmAPY16* (chr9_124751424) regulates plant length (**B**), ear-height (**C**), drought resistance (**D**) and *ZmAPY5* expression (**E**) in maize. **F** Association of SNPs from *ZmAPY5* with day to silking (DTS). Chr1_54070081 is most significantly associated with DTS compared with other SNPs. SNPs significant associated with DTS ($p < 0.01$) were marked in red. **G-I** Genetic variation of *ZmAPY5* (chr1_54070081) regulates silking period (**G**), drought resistance (**H**) and *ZmAPY5* expression (**I**) in maize. Statistical significance was determined by Student's *t* test

Analysis of *ZmAPY* genes expression in maize under abiotic stresses

Expression profiles data of *ZmAPY* genes under well-water and drought treatment were collected from qTeller and previous study (Zhang et al. 2019). The FPKM / TPM values of the *ZmAPY* genes were used to draw a heat map by using

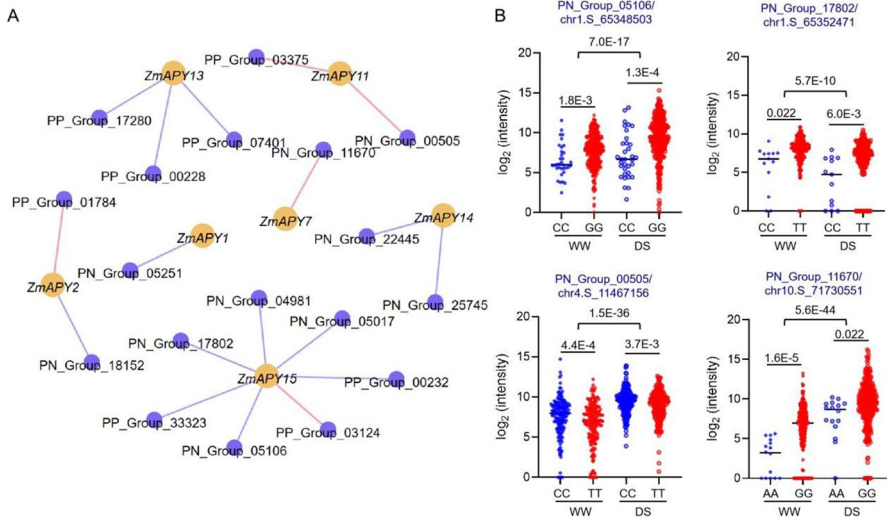


Fig. 7 Genetic variation in the *ZmAPYs* regulate the content of drought-induced metabolites **A** Co-localization analysis of mQTLs with *ZmAPY* genes. The red lines represent the collinearity relationship of mQTLs under drought stress with *ZmAPY* genes, while the blue lines represent the collinearity relationship of mQTLs under control conditions with *ZmAPY* genes. **B** Genetic variations in the *ZmAPY* gene region regulate the contents of metabolites induced by drought. Statistical significance was determined by Student's *t* test

HEMI. For qPCR validation, the FastPure Universal Plant Total RNA Isolation kit (Vazyme) was employed for RNA extraction. HiScript III qRT SuperMix for qPCR (Vazyme) was used for reverse transcription to synthesize cDNA. Specific primers were designed for the 16 *ZmAPY* genes and utilized for qRT-PCR analysis. The qRT-PCR reactions were performed using the SYBR Green master mix (ChamQ SYBR qPCR Master Mix, Vazyme). Three independent replicates were performed for each treatment. The primers used are described in Table S5.

Association analysis of *ZmAPY* genes

Association analysis for *ZmAPYs* was performed by using a maize association mapping population containing 540 inbred lines in a previous study (Liu et al. 2017). Among 1,227,480 high-quality SNP data with minor allele frequency (MAF) ≥ 0.05 , 502 SNPs were found in the gene region of all 16 *ZmAPYs*. maize agronomic traits and drought tolerance trait were also collected from previous studies (Yang et al. 2014). The MLM model were chosen to detect the SNPs significantly associated with maize agronomic traits and drought tolerance by using the TASSEL5.0 program (Bradbury et al. 2007).

