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Genome-wide identification, phylogenetic and biochemical analysis of the APYRASE family members, and gene expression analysis in response to the abiotic and biotic stresses in bread wheat (*Triticum aestivum*)

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*APYRASE*s, which directly regulated the intra- and extra-cellular ATP homeostasis, plays a pivotal role in the regulation of the adaptations to various stresses in mammals, bacteria and plants. In the present study, we identified and characterized the wheat *APYRASE* family members at the genomic level. The results showed that a total of eight APY homologs with conserved ACR domains were identified. The wheat APYs were further analyzed bioinformatically of their sequence alignment, phylogenetic relations and conserved motifs. Although they share highly conserved secondary structure and tertiary structure, the wheat APYs could be mainly categorized into three groups, according to the phylogenetic and structural analysis. Further, these APYs exhibited similar expression patterns in the root and shoot, among which *TaAPY3-1* and *TaAPY3-3* had the highest expression level. The time-course expression patterns of the eight *APY*s in the wheat seedlings in response to the biotic stress and abiotic stress were also investigated. *TaAPY3-2*, *TaAPY3-3*, and *TaAPY6* exhibited strong sensitivity to all kinds of stresses in the leaves. Some *APY*s showed specific expression responses, such as *TaAPY6* to the heavy metal stress, and *TaAPY7* to the heat and salt stress. These results suggested that the stress-inducible *APY*s could have potential roles in the regulation of the adaptation to the environmental stresses. Moreover, the catalytic activity of TaAPY3-1 was further analyzed in the in vitro system. The results showed that TaAPY3-1 protein exhibited high catalytic activity in degradation of ATP and ADP, but not GTP, CTP and TTP. It also has an extensive range of temperature adaptability, but rather preferred relative acid pH conditions. In this study, the genome-wide identification and characterization of the *APY*s in wheat could be

useful for further genetic modifications to generate high-stress tolerant wheat cultivars.

Genome-wide identification, phylogenetic and biochemical analysis of the APYRASE family members, and gene expression analysis in response to the abiotic and biotic stresses in bread wheat (*Triticum aestivum*)

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ABSTRACT

*APYRASE*s, which directly regulated the intra- and extra-cellular ATP homeostasis, plays a pivotal role in the regulation of the adaptations to various stresses in mammals, bacteria and plants. In the present study, we identified and characterized the wheat *APYRASE* family members at the genomic level. The results showed that a total of eight APY homologs with conserved ACR domains were identified. The wheat APYs were further analyzed bioinformatically of their sequence alignment, phylogenetic relations and conserved motifs. Although they share highly conserved secondary structure and tertiary structure, the wheat APYs could be mainly categorized into three groups, according to the phylogenetic and structural analysis. Further, these APYs exhibited similar expression patterns in the root and shoot, among which *TaAPY3-1* and *TaAPY3-3* had the highest expression level. The time-course expression patterns of the eight *APY*s in the wheat seedlings in response to the biotic stress and abiotic stress were also investigated. *TaAPY3-2*, *TaAPY3-3*, and *TaAPY6* exhibited strong sensitivity to all kinds of stresses in the leaves. Some *APY*s showed specific expression responses, such as *TaAPY6* to the heavy metal stress, and *TaAPY7* to the heat and salt stress. These results suggested that the stress-inducible *APY*s could have potential roles in the regulation of the adaptation to the environmental stresses. Moreover, the catalytic activity of TaAPY3-1 was further analyzed in the in vitro system. The results showed that TaAPY3-1 protein exhibited high catalytic activity in degradation of ATP and ADP, but not GTP, CTP and TTP. It also has an extensive range of temperature adaptability, but rather preferred relative acid pH conditions. In this study, the genome-wide identification

and characterization of the *APY*s in wheat could be useful for further genetic modifications to generate high-stress tolerant wheat cultivars.

INTRODUCTION

The environmental stresses, such as salt, cold, drought and the pathogens would cause significant damage to the plant cells, which could lead to disturbed intra- and extra-cellular ATP homeostasis (Cao et al., **2014**). The cellular ATP level was tightly controlled by the GDA1-CD39 nucleoside phosphatases, which were widely existed in the plants, animals, fungi and bacteria (Matsumoto et al., **1984;** Tong et al., **1993**). The APYRASEs (APYs), which were a class of nucleoside triphosphate diphosphohydolases (NTPDases), played an important role in maintaining NTP homeostasis. APYs can be generally divided into ecto- and endo-APY, according to their subcellular locations (Matsumoto et al., **1984;** Tong et al., **1993;** Thomas et al., 1999; Dunkley et al., **2004**). Ecto-apyrases were localized on cell surface while endo-apyrase were usually on Golgi, endoplasmic reticulum (ER) and intracellular vesicles (Leal et al., **2005**). Some ecto-apyrases had membrane-spanning domains at their N- and C-terminal, and endo-apyrases usually has glycosylation on the amino acid, which was important for its correct protein folding, membrane targeting, cellular allocation and enzyme activity (Smith and Kirley, 1999; Murphy and Kirley, **2003;** Wu et al., **2005;** Knowles, **2011**). Different from ATPases using Mg^{2+} as a co-factor, APYs can use variety of divalent, including Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (YangJ and Y, **2011**). The cellular ATP level not only plays a pivotal role for the energy supply, but also regulated various cellular processes related to the abiotic stress responses, including Na^{+}/H^{+} exchange, vacuolar Na+ distribution, K^{+} homeostasis, reactive oxygen (ROS) species regulation, and salt-responsive expression of K^{+}/Na^{+} homeostasis and plasma membrane reparation (Sun et al., **2012a**). Thus, the cellular ATP homeostasis regulated by the APYs was important for maintaining the normal cell function.

In plants, it had been reported that *APY*s participate in various processes, including growth regulation, and stress responses (Clark et al., **2014**). So far, seven *APY* members have been identified and functionally characterized in *Arabidopsis* (Tsan-Yu et al., 2015). AtAPY1 and AtAPY2, which were located at the Golgi, were involved in the regulation of many aspects of plant growth and development, such as pollen germination, root growth and stomata closure (Iris et al., **2003;** Jian et al., 2007; Tsan-Yu et al., 2015; Yang et al., **2015**). Although AtAPY1 and AtAPY2 were endo-APYs, the mutation of which could cause significant elevation of extracellular ATP (eATP) (Wu et al., **2007;** Hui et al., 2014), demonstrating that the intracellular located APYs could also regulate the eATP homeostasis. *AtAPY6* and *AtAPY7* also played pivotal roles in pollen development through the regulation of polysaccharides synthesis (Yang et al., 2013). Overexpression of *PeAPY2* in *Arabidopsis* led to significant cleavage of the reactive oxygen species (ROS) (Sun et al., **2012a**), which would make it more tolerant to the abiotic stresses. Recently in some other species, it had been suggested that the *APY*s were directly involved in the regulation of biotic and abiotic stress resistance, such as drought and salt tolerance in *Populus euphratic* (Sun et al., **2012b;** Shurong et al., **2015**), pathogen resistance in pea and tobacco (Shiraishi and Tomonori, **2013;** Sharma et al., **2014**), water logging response in soybean (Alam et al., 2010). These results demonstrated that APY played an important role in the regulation of stress adaptation. Nevertheless, the

molecular mechanism still largely remained unclear.

