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Molecular cloning of a new wheat calreticulin gene *TaCRT1* and expression analysis in plant defense responses and abiotic stress resistance

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ABSTRACT. Calreticulin proteins play essential roles in regulating various metabolic processes and in molecular signal transduction in animals and plants. Using homologous PCR, we screened a cDNA library of the wheat resistance gene Yr5 from a near-isogenic line in the susceptible common wheat variety Taichung 29, which was inoculated with an incompatible race CYR32 of Puccinia striiformis. We isolated a novel full-length cDNA encoding calreticulin protein, which we named TaCRT1. Sequence analyses indicated that TaCRT1 contains an open reading frame of 1287 bp in length; it was deduced to encode 428 amino acids. Clustering analysis showed that TaCRT1 belongs to group III of the calreticulin protein family. Semi-quantitative RT-PCR was used to analyze expression profiles of the isolated gene under biotic and abiotic stresses. Expression of TaCRT1 was suppressed by exogenous application of phytohormones, such as abscisic acid and methyl jasmonate, and by dehydration; but it was induced by CYR32 infection and cold treatment. Based on the expression patterns,

we propose that TaCRT1 participates in regulatory processes involved in defense responses and stress resistance in wheat.

Key words: Wheat; Calreticulin; *Puccinia striiformis*; Defense response; Abiotic stress resistance

INTRODUCTION

Calreticulin (CRT), a Ca^{2+} binding protein, was first detected in rabbit skeletal muscle sarcoplasmic reticulum (Ostwald et al., 1974). In animals, the protein has demonstrated many regulatory functions, such as protein processing, inner cellular Ca^{2+} balance regulation, cell adhesion and control of expression of steroid-sensitive genes and apoptosis. Calreticulin genes were initially isolated from barley (Chen et al., 1994) and spinach (Menegazzi et al., 1993), additional calreticulin cDNA sequences were subsequently cloned from tobacco (Denecke et al., 1993), corn (Kwiatkowski et al., 1995, Dresselhaus et al., 1996), Chinese cabbage (*Beassica pekinensis*) (Lim et al., 1996), Arabidopsis (Nelson et al., 1997), rice (Li and Komatsu, 2000), pea (*Pisum sativum*) and *Ginkgo biloba*.

There were approximately 124 plant calreticulin gene sequences found in current gene databases, of which 32 are from rice, 12 from corn, and only 2 from common wheat genomes. Therefore, calreticulin proteins were universally present in animals and higher plants, furthermore the structure of this soluble protein was highly conserved (Saito et al., 1999). It was shown that calreticulins participated in plant development regulations and in defense and stress responses. Chinese cabbage calreticulin gene *BrCRT1* has been reported to increase sensitivity of plant tissues to hormones related to plant stem and root regeneration (Jin et al., 2005). The rice gene *OsCRO1* from CRT family was induced by low temperature as a response mechanism to cold stress (Li et al., 2003). Wheat gene *TaCRT* enhanced tobacco drought tolerance whereas it was overexpressed in tobacco (Jia et al., 2008). *AtCRT3* expression was inducible by high salt treatment (Persson et al., 2003). In addition, several calreticulins were found to play important roles in plant resistance to virus. For examples, papaya (*Carica Papaya*) *CpCRT* gene increased plant resistance to papaya ringspot virus (Shen et al., 2010), and the intra-cellular transportation of tobacco mosaic virus was suppressed in tobacco plants overexpressing a corn gene *ZmCRT1* (Chen et al., 2005). These results indicated that calreticulins played key roles in plant defense responses and resistance to environmental stresses (Jia et al., 2009).

Disease and environmental stress are the main factors causing lower wheat yields. Improving resistance to disease and adverse environmental conditions is one of the main goals of wheat breeding. Calreticulin, as one form of chaperone proteins, is involved in the regulations of plant gene expressions and many metabolic reactions. Therefore, cloning calreticulin gene and defining its functions will provide a novel approach for wheat breeding for disease resistance and stress tolerance. In this study, a full-length cDNA coding calreticulin was isolated from a Taichung29*6/*Yr5* cDNA library. Its functions were analyzed in wheat yellow rust resistance and abiotic stress responses.