Results

Identification of maize *APY* genes

Totally, we identified 16 *ZmAPY* family members from the Ensembl Plant database (<https://plants.ensembl.org/index.html>), named *ZmAPY1-ZmAPY16* (Table S1). Their physicochemical properties, including gene ID, protein size, molecular weight (MW), isoelectric point (pI), the grand averages of hydrophobicity (GRAVY), instability index and localization prediction, were characterized and shown in Table S1. The *ZmAPY* proteins varied in length from 81 to 701 amino acids, with molecular weights ranging from 8.74822 kDa to 76.9779 kDa. Isoelectric points ranged from 4.70 to 11.62 acidic. Most *ZmAPY* proteins were hydrophilic (GRAVY < 0) except for *ZmAPY6* and *ZmAPY7*, which were hydrophobic (GRAVY > 0). The estimated instability index ranged from 30.91 to 72.05. Subcellular localization predictions indicated that nine *ZmAPY* proteins were likely localized in the chloroplast, four in the plasma membrane, three in the mitochondria, one in the extracellular space, and one in the nucleus (Table S1), indicating diverse functional roles for these *ZmAPY* genes.

To explore the phylogenetic relationships of *ZmAPYs* with other species, a phylogenetic tree was constructed, incorporating 16 *ZmAPYs*, 7 *AtAPYs*, and 9 *OsAPYs* (Fig. 1A; Table S2). The phylogenetic tree topology classified these *APY* proteins into three Groups: Group I, II, III. In addition, there are two *APY*-like proteins (*ZmAPYL1*, *ZmAPYL2*) in maize that do not belong to Group I-III. Therefore, we classify them as an outgroup (Fig. 1A, Table S2). The expansion of *APYs* in maize, compared to *AtAPYs* and *OsAPYs*, suggests the potential importance of this gene family to regulate biological processes.

Distribution and collinearity analysis of maize *APY* genes

To investigate features of the *ZmAPYs* gene family, we analyzed the chromosome distribution of each *ZmAPY* gene. Our investigation showed that the *ZmAPY* genes in the maize genome were unevenly distributed across all 9 chromosomes, with the exception of chromosome 8 (Fig. 1B). The number of *APY* genes varied on each chromosome. Specifically, there is a single *APY* gene located on chromosomes Chr5, Chr6, Chr7, and Chr9. Chromosomes Chr2, Chr3, and Chr10 each contain two *APY* genes, while chromosomes Chr1 and Chr4 each have three *APY* genes. Gene duplications play a crucial role in the expansion of gene families (Konrad et al. 2011). Segmental duplications lead to the presence of large repetitive chromosomal blocks in the genome and are often associated with chromosomal rearrangements and polyploid events (Lallemand et al. 2020). Collinearity analysis indicated the occurrence of three segmental duplication events involving five *ZmAPY* genes across the maize genome, while no tandem duplications were observed (Fig. 1B).

APY gene structures and predicted protein motifs

Structural differences in exon–intron arrangement serve as sources of gene family variation and species diversity, leading to alterations in gene expression and function. To investigate the conservation and diversity of gene structure within the maize APY gene family, the exons and introns of 16 APY genes were analyzed based on their coding sequences and genomic data. The number of exons varied among the APY genes, ranging from 1 to 10 (Fig. 2A). Most members contained 6–10 exons, with two members in Group I (*ZmAPY5* and *ZmAPY6*) and one member in Group III (*ZmAPY13*) having only 1 and 2 exons, respectively (Fig. 2A). The number of introns ranged from 0 to 9. Two transposon insertions of 9.56 kb and 33.57 kb were identified in the intron regions of *ZmAPY5* and *ZmAPY10*, respectively. *ZmAPY* genes with collinearity exhibited similar gene structures. Additionally, members within the same subgroup typically displayed similar motifs and lengths, suggesting functional similarities (Fig. 2A). Analysis of the protein sequences of all *ZmAPY* gene family members revealed a conserved GDA1_CD39 domain in all proteins, with varying numbers of transmembrane regions. Specifically, members of Group I had one transmembrane region, most members of Group II had two transmembrane regions, and family members of Group III had no transmembrane regions (Fig. 2B). This structural variation may contribute to the functional distinctions observed among different subgroups.