Due to the novel functions of APY in the stress responses, the identification and functional characterization of the *APY* family genes in the crops, were important for the improvement of the stress tolerance via genetic modifications using *APY*s as the molecular targets. So far in wheat, there is still in lack of the APY-related studies. Here, we firstly identified the *APY* members in wheat in the genomic level. A comprehensive characterization and phylogeny of the *TaAPY*s using the bioinformatic and biochemical methods were further performed. Meanwhile, the time-course expression pattern of these *APY*s genes in response to various abiotic and biotic stresses was investigated. Additionally, the in vitro enzymatic analysis was also performed to further prove the APY enzymatic activities in degradation of the NTPs. Conclusively, these results provided valuable insights in the bioinformatic and functional characteristics of the *APY* gene family in wheat, which would further benefit the molecular breeding aiming at generating the stress-tolerant wheat cultivars.

Materials and methods

Screening of gene sequences

The amino acid sequence of the seven *Arabidopsis thaliana* APYs, obtained from TAIR (https://www.arabidopsis.org/), were used to blast against the wheat transcriptome and genome databases (Appels et al., **2018**). The APY candidates were accepted only if the protein contains the conserved Apyrase Conserved Region (ACR) (Steinebrunner et al., **2000**).

Phylogenetic analyses of APYs

The protein sequences of APYs from other species were obtained from the NCBI database. The phylogenic analysis was carried out using the MEGA5 with maximum likelihood method with 1000 bootstrap replicates and other parameters were set as default (Jones et al., **1992;** Tamura et al., **2011**).

Structure analysis of TaAPYs gene and amino acid sequences

The DNAman software was used to analyze the conservation property of CDS and amino acid (AA) sequences. The secondary structures of these proteins were predicted using the online tool NPS@SOPMA (https://npsa-prabi.ibcp.fr/). The structure analysis included percentage of each amino acid, position and number of alpha helix, Beta Bridge, Random coli, as showed in different colors. The motif analysis was carried out using the MEME motif analysis. The membrane spanning motif method was analyzed using the online tool TMHMM (Jianyi et al., **2013b;** Jianyi et al., **2013a**). The 3D models of tertiary structures were simulated using the Swiss-Model (https://www.swissmodel.expasy.org/) which is based on the automatic ExPASy (Expert Protein Analysis System) web server (Guex et al., **2010;** Pascal et al., **2011 ;** Waterhouse et al., **2016;** Bertoni et al., **2017;** Waterhouse et al., **2018**).

Abiotic and biotic stress treatment.

Two-week-old wheat seedlings (Yangmai 158) were used for the stress treatment. 300

mM NaCl, 42℃, 200 mM $CdCl_2$, and 300 mM mannitol were separately used as salt, heat, heavy metal and drought treatment as previously described (Ni et al., **2018**). The wheat leaf and root were collected at 1, 3, 6, 12 and 24 h post treatment. The *Bgt* spores were inoculated on the wheat leaves as previously described. The leaf sample was taken at 24, 48, 72 and 96 h post inoculation. The samples were immediately frozen in the liquid nitrogen and stored at -80℃. Each sample was prepared with three biological replicates.

RNA isolation and quantitative real-time PCR

The Plant RNA kit (Omega, Shanghai, China) and TransScript one-step gDNA Removal and cDNA synthesis SuperMix (Transgen, Beijing, China) were separately used for RNA and cDNA synthesis. The qPCR was carried out using the SYBR Green PCR Kit (Qiagen, China) on the Lightcycler96 system (Roche, Swiss). Raw data were calculated using the software given in the Lightcycler96 system. The primer information was listed in the Table S3.

Vector construction, recombinant protein expression and protein purification

The CDS of *TaAPY3-1* with removed region of the membrane spanning domain, was cloned intro the pET22a vector. The expression vector was then transformed into the *Escherichia coli* BL21. The transformed cells were cultured in the Luria-Bertani (LB) broth at 4℃ till the OD600 reaches 0.8, and then induced with 0.5 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG). The induced cells were cultured at 37℃ for 5 hours. The cells were then harvested by centrifugation at 10000×g and resuspended with 20 ml lysis buffer (20 mM Tris, 500 mM NaCl, pH 7.6). The cells were then lysed by sonication as previously described (Ye et al., **2015**).

The recombinant protein was further isolated from the denatured inclusion body by using the Inclusion Body Protein Extraction Kit (Shengong, Shanghai, China). Briefly, the denatured protein was renatured by urea gradient dialysis in 500 ml renaturing buffers (10 mM Tris–HCl, 100 mM NaCl, 2 mM reduced glutathione, 0.2 mM oxidized glutathione, pH 8.5). The concentrations of urea in the renaturing buffers were 6.0, 4.0, 2.0, 1.0, and 0 M, respectively. Every dialysis step was carried out at 4°C for 12 h. After the refolding process, the insoluble protein was removed by centrifugation at 10,000×g for 30 min at 4°C. The amount and purity of recombinant protein were assessed by using the BCA Protein Assay Kit (Shengong, Shanghai, China).

Protein catalytic activity assay

The reaction system was set as: 50 mM Tris-HCl, 8 mM $CaCl_2$, 0.25 ng/ul BSA, 2.5 mM DTT, 150 mM NaCl, and 0.05% Tween 20 (100 ul) as previously described (Dong et al., **2012**). Then, 10 mM ATP (or ADP, TTP, CTP and GTP) and 4 ug purified recombinant APY was added into this system. The catalytic activity was analyzed under different conditions (Temperature, pH, substrates, and ions) by using the Phosphate Assay Kit (Jiancheng, Nanjing, China).

Results

Genome-wide identification and phylogenetic analysis of the APY family members

in wheat

The protein sequences of the seven *Arabidopsis* APYs and the conserved ACR domains were used as the query sequences to BLAST against the recently published wheat genome and transcriptome database (Appels et al., **2018**). After careful validation of the candidates, a total of 24 APY members were identified with top hits for the AtAPY homologs (AtAPY1-7) in the wheat genome. These wheat APY candidates exhibited high sequence similarity and all contained five Apyrase Conserved Region (ACR) domains (Figure S1). The twenty four wheat APYs were further divided into eight groups, each group with three homologs located at different genome sets (A, B and D) (Table 1). The CDS information of these APYs was listed as supplementary Table S1. Based on the sequence similarity to the *Arabidopsis APY* homologs, the identified wheat *APY*s were separately named as *TaAPY1*, *TaAPY2*, *TaAPY3-1*, *TaAPY3-2*, *TaAPY3-3*, *TaAPY5*, *TaAPY6* and *TaAPY7*. As show in Table 1, the *APY* genes were predicted to encode 430 to 706 amino acids in length, with putative molecular weights ranging from 46.446 to 77.557 kD, and the protein isoelectric points (PIs) from 5.93 to 9.2 (Table. 1). As the three copies of the APYs in different wheat genome set (A, B and D) had very high CDS sequence similarity (Table 1), thus the APYs from the genome set A were used in the following bioinformatic and biochemical analysis.

To investigate the evolutionary relationships among the APYs, a phylogenetic tress was constructed using the APY homologs from *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Aegilops tauschii*, *Phoenix dacylifera*, and some other species (Table S2). The APYs can be mainly divided into three distinct groups (I, II, and II). Specifically, TaAPY1, TaAPY2, TaAPY3-1, TaAPY3-2 and TaAPY3-3 in Group I, TaAPY5 and TaAPY6 were clustered in Group II, and TaAPY7 in Group III (Figure 1).

Gene structure and conserved motif analysis of the APY genes in wheat.