MATERIAL AND METHODS

Experimental materials

The near isogenic line of wheat yellow rust resistance gene *Yr5* Taichung29*6 /*Yr5*

was used as the experimental material in this study. The wheat near isogenic line seedlings were inoculated with an incompatible race CYR32 of *Puccinian striiformis*. After inoculation of 4, 8, 12, 24, 36, 48 and 72 hours, seedling leaves were collected respectively and then mixed, mRNA was isolated to construct a cDNA library as the protocol of Stratagene (2004). Primers were synthesized in Beijing SunBiotech, China. EasyTaq enzymes, agarose gel DNA extraction kit, Peasy-T3 vector, plasmid extraction kit, and RNA reverse transcription kit were manufactured by TransGen Biotech (Beijing, China). DH5α competent cells were the products from Tiangen Biotech (Beijing, China) and DNA sequence analysis was performed by Beijing Biosune Technology (Beijing, China).

Cloning of *TaCRT1* cDNA

Primers were designed against sequences encoding the conserved structural domains of calreticulin family to amplify homologous fragments from wheat cDNA library. The PCR products were used as probes to screen the library for the full length calreticulin cDNA clones.

Analysis of TaCRT1 sequence

The one clone containing the longest cDNA fragment among the positive ones was sequenced, and was submitted to do BLAST search at NCBI web site (http://www.ncbi.nlm.nih.gov/) online service. Both DNAMAN program and NCBI on line tools were used to deduce amino acid sequence and the basic properties and structural domains of the predicted protein. The dendrogram relationship of the TaCRT1 and other members in CRT protein family was constructed using Clustal X and the minimum evolution function in the Mega 4.0 program. The CRT protein sequences used in this study were listed following as wheat TaCRT1 (ADG85705), TaCRT (ABR15365) and AAW02798; rice OsCRO1 (BAA88900), Q9SP22, BAG89479, BAC06263 and EAY76813; corn ZmCRT1 (CAA86728), ZmCRT2 (NP-001105712), ZmCRT3 (ACG33961), AAF01470 and CAA86728; barley HvCRH1 (AAA32948) and HvCRH2 (AAA32949); *Arabidopsis* AtCRT1 (AAC49695), AtCRT2 (AAK74014) and AtCRT3 (AAC49697); tobacco NtRPL60 (CAA59694), ACH72686 and NpCRT1 (CAA95999); rape (*Brassica napus*) AAB70919; sorghum (*Sorghum bicolor*) XP-002456774, EES18468 and EES01894; Syringa pekinesis (*Brassica rapa* subsp. *pekinensis*) PeCRT (AAQ19995); papaya CpCRT (ACQ91203); castor bean (*Ricinus communis*) AAB71420; alfalfa (*Medicago truncatula*) ACJ85691; and soybean (*Glycine max*) ACU24486.

Semi-quantitative RT-PCR analysis

The 7d old Taichung29*6/*Yr5* seedling growing at room temperature (15-18°C) were subjected to the treatments described below. Cold treatment was done by incubating the seedlings at 4°C. The phytohormone treatment was by spaying the plants with 100 µmol/L (MeJA) or 100 µmol/L ABA solution, the control plants were sprayed with 0.025% (V/V) Tween20. Leaf tissues were collected after 1, 3, 6, 12 and 24 h of treatments. For drought treatment, after removed residual soil, the seedlings were placed on filter papers and sampled after 2, 4, 6, and 8 h treatment respectively. When the first seedling leaf was fully expanded (about 7 d after seedling emergence), the seedlings were inoculated with the incompatible race CYR32

of *Puccinia striiformis* f. sp. *tritici* by means of the ring-brush method. After it was kept in 100% humidity for 24 h at 10-12°C in an incubation room, wheat seedlings were transferred to a greenhouse at 15-18°C. Tissues were collected after inoculation of 4, 8, 12, 16, 24, 36, 48 and 72 h respectively. The controls were sprayed with 0.025% (V/V) Tween20. All the samples were frozen in liquid nitrogen immediately after collection, and then stored at -80°C. Total RNA was extracted using Trizol reagent, and cDNA samples were produced by reverse transcription. Primers specific to *TaCRT1* were used in order to assess expression patterns of *TaCRT1* with semi-quantitative PCR after infected by yellow rust or under different stress conditions. TaCRT1-RT-F: 5'-ATACCGAATCCAGCATACAAAGG-3'; TaCRT1-RT-R: 5'-TCTC CTTCCTCCCTTGCTTGTT-3'. Meanwhile, wheat actin primers were synthesized and its PCR product was used as an endogenous control. Actin1: 5'-GTTCCAATCTATGAGGGATACACGC-3'; Acti2: 5'-GAACCTCCACTGAGAACAACATTACC-3'. (actin primers accession number: AB181991). All semi-quantitative RT-PCR analysis results were repeated twice.