Expression patterns of APY genes in maize

The investigation into tissue-specific gene expression patterns provides valuable insights into the potential biological roles of the *ZmAPY* genes. Analysis of the expression patterns within the *ZmAPY* gene family revealed distinct expression profiles among different members (Fig. 3), highlighting their diverse functions. Specifically, *ZmAPY8*, *ZmAPY9*, and *ZmAPY12* exhibited specific expression in roots, indicating a potential role for these genes in root-related processes. On the other hand, *ZmAPY15* and *ZmAPY16* showed significantly higher expression levels in anthers compared to other tissues, suggesting their involvement in anther-related functions. Moreover, there was a notable trend of high expression of *ZmAPY11* in seeds and endosperm, implying a potential role in seed development and maturation. Conversely, minimal to no detectable expression was observed for *ZmAPY3*, *ZmAPY4*, *ZmAPY6*, and *ZmAPY7* across all tissues and organ. Interestingly, similar tissue-specific expression patterns were observed between collinear *ZmAPY* genes, such as *ZmAPY1/ZmAPY2/ZmAPY5* and *ZmAPY15/ZmAPY16*, suggesting potential functional conservation or shared regulatory mechanisms among these gene clusters. The differential tissue-specific expression patterns observed among the *ZmAPY* genes indicate their diverse biological roles and potential contributions to various developmental processes and physiological functions in maize.

Prediction of upstream regulators of *APY* gene

The analysis of variations in expression patterns among different *ZmAPY* genes has led to the identification of potential upstream regulators that may control *APY* gene transcription in maize. By utilizing plantfdb software and existing ChIP-seq data of 104 transcription factors (Tu et al. 2020), a total of 251 upstream regulators were predicted (Fig. 4A, Table S3). Subsequently, the correlation between the expression levels of these predicted regulators and *ZmAPY* gene expression was examined. Significantly, NACTF78, ZIM36, bZIP79, and CCHH26 displayed strong correlations with the transcription levels of *ZmAPY2* ($R^2=0.51$, $P=5.4 \times 10^{-19}$), *ZmAPY5* ($R^2=0.56$, 2.4×10^{-21}), *ZmAPY9* ($R^2=0.67$, $P=1.1 \times 10^{-16}$), and *ZmAPY14* ($R^2=0.60$, $P=1.0 \times 10^{-23}$), respectively (Fig. 4B). Notably, NACTF78 has been previously reported to regulate Fe concentrations in maize kernels, potentially enabling the cultivation of maize varieties with both high yield and high Fe concentrations in their kernels using a molecular marker in the *NACTF78* promoter (Yan et al. 2023). Additionally, Vélez-Bermúdez et al. reported that ZML2 (ZIM36) regulates wound-induced lignin genes in maize (Vélez-Bermúdez et al. 2015), while ZmTGA9-1 (bZIP79) has been shown to regulate male sterility in maize (Jiang et al. 2021). The observed correlations between the expression levels of *ZmAPY* genes and these transcription factors suggest that *ZmAPY* genes may also play a role in regulating these biological processes, indicating a potential link between *APY* gene expression and the modulation of Fe concentrations, lignin gene regulation, and male sterility in maize. These findings provide valuable insights into the regulatory network involving *ZmAPY* genes and their upstream regulators in maize, shedding light on the diverse biological processes influenced by these genes.