To investigate the structural diversity of the APYs, the online structural analysis tool NPS @ SOPMA (Deléage, **2017**) was used for conserved motif analysis. As showed in Figure 2, the amino acid sequences of those eight APYs were highly conserved. Additionally, a total of 16 motifs can be detected among the APYs (Figure S2). Generally, the eight APYs all contained motif 1, 3, 5 and 8 (Figure 2). APYs in the same group had specific motifs, such as motif 12 to TaAPY5 and 6 (Group II), motif 8 to TaAPY3-1 and 3-3 (Group I), motif 15 to TaAPY1 and 2 (Group I), and motif 6 to TaAPY 1, 2, 3-1, 3-2 and 3-3 (Group I) (Figure 2). Further, the membrane spanning motif (MSM) analysis showed that all the Group I APYs (TaAPY1, 2, 3-1, 3-2 and 3-3) were predicted to contain only one MSM at N-terminal, whereas Group II APYs (TaAPY5 and 6) had two MSMs, separately located at N- and C- terminal (Figure 3). Interestingly, it was predicted that the Group III APY (TaAPY7) contained three MSMs (Figure 3). As comparison, the MSM of the seven APY members from *Arabidopsis* were also analyzed. The results showed that except AtAPY5 and 7, which separately had two and three MSMs, the others only exhibited one MSM at the N-terminal, which was similar to the wheat APYs (Figure S3). The membrane-spanning was closely related to the protein allocation and transportation. Thus these results provided important information of their potential cellular roles. Moreover, the 3D structure analysis of the eight proteins showed that TaAPY5, 6 and 7 contains four subunits while other APYs only have two. Two similar subunits of the APY was linked with extended strand surrounded by alpha helix (Figure 4), which was a

signature character of GDA1-CD39 nucleoside phosphatase super family. Based on the results of 3D protein structure simulation (Figure 4), the eight wheat APYs can be also divided into two groups, Group I (TaAPY1, 2, 3-1, 3-2 and 3-3), and Group II (TaAPY5, 6 and 7). Although TaAPY7 and TaAPY5, 6 were categorized into different groups in the phylogenetic tress, they shared high 3D structure similarity (Figure 1).

Gene expression pattern of the APYs in response to the abiotic and abiotic stresses in the wheat seedlings

To investigate the expression pattern of the eight *APY*s in the wheat, the gene expression in shoot and root, and their responses to the abiotic stresses (salt, heat, heavy metal and osmotic stresses), were further analyzed by the quantitative real-time PCR. The results showed that all the eight *APY*s had similar expression level at the seedling shoot and root, with the highest expression level of *TaAPY3-1* and *TaAPY3-3*, and the lowest level of *TaAPY6* both in the shoot and root (Figure 5), suggesting *TaAPY3-1* and *TaAPY3-3* could be the predominant *APY*s in wheat.

Previous studies had shown that several *APY*s could be involved in the regulation of the abiotic stress adaptation (Clark et al., **2014**). Thus we further investigated the time-course expression profile of *APY*s in the leaf and root of the wheat seedlings in response to different abiotic stresses. The results showed that in the leaves, most of the *APY*s could be upregulated after subjected to the cadmium treatment (Figure 6A). Specifically, the expression of *TaAPY3-1* reached a peak at 6 h in the leaves. The expression of the other *APY*s in the root was not as sensitive as that in the stem, only *TaAPY6* exhibited a significant upregulation at 6 h (Figure 6A). Further, mannitol treatment was used to produce an artificial drought stress condition in the wheat seedlings. The results showed that, all the *APY*s could be up-regulated within 24 h, among which, *TaAPY1* and *TaAPY6* reached an extremely high expression level in the leaves (Figure 6B). As contrary, very few genes exhibited significant changes at different time post mannitol treatment in the root (Figure 6B), suggesting these *APY*s regulated the drought responses in the shoot, but only in the root. Under heat stress, not until at 12 h, the expression of *TaAPY7*, *TaAPY6*, *TaAPY5* and *TaAPY3-2* began to increase both in the leaves and root, with the highest increase fold of *TaAPY3-2* (Figure 6C). For salt stress, the expression of all the *TaAPY*s was significantly increased in the leaves at 12 h, whereas in the root, only *TaAPY1*, *TaAPY7*, and *TaAPY5* were shortly up-regulated, while others remained unaffected all the time (Figure 6D).

As for biotic stress, powdery mildew pathogen was used to stress the wheat seedlings. The results showed that seven *APY*s (except *TaAPY1*) showed significant sensitivity to *Blumeria graminis* infection. Specifically, the significant up-regulation of *TaAPY2*, *TaAPY3-1*, *TaAPY3-2*, *TaAPY5* and *TaAPY6* could be detected at the pre-penetration stage (24 h), whereas *TaAPY3-3* and *TaAPY7* were significantly up-regulated at the late infection stages (Figure 7). These results suggested that the wheat APYs could be involved in the regulation of biotic stress responses. Nevertheless, different APYs may have diverse roles at different infection stages.

Enzymatic analysis of the recombinant APY3-1 in wheat

To further validate the enzymatic activity of the wheat APYs, the recombinant APY3-1

without the cross-membrane domain was cloned and purified using the *Escherichia coli* expression system (Figure 8A and B). The production of the inorganic phosphate in the system was used to determine ATP hydrolyzation as previously described (Dong et al., **2012**). The results showed that the recombinant TaAPY3-1 exhibited high enzymatic activity with a relatively wide range temperature from 25 to 52℃, and had the highest catalytic activity at 37℃ (Figure 8C). Further, the APY3-1 had relatively high enzyme activities at the acid conditions, with maximal activity detected at pH 4.5 to 5.5 (Figure 8D). Moreover, the hydrolyzation efficiency of APY3-1 on different substrates was also evaluated. The results showed that the APY3-1 exhibited slightly lower enzymatic activity on degrading the ADP compared with ATP, while had very lower activity on the degradation of TTP, GTP and CTP, suggesting that the TaAPY3-1 had high substrate preference (Figure 8E). Moreover, it has been demonstrated that all the NTPDase family members require divergent metal ions as cofactors for their enzymatic activity. Thus, we also evaluated different ions on the enzymatic activity in degrading the ATP. The results showed that Ca^{2+} was proved to be the most effective cofactor than others. The preference order was as follows: $Ca^{2+} > Mg^{2+} > Zn^{2+}$ (Figure 8F). Without the ions, the APY3-1 completely lost the enzymatic activity, suggesting that the apyrase activity is dependent on the ions as cofactors, with the preference for Ca^{2+}. The catalytic activity of TaAPY3-1 can reach a peak of Vmax=61 (Pi uM/h/ug protein), Km=8.7 mM under the most appropriate conditions (37℃, pH 5.5, 8 mM Ca^{2+}) (Figure 8G). Conclusively, these results suggested that the APY3-1 had high and specific ATP and ADP degradation activities, which was consistent with the functions of the APY homologs reported in other species.

Discussion

The cellular ATP homeostasis not only played a pivotal role in maintaining normal cell growth and development, but was also important for the regulation of stress responses (Clark and Roux, 2018). APYRASEs (APYs) played a key role in maintaining regular cell growth and stress responses, mainly by the regulation of the extra- and intra-cellular ATP level, and the Golgi-based glycoprotein synthesis (Kiwamu et al., 2010; Clark et al., **2014**). Stresses could cause significant eATP efflux from the cell, which led to increased ROS accumulation and further induce cell apoptosis (Jeter et al., 2004; Song et al., 2006; Kiwamu et al., 2010; Vadim et al., 2010). Thus the efficient cleavage of the over-dosed eATP could be important for prevention of the stress-induced cell apoptosis. Recent researches showed that overexpression of the APY could significantly inhibit the ROS production and promote the stress resistance (Shurong et al., **2015**). Thus, the regulation of the endo- and extracellular ATP level manipulated by the APYs could be the potential target to improve the stress resistance. In this paper, we firstly identified and characterized the *TaAPY* family members at the genomic level. The results showed that a total of eight APY genes, which all contained the conserved ACR domains, were identified in the wheat genome (Table 1). The identification and characterization of the APYs could provide valuable insight into the physiological and biochemical functions of wheat APYs in the stress responses.