RESULTS

TaCRT1 gene cloning and sequence analysis

A full-length calreticulin cDNA of 1657 bp in length was isolated from a wheat cDNA library. This cDNA clone contains a 1287 bp open reading frame from 66 bp to 1352 bp. The deduced peptide consists of 428 amino acids with a molecular mass of 50278.0 Da and pI of 7.32, it is a basic protein. Similar to other calreticulin proteins from plants of different species, TaCRT1 has three structurally conserved domains N, P, and C of calreticulin family in the regions between amino acid 24 to 210, 211 to 312, and 313 to 428 respectively (Figure 1A). Within the N domain there are two conserved family specific motifs: motif 1 and motif 2, at regions of amino acid 109 to 126 and 142 to 149 respectively. The cysteine residues in this domain are critical for protein folding (Matsuoka et al., 1994). In the P domain, there are three copies of Repeat A (PXXIXDPXXKKPEXWDD) and Repeat B (GXWXAXXIXNPXYK) at the site of amino acid 216 to 268, and 271 to 312 (Figure 1A, B), facilitating binding of Ca^{2+} ions at lower concentration levels.

Cluster analysis of CRT proteins

The analyzed calreticulin proteins were from monocotyledon plants including wheat, rice, corn, barley, and sorghum, and dicotyledon ones such as tobacco, rape, *Syringa pekinesis*, papaya, caster bean, alfalfa and soybean. Based on cluster analysis, calreticulin family proteins from higher plants were divided into Group I, Group II and Group III (Figure 2). Group I included only the calreticulins from monocots of rice, corn, wheat and barley. Group II merely contained those from dicots species of tobacco, papaya, caster bean, canola, and *Arabidopsis*, but group III consisted of ones from both monocots and dicots. TaCRT1 was clustered in Group III, and it was much closer to wheat, another calreticulin protein TaCRT. The identity between TaCRT1 and TaCRT was 97.2%.

Plant calreticulin family members can also be divided into three categories according to their functions of development regulations, stress resistance and defense responses. In Group I, barley HvCRH1 and HvCRH2 involve in plant development regulation (Chen et al., 1994),