Expression analysis of *ZmAPYs* under drought, cold, heat stresses

To investigate the potential role of *APY* genes in regulating maize abiotic stress responses, we analyzed the transcription levels of *APY* genes under drought, cold, and heat stress using RNA-seq data from the maize inbred line B73. Our findings revealed the transcription of the *ZmAPY* gene is responsive to drought, cold, and heat stress, displaying distinct response profiles (Fig. 5A; 5B). Subsequently, we validated the transcription of *ZmAPY* genes under drought, cold, and heat stress through qRT-PCR. The results revealed significant upregulation of 6 *ZmAPY* genes, *ZmAPY1*, *ZmAPY2*, *ZmAPY8*, *ZmAPY13*, *ZmAPY14* and *ZmAPY15* under severe drought (DT4), while the transcription of *ZmAPY15* was suppressed by drought (Fig. 5C). Cold stress induced the transcription of *ZmAPY1* but inhibited the transcription of *ZmAPY5*, *ZmAPY8*, *ZmAPY11*, *ZmAPY13* and *ZmAPY15* (Fig. 5C). Moreover, heat stress significantly inhibited the transcription of 7 *ZmAPY* genes, *ZmAPY1*, *ZmAPY2*, *ZmAPY5*, *ZmAPY8*, *ZmAPY11*, *ZmAPY14*, and *ZmAPY16*, while the transcription of *ZmAPY15* was induced by heat (Fig. 5C). It is noteworthy that some of the expression analysis results for the *ZmAPY* genes are absent from Fig. 5C due to their expression levels falling below the detection threshold of qRT-PCR. The

stress-responsive expression patterns of *ZmAPY* genes suggest their potential regulatory roles in drought, cold, heat, and salt stress responses.

***ZmAPYs* was associated with agronomic traits and drought resistance of maize**

To further investigate the impact of *APY* on maize agronomic traits and drought resistance, we examined the relationship between SNPs in the *ZmAPY* gene region and 17 agronomic traits as well as drought phenotypes using the MLM model. Our analysis revealed a significant association between the SNP (chr9_124751424, CC/TT) in *ZmAPY16* and maize plant height (Fig. 6A). Subsequent analysis indicated notable differences not only in plant height but also in ear height, drought resistance, and *ZmAPY16* expression between the "CC" and "TT" genotypes. Plants with the "TT" allele, showing high *ZmAPY6* expression, exhibited greater plant height, ear height, and drought survival rates compared to those with the "CC" allele (Fig. 6B-E), suggesting a positive regulatory role of "TT" allele in maize plant height, ear height, and drought resistance. Additionally, we observed a significant association between the SNP (chr1_54070081, CC/TT) in *ZmAPY5* and the spinemaking period in maize (Fig. 6F). Plants with the "TT" allele exhibited lower *ZmAPY5* expression levels, delayed flowering, and increased drought survival compared to those with the "CC" allele (Fig. 6G-I), indicating a negative regulation of maize spinemaking and a positive regulation of maize drought resistance by *ZmAPY5* "TT" allele.

Genetic variation within *ZmAPYs* regulate the content of drought-induced metabolites

Metabolites, as small molecules that serve as the end products of metabolic processes and physiological pathways, are known to play crucial roles in plant drought resistance (Kim et al. 2017; Todaka et al. 2017). These compounds can act as osmo-protectants, antioxidants, signaling molecules, and regulators of various stress-responsive pathways in plants (Nakabayashi et al. 2014; Obata et al. 2013; Fàbregas et al. 2018). Analyzing the genome-wide metabolite profiles of 385 maize natural inbred lines grown under well-watered and drought-stressed conditions (Zhang et al. 2021a), we identified metabolite quantitative trait loci (mQTL) for 18 metabolites that co-located with the *ZmAPY* genes, indicating a potential relationship between *ZmAPYs* and the levels of these metabolites (Fig. 7A, Table S4). Further investigation into these metabolites revealed that four drought-induced metabolites were influenced by genetic variations within the *ZmAPYs* gene region. Specifically, genetic variations chr1.S_65348503 and chr1.S_65352471 within the *ZmAPY15* gene region were found to regulate the contents of metabolites PN_group_05106 and PN_group_17082, while another genetic variation chr1.S_65352471 within the *ZmAPY11* gene region was associated with the regulation of the metabolite PN_group_00505. Additionally, a genetic variation chr10.S_71730551 within the *ZmAPY7* gene region was linked to the regulation of the metabolite PN_group_11670 (Fig. 7B). These findings suggest that *ZmAPY* genes may impact maize drought resistance by modulating the contents of these drought-induced