In wheat genome, a total of eight APY members were identified. Similar to the categorization of the AtAPY1-7 in *Arabidopsis* (Tsan-Yu et al., 2015), the identified wheat APYs can be also divided into three groups. It has been postulated that the

transmembrane character could be associated with the subcellular locations of the proteins. TaAPY5 and TaAPY6 contained both N- and C-membrane spanning motifs (MSMs), and TaAPY7 had three MSMs (Figure 3). It has been reported that all four ecto-APYs from the human contained both N- and C-terminal transmembrane domains, others which contained only C-terminal transmembrane domains were endo-APYs (Knowles, **2011;** Tsan-Yu et al., 2015). In *Arabidopsis*, although AtAPY6 and AtAPY7 contained both N- and C-terminal MSM (Figure S3), it was localized to the ER, similar to the other AtAPYs (Tsan-Yu et al., 2015). These results suggested that the MSMs cannot be considered as the protein location marker as it did in the mammalian. Although *APY1-7* in *Arabidopsis* were proved to be inter-cellular located, they could also affect the extra-cellular ATP level, which was important for the stress responses. It has been revealed that *APY1* and *APY2* mutation in *Arabidopsis*, could cause significant elevation of the eATP level (Wu et al., **2007;** Hui et al., 2014), suggesting the eATP homeostasis could also be regulated by the endo-APYs in plants.

The investigation of the gene expression pattern in response to the stresses could help to identify the gene function (Wang et al., 2018; Wang et al., 2019). In wheat, the correlation between the diversely regulated APY expression and the stresses, provided evidences that the APYs could be directly or indirectly involved in the regulation of the stress adaptation (Figure 6 and 7). The results showed that different *APY*s exhibited various expression patterns in response to different stresses, and even varied in different organs (Figure 5-7). Generally, the expression of most *APY*s (including *TaAPY1*, *TaAPY2*, *TaAPY3-1*, *TaAPY3-3*, *TaAPY7* and *TaAPY6*) was up-regulated in the leaf under drought stress, and barely upregulated in root except *TaAPY5* and *TaAPY6* (Figure 6). For salt stress, except *TaAPY3-1*, *TaAPY3-2*, and *TaAPY3-3*, other genes exhibited significant up-regulation in the root (Figure 6). Specifically, the significant up-regulation of 20 times can be detected in the wheat seedlings in response to various stress conditions, such as *TaAPY1*, *TaAPY6* to drought stress, *TaAPY3-2*, *TaAPY6* and *TaAPY7* to heat stress (Figure 7). Specifically, our results also showed that the expression of most *APY*s was biotic stress-responsive. The expression of *TaAPY2*, *TaAPY3-1*, and *TaAPY5* exhibited significant up-regulation at 24 h after powdery mildew inoculation (Figure 7). As shown in figure 7, the powdery mildew spores started to colonize at the cell surface at 24 h. The transient up-regulation of these three *APY*s indicated that they were involved in the primary defense in the wheat leaves, after subjected to the powdery mildew. The expression of almost all the APYs was inhibited at 48 to 72 hours, while increased at 96 h, when new *Bgt* spores were formed and started to infect the leaves again. Thus the expression pattern of these APYs indicated that the APYs could function at the pre-invasive stages in response to the biotic stresses. Conclusively, the wheat APYs were mostly stress-responsive, and some exhibited the stress specificity.

The recombinant protein of TaAPY3-1was purified and its enzymatic activity was further evaluated under different conditions. Unlike the ecto-APY (NTPDase1) from human lymphocytes (Leal et al., **2005**), the TaAPY3-1 protein exhibited high stability within a much wider temperature range from 4℃ to 60℃, and reached the highest activity at 37℃ (Figure 8). Further, a relative alkaline conditions were required for the enzymatic activities of NTPDase1 (Leal et al., **2005**), while the appropriate reaction pH for TaAPY3-1 was only 4.5 to 5.5. The buffer pH over 7 in the reaction buffer almost diminished the

enzymatic activity of TaAPY3-1 (Figure 8). This could be due to the different subcellular locations of the APY members, where the pH of different cell compartment was different. Meanwhile, in *Arabidopsis*, the AtAPY3 showed relative high enzymatic activity in hydrolyzing the ATP, UTP, GTP and CTP, and also can hydrolyze ADP and GDP (Tsan-Yu et al., 2015). Although TaAPY3-1 and AtAPY3 were homologous proteins, the TaAPY3-1 only exhibited high enzymatic activity to ATP and ADP, low activity to TTP and GTP, and no activity to CTP. These results demonstrated that the homologs in different species, might function differently, and TaAPY3-1 in wheat was an ATP/ADP-specific APY.

CONCLUSION

In this study, we identified and characterized the APY family members in wheat at the genome-wide level. The phylogenetic, structural and expression analysis provided a theoretical basis for further functional study and the genetic improvement during the molecular breeding for generation of the stress-resistant wheat cultivars.

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Figure 1

Phylogenetic analysis of the putative APYs in wheat and other plant species.

The phylogenetic tree was created using the MEGA5 software with maximum Likelihood method.