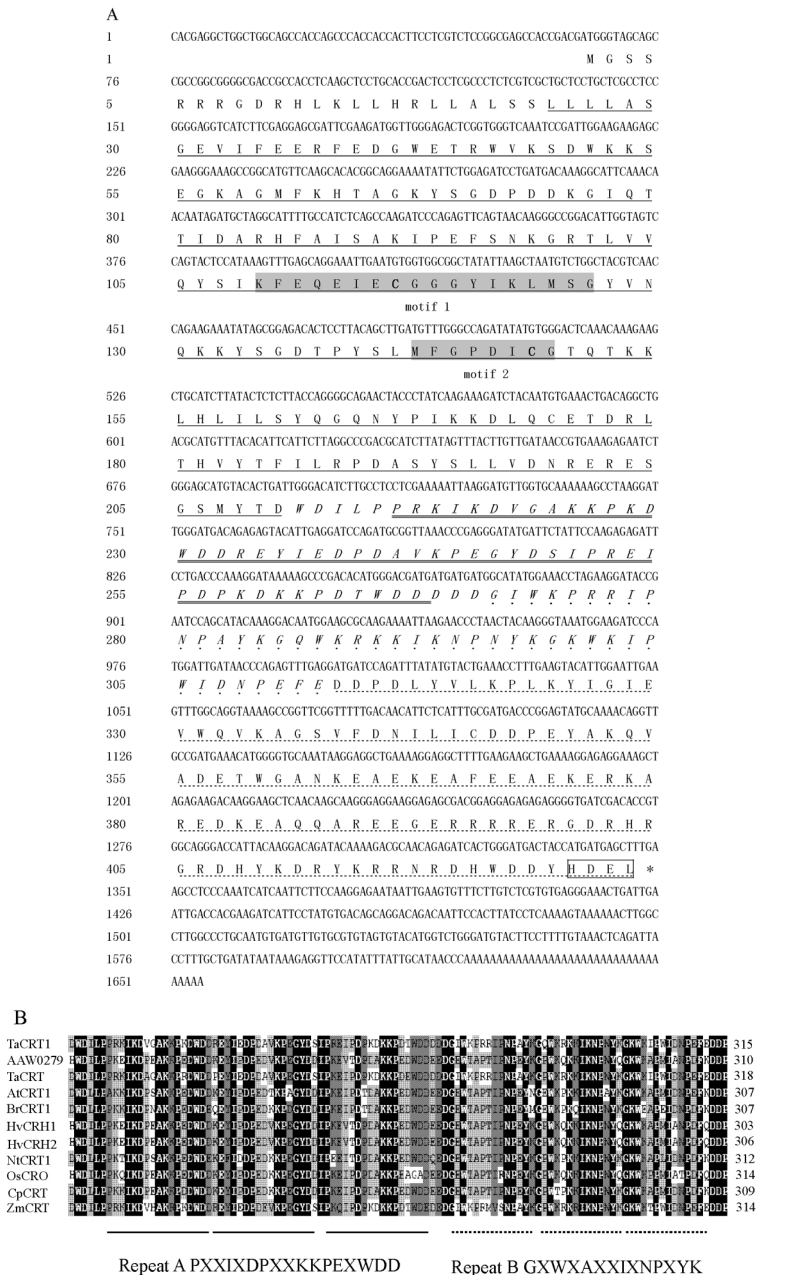

Figure 1. Analysis of the nucleotide and deduced amino acid sequences of *TaCRT1*. (A) Nucleotide and deduced amino acid sequence of *TaCRT1*: N domain is underlined with single line, including conserved motif 1 and motif 2 shown in shade, which are specific to CRT family, the two conserved key Cys residues in motif 1 and motif 2 are shown in bold C. P domain are shown in Italic, which included Repeat A and Repeat B motifs, marked with double underline and dot accent respectively. C domain is underlined with a dash, and a conserved endoplasmic reticulum-retention motif of plant calreticulin at C-end is boxed. (B) Analysis of repeat A and repeat B motifs of plant-specific calreticulin family proteins.

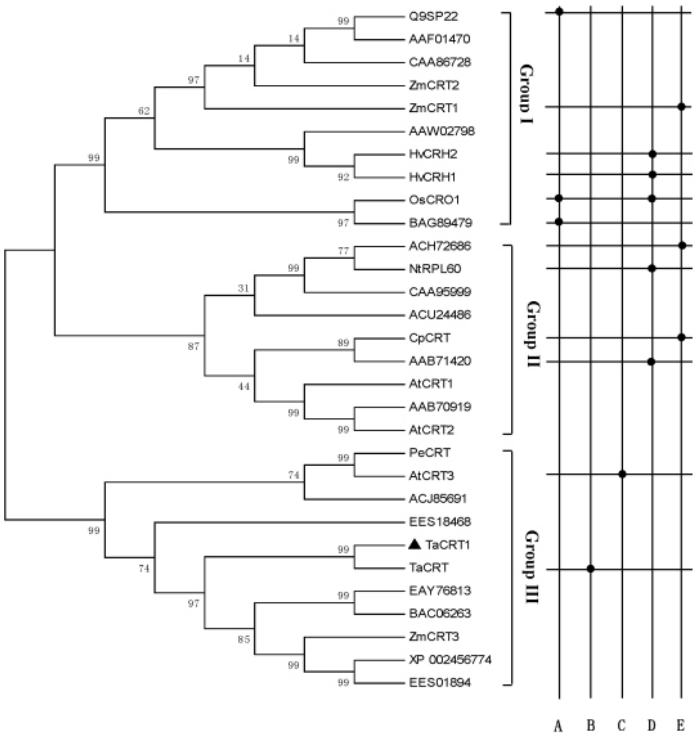

Figure 2. Cluster analysis of the calreticulin proteins in wheat and other plant species. The function-known calreticulin genes involved in abiotic stress resistance to (A) Cold, (B) Drought, (C) Salt, (D) Plant development or (E) defense responses are shown at the right side of the figure.