metabolites. This insight highlights the potential role of *ZmAPY* genes in mediating maize response to drought stress through the regulation of key metabolites involved in stress adaptation and tolerance.

Discussion

Apyrase (APY) is widely existed in eukaryotes and is highly conserved throughout the evolution of eukaryotes. In plants, apyrase genes have been reported to regulate a variety of biological processes, including root hair development, stomatal movement, and defense responses (Clark et al. 2014; Lim et al. 2014; Wu et al. 2007). However, no apyrase genes have been identified in maize, and their functions remain unclear. In this study, through sequence alignments and phylogenetic analysis, we identified 16 apyrase genes in maize genome and further divided these genes into three subgroups (Fig. 1A). The number of group I *ZmAPY* genes far exceeds the number of group I apyrase genes in *Arabidopsis* and rice that identified in a previous study (Clark et al. 2014; Chowdhury et al. 2023). Syntenic analysis revealed that most *ZmAPY* genes are of the dispersed repeat type, but segmental duplication also played a significant role in the expansion process of group I *ZmAPY* genes (Fig. 1B). Meanwhile, genes with synteny show higher similarity in expression patterns (Fig. 3). These results suggest that *APY* genes in maize may have produced functional redundancy through expansion, and since apyrase plays an important regulatory role in plant growth and development and stress responses, the expansion and functional redundancy of gene subgroups generated by segmental and dispersed duplication may enhance the robustness of the maize regulatory network.

The members of the *ZmAPY* genes are predicted to encode proteins ranging from 81 to 701 amino acid in length, and the PI ranging from 4.70 to 11.62 (Table S1). These results suggest that the differences among *ZmAPY* genes may be more pronounced than those in rice (in which *OsAPY* proteins ranging from 451 to 702 amino acid in length, and the PI ranging from 5.44 to 9.34) (Chowdhury et al. 2023). The predicted gene structures of the *ZmAPY* genes contain one exon to ten exons (Fig. 2A), similar to the gene structures in rice, with two to twelve exons (Chowdhury et al. 2023). Similar to *OsAPY* proteins, the *ZmAPY* proteins were predicted to localize to various cellular compartments, including the chloroplast, mitochondrial, plasma membrane, cytoplasm, nuclear and extracellular (Table S1). In summary, *ZmAPY* genes share similar characteristics with apyrase in rice, but exhibits greater diversity among family members, suggesting that although *ZmAPY* genes maintain functional conservation, they may also have undergone functional divergence.

Transcription factors are core elements that regulate transcriptional levels during various stages of plant life processes. Analysis of the promoter regions of all *ZmAPY* genes revealed the presence of diverse upstream transcription factors which may regulate *ZmAPY* genes expression (Fig. 4). Among the 251 potential upstream transcription factors, several genes have been reported to regulate maize growth and stress response progresses (Table S3). For example, *WRKY48 / ZmWRKY40* was predicted to binding the promoter sequences of seven *ZmAPY* genes, overexpression *ZmWRKY40* could enhance drought tolerance in transgenic *Arabidopsis* by