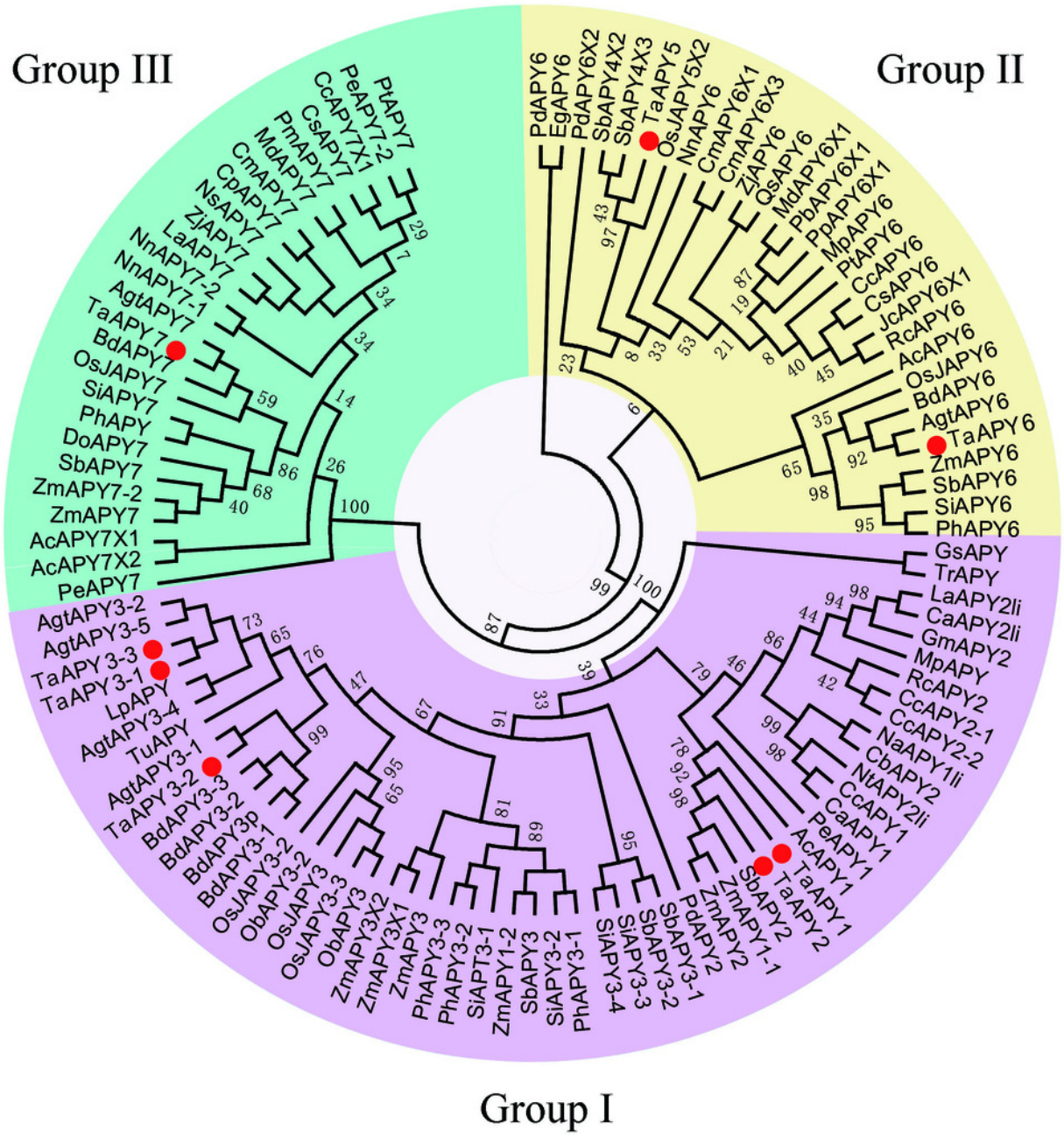

Figure 2

Conserved motif analysis of the wheat APYs.

(A) The motif analysis of the eight APYs was carried out by using the online software MEME suite 5.0.2. **(B)** The details of the conserved motifs (1, 3, 5 and 8) of the eight APYs were represented, and other motifs were listed as supplementary material Figure S2. Different colors represent different motifs of the protein.

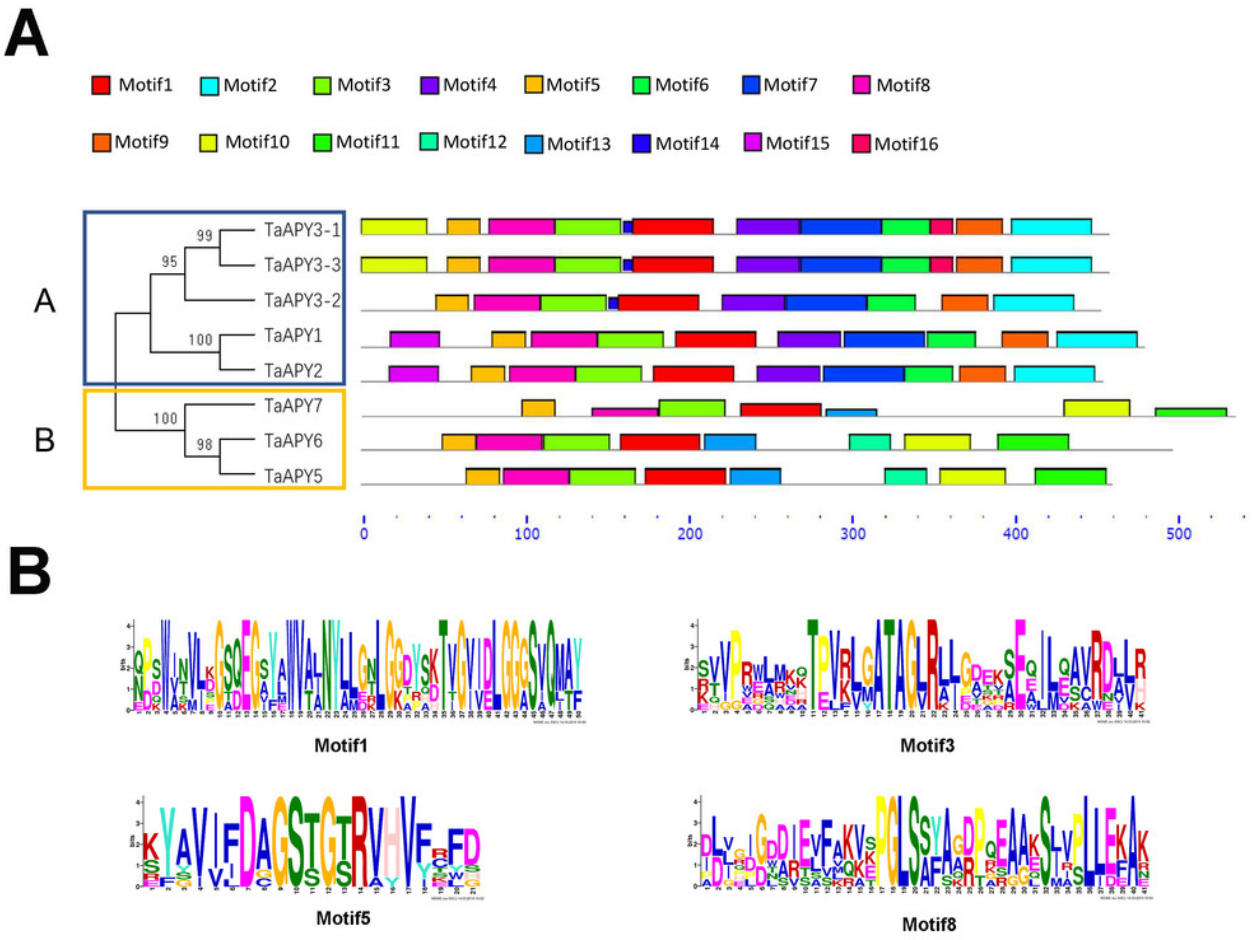

Figure 3

Secondary structure analysis of the eight APYs.

Alpha helix was colored in red, Extend strand in blue and Random coli in purple. The cross membrane domain was predicted and marked with the red box.

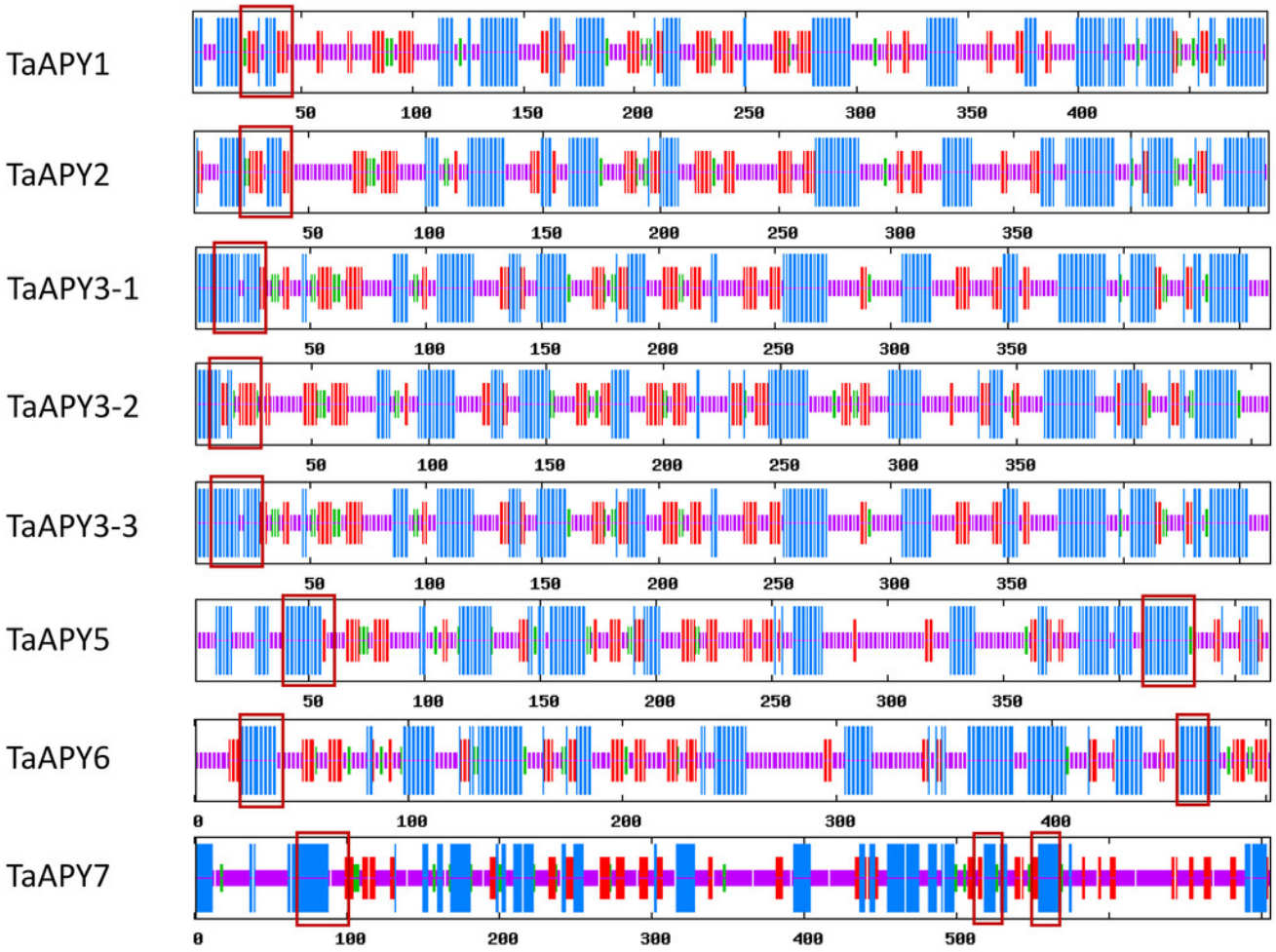

Figure 4

3D structure analysis of the eight wheat APYs.

Models were constructed using the Swiss-model website (https://www.swissmodel.expasy.org/).

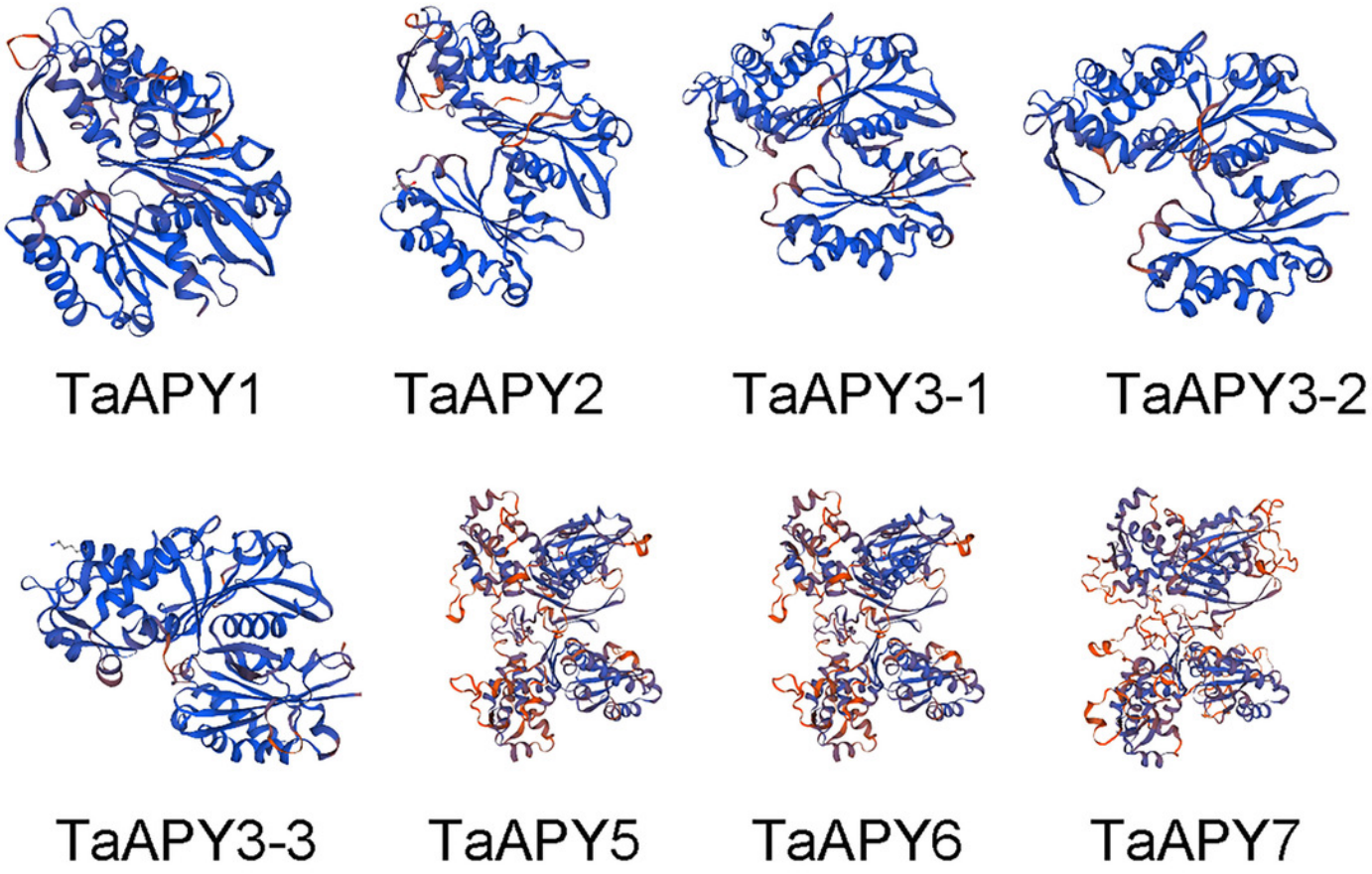

Figure 5

Expression pattern of the eight *TaAPY*s in the root and leaf of the 10-d-old wheat seedlings.

TaACT was used as internal control. Data are presented as means ± SD of three biological replicates.