regulating stress-related genes, and the reactive oxygen species (ROS) content in transgenic lines was reduced compared with wild-type plants under drought stress (Wang et al. 2018). ZmBES1/BZR1-5 was predicted to bind the promoter sequences of six *ZmAPY* genes, ZmBES1/BZR1-5 decreases ABA sensitivity and confers tolerance to osmotic stress in transgenic *Arabidopsis*. Meanwhile, ZmBES1/BZR1-5 can also positively regulates kernel size (Sun et al. 2020, 2021). A bZIP transcription factor *id1* was predicted to binding the promoter sequences of ten *ZmAPY* genes, previous study showed that *id1* can regulate maize flowering time and floral inductive signals (Colasanti et al. 1998; Muszynski et al. 2006). These results further suggested potential roles of *ZmAPY* genes in maize growth and stress response progresses.

Maize is a world major food crop, and its yield is seriously threatened by abiotic stresses (Lesk et al. 2016). Although a large number of abiotic stress response genes and loci have been identified in recent years through genetic and molecular biology techniques, due to limited understanding of the plant abiotic stress response system, only a few genes have been successfully applied in commercialization (Zhang et al. 2020). Therefore, it remains essential to further elucidate the abiotic stress response system of maize. Previous studies have shown that the extracellular ATP (eATP) level rises when plants are subjected to stress, leading to the production of reactive oxygen species (ROS) and cell death. Apyrase can regulate the production of ROS by hydrolyzing eATP, and participate in various stress response processes in plants (Deng et al. 2015). By analyzing the transcriptomic data from previous studies, we investigated the expression patterns of *ZmAPY* genes under different abiotic stresses (Makarevitch et al. 2015; Zhang et al. 2019). The results showed that seven *ZmAPY* genes responded to drought stress, six to cold stress, and eight to heat stress, and the stress responsive *ZmAPY* genes were further confirmed by qPCR (Fig. 5C). These results indicated that, like other plants, *ZmAPY* genes could also be involved in regulating maize's abiotic stress response. Of course, more researches are required to clearly elucidate the function of *ZmAPY* genes in maize abiotic stress responses.

Natural variations significantly affect maize agronomic traits and stress resistance, and numerous elite alleles have been identified through genetic approaches which significantly accelerated the breeding process (Xiao et al. 2017). To explore the potential application value of *ZmAPY* genes and their genetic variations in maize breeding, we collected drought tolerance and agronomic traits data of maize association panels published in previous study (Zhang et al. 2019; Yang et al. 2014), and utilized high-density SNP markers to perform association analyses on the *ZmAPY* gene and its flanking regions. The result indicated that natural variations in *ZmAPY16* gene and flanking region were significantly associated with maize plant height (Fig. 6A), further analysis revealed that these variations were also correlated with the *ZmAPY16* expression and maize drought tolerance (Fig. 6B-E). Additionally, we found that *ZmAPY5* was significantly associated with maize silking time (Fig. 6F), and associated variations were also correlated with the *ZmAPY5* expression and maize drought tolerance (Fig. 6G-I). These findings suggest that variations of *ZmAPY* gene expression may influence maize agronomic traits and drought tolerance. Furthermore, since many metabolic pathways involve energy conversion, while apyrase can affect the levels of ATP/ADP. To investigate the effects of apyrase

on maize metabolites, we performed co-localization analysis between *ZmAPY* variations and previously reported metabolite regulation sites (Zhang et al. 2021a), which revealed that seven *ZmAPY* genes were co-localized with 18 metabolite regulation sites (Fig. 7A). Some of these metabolites showed significant content change under drought stress (Fig. 7B). Although the functions of these metabolites are not well understood at present, these results suggest that *ZmAPY* genes and their genetic variations may participate in maize drought response by affecting the levels of drought-responsive metabolites. In summary, we revealed the variations of *ZmAPY* gene expression among maize populations, which were associated with multiple important maize traits. These results indicate that *ZmAPYs* and their genetic variations may have potential applications in maize breeding.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval We declare that these experiments comply with the ethical standards in China.

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