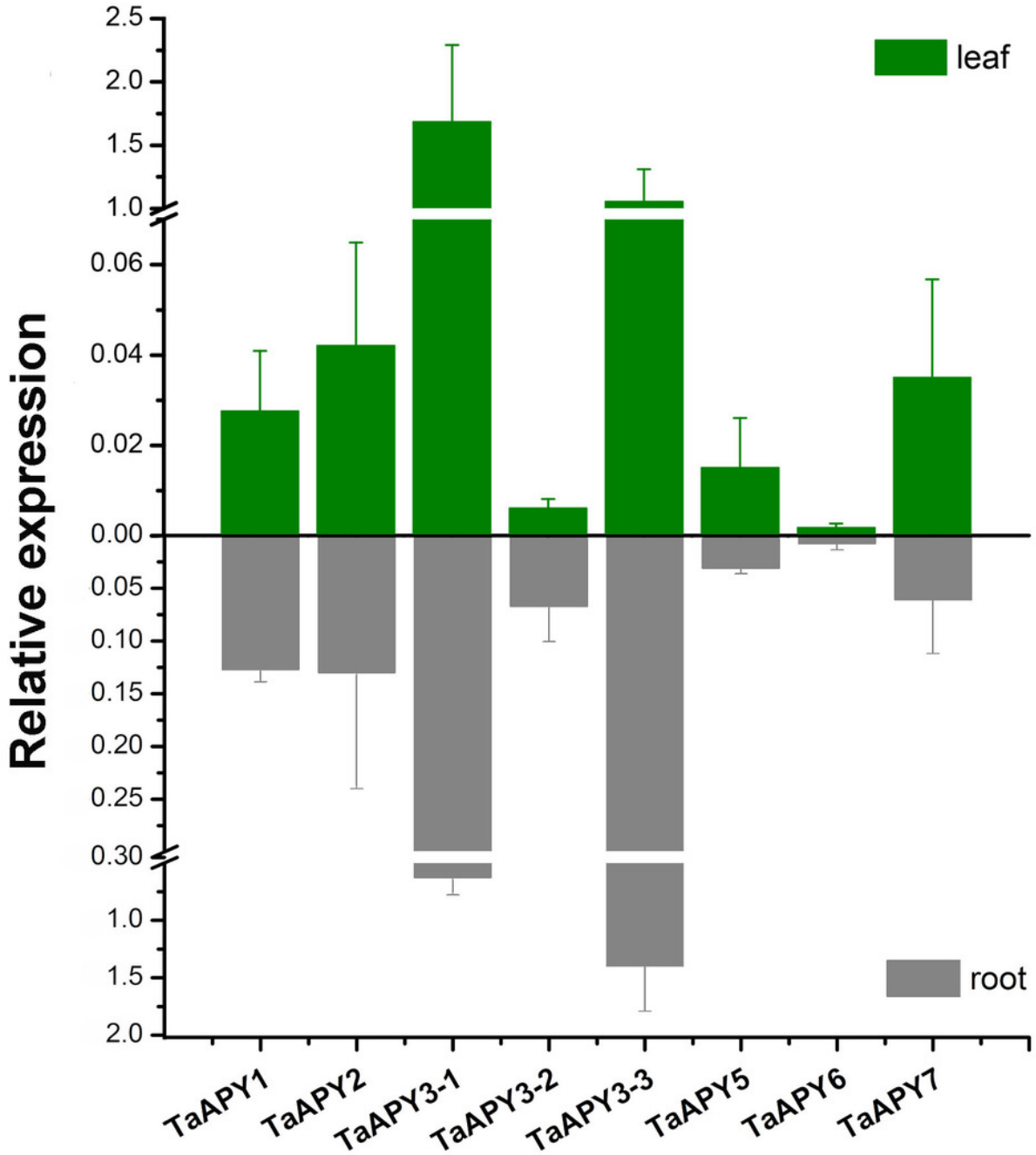

Figure 6

Expression pattern of the wheat *APY*s in response to the abiotic stresses by qPCR.

(A) Heavy metal (200 mM $CdCl_2$). **(B)** Drought (300 mM mannitol). **(C)** Heat (42℃). **(D)** Salt (300 mM NaCl). *TaACT* was used as internal control. Green and red colors represented decreased or decreased expression level.

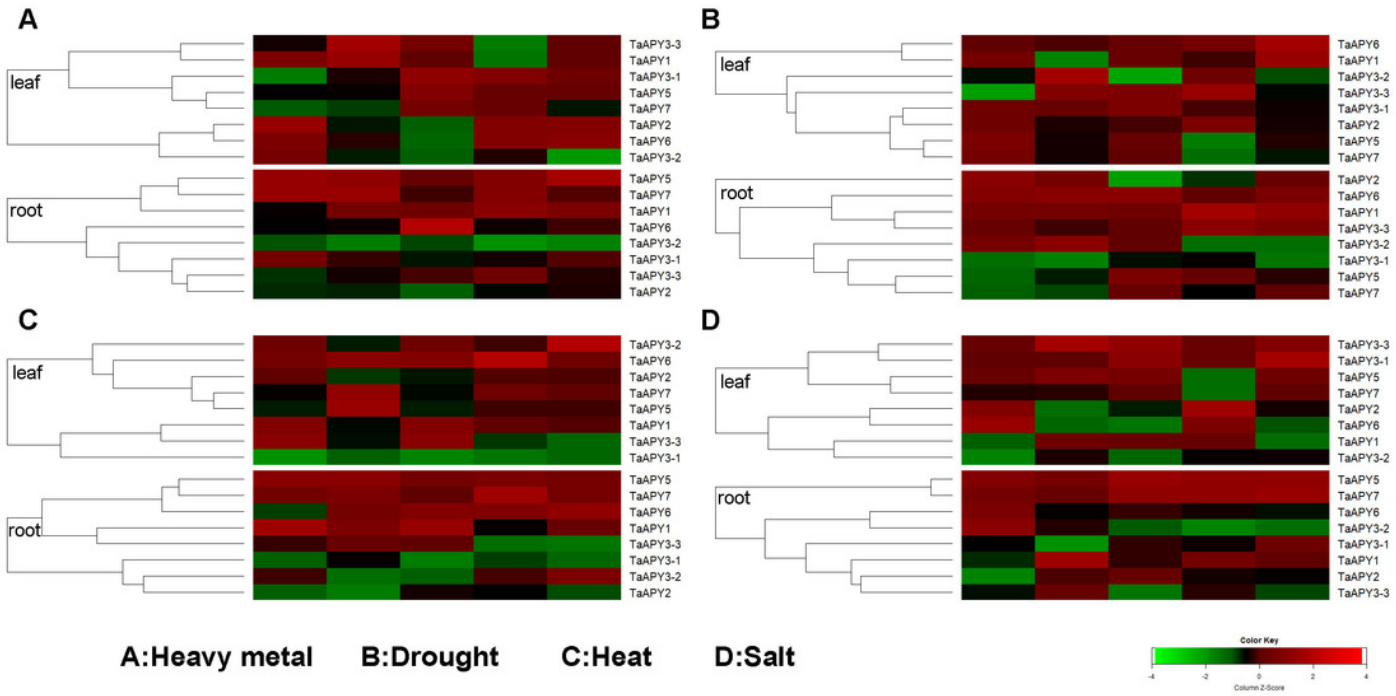

Figure 7

Expression pattern of the *APY*s in response to the *Bgt* infection.

The expression of the *APY*s was analyzed separately at 24, 48, 72 and 96 h post *Bgt* infection. *TaACT* was used as the internal control. Green and red colors represented decreased or decreased expression level.

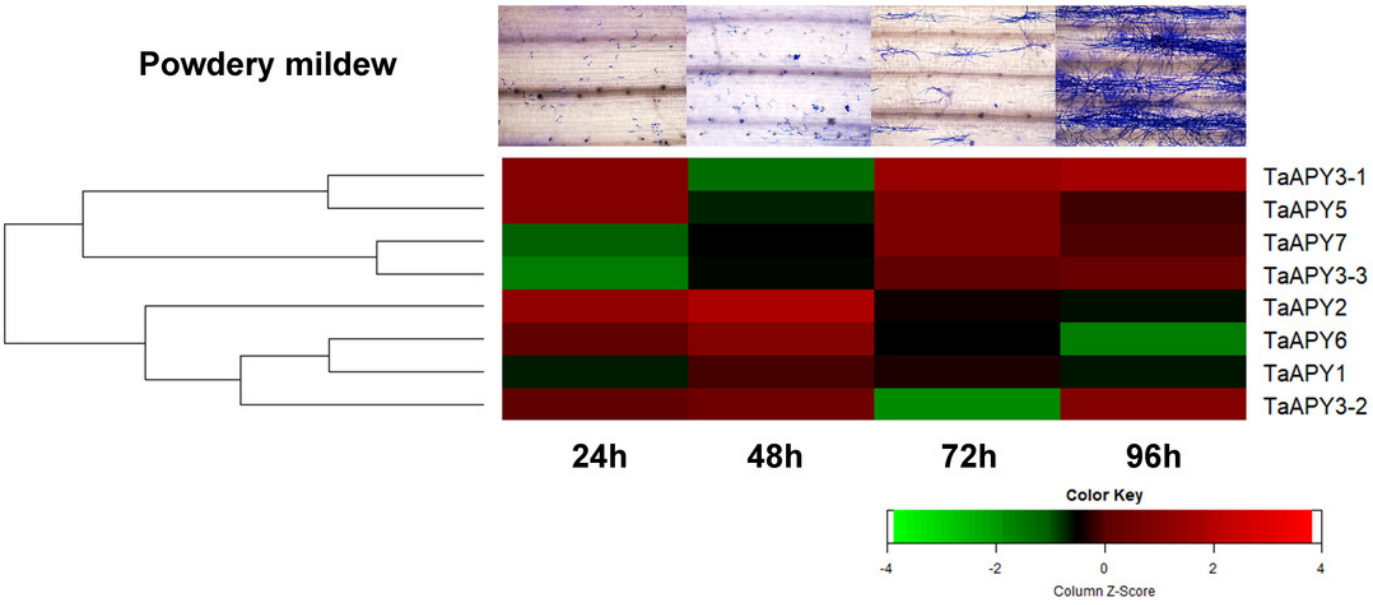

Figure 8

Enzymatic activity analysis of recombinant TaAPY3-1.

(A) Scheme of the purified TaAPY3-1 without the membrane spanning domain. **(B)** SDS-PAGE analysis of the protein purification. **(C)** Enzymatic activity of TaAPY3-1 in degradation of ATP under different temperature. **(D)** Activity of TaAPY3-1 under different pH. **(E)** Activity of TaAPY3-1 under different substrates. **(F)** Different ions on the enzymatic activity of TaAPY3-1. **(G)** Enzymatic activity analysis of TaAPY3-1 with different concentrations of ATP. Data are presented as means ± SD of three biological replicates.

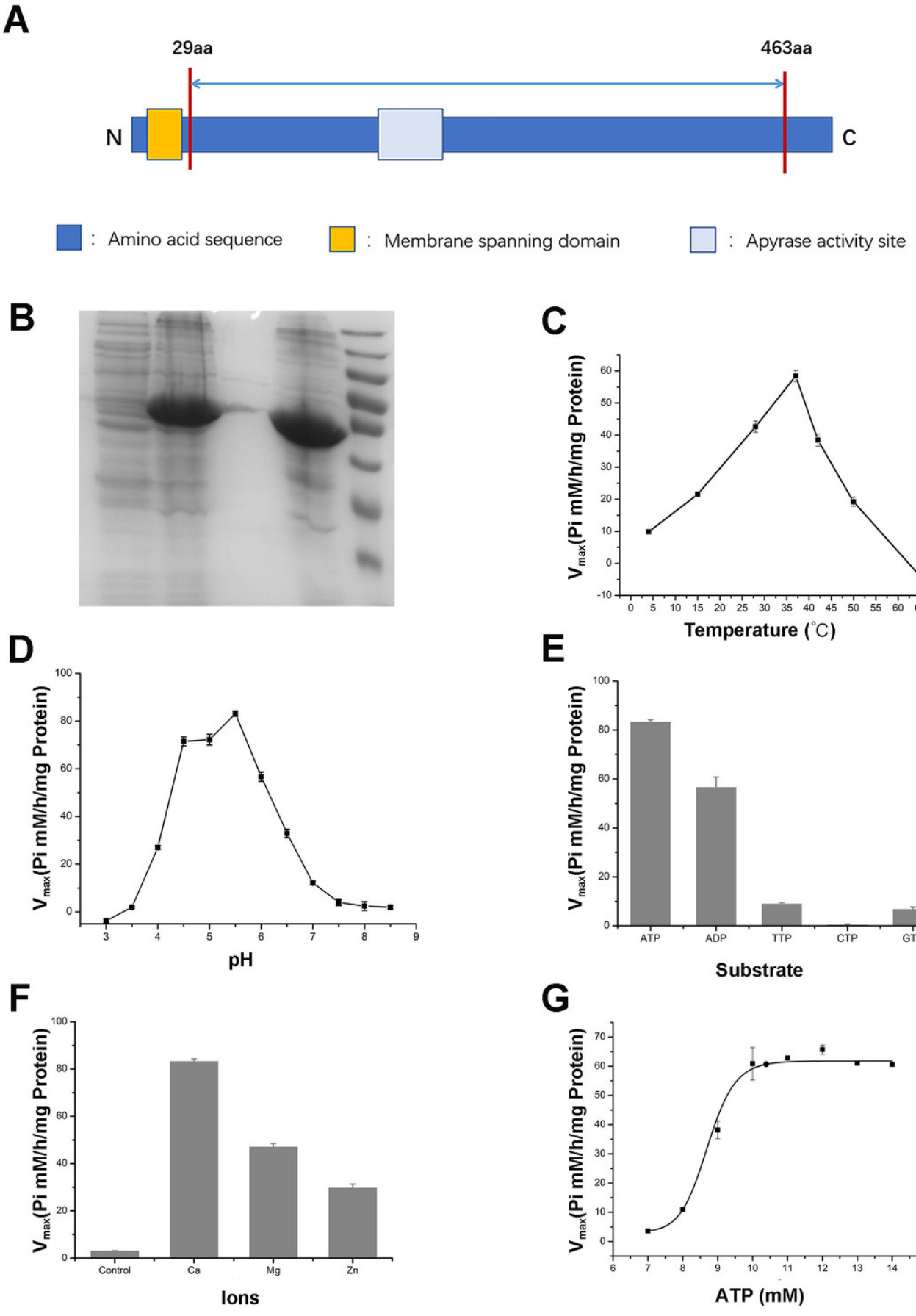

Table 1(on next page)

Table 1. Characteristics of the APY members in wheat.

Table 1. Characteristics of the APY members in wheat.

Name	Gene ID	Protein length (AA)	CDS length (bp)	MW (kDa)	PI	Exon number	CDS similarity
TaAPY1	TraesCS4A01G131300.1	485	1458	52.225	5.93	10	
	TraesCS4B01G173300.1	485	1458	52.261	6.05	10	98
	TraesCS4D01G175400.1	485	1458	52.25	6.34	9	98
TaAPY2	TraesCS2A01G102100.1	457	1374	48.91	6.68	9	
	TraesCS2B01G119200.1	459	1380	49.08	6.68	9	97
	TraesCS2D01G101500.1	469	1410	50.034	7.04	9	98
TaAPY3-1	TraesCS5A01G532000.1	462	1389	49.471	6.05	7	
	TraesCS4B01G363700.1	462	1389	49.555	6.36	7	95
	TraesCS4D01G357100.1	463	1392	49.493	6.22	7	95
TaAPY3-2	TraesCS5A01G547700.1	457	1374	48.963	8.89	6	
	TraesCS4B01G381600.1	430	1293	46.446	8.81	6	94
	TraesCS4D01G357100.1	452	1359	49.036	6.06	10	83
TaAPY3-3	TraesCS7A01G160900.1	454	1365	49.196	5.96	7	
	TraesCS2B01G025000.1	449	1350	49.178	6.76	7	95
	TraesCS2D01G020200.2	448	1347	49.979	7.07	7	95
TaAPY5	TraesCS6A01G105900.1	502	1509	54.652	8.81	8	
	TraesCS7B01G178800.1	465	1398	51.287	8.96	8	96
	TraesCS7D01G280900.1	447	1344	49.209	8.02	6	96
TaAPY6	TraesCS6A01G105900.2	340	1023	36.015	8.13	8	
	TraesCS6B01G135200.1	502	1509	54.54	9.05	8	92
	TraesCS6D01G094400.2	502	1509	54.535	8.86	8	94
TaAPY7	TraesCS1A01G288900.1	706	2121	77.499	9.2	2	
	TraesCS1B01G298200.1	706	2121	77.557	9.19	2	98
	TraesCS1D01G287900.1	706	2121	77.472	9.2	2	98

CDS: coding sequence; AA: amino acid; MW: molecular weights; PI: protein isoelectric points.