and corn ZmCRT1 in defense responses (Chen et al., 2005), but rice Q9SP22, OsCRO1 and BAG89479 are associated with cold stress resistance (Arun et al., 2004; Komastu et al., 2009). In Group II, tobacco ACH72686 and papaya CpCRT participate in plant defense responses (Lu et al., 2010; Shen et al., 2010), while tobacco NtRPL60 and caster bean AAB71420 play roles in plant growth and development (Denecke et al., 1995; Coughlan et al., 1997). In Group III, only *Arabidopsis* AtCRT3 and wheat TaCRT have been demonstrated to function in plant response to salt and drought stress (Persson et al., 2003; Jia et al., 2008). Therefore, the members in each group possess diverse biological functions (Figure 2).

Expression pattern of TaCRT1

Since repeat A and Repeat B in P domain of calreticulin are high-through Ca^{2+} binding motifs (Michalak et al., 2009), calreticulin is a regulator in Ca^{2+} signal pathway. In order to understand the roles of *TaCRT1* in defense and stress resistance, its expression patterns under different conditions were evaluated using semi-quantitative RT-PCR method. When wheat seedlings were exposed to cold (4°C), *TaCRT1* transcript level began to decline dramatically, and then 3h later *TaCRT1* expression level started to recover to the original level (0 h control)

and then declined again, indicating that normal transcription of *TaCRT1* was very susceptible to cold (Figure 3A). *TaCRT1* expression started to decrease significantly after treated 6 h by ABA application, and dropped to a very lower level after 24 h treatment (Figure 3A). MeJA treatment could initially induce *TaCRT1* expression to decline slightly, and then it rose to control level at later stages of treatment (12 and 24 h) (Figure 3A). In addition, drought also significantly suppressed *TaCRT1* expression. After 8 h treatment, the gene transcripts were hardly detected (Figure 3B). We also tested *TaCRT1* response to infection by an incompatible race of *Puccinia striiformis*. After inoculation with a race of CYR32, *TaCRT1* was induced to express at a higher level than control and maintain it more than 40 h (Figure 3C).

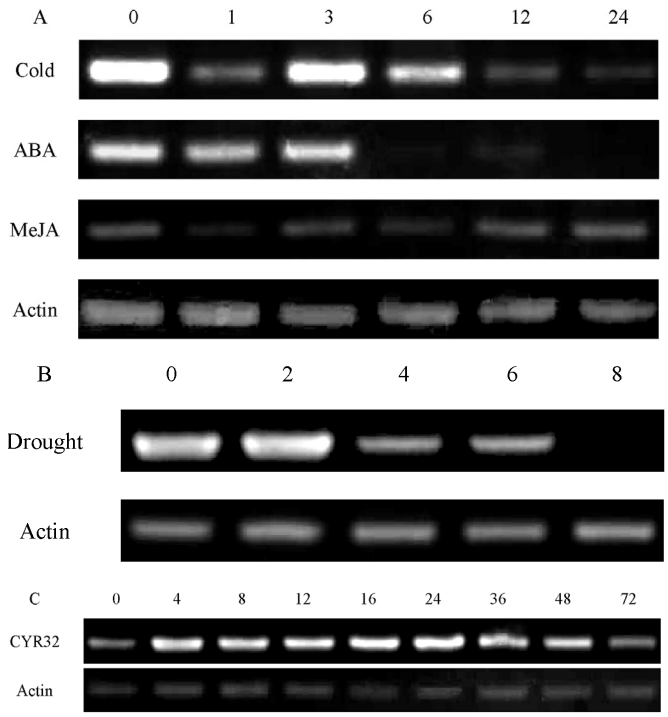

Figure 3. Expression patterns of *TaCRT1* after CYR32 inoculation and stress treatments. (A) Cold (4°C), ABA and MeJA; (B) Drought; (C) Inoculation with race CYR32 of *Puccinian striiformis*. Numbers above are the hours after treatments.

DISCUSSION

Calreticulin proteins are highly abundant and structurally conserved soluble proteins in endoplasmic reticulum. They are universally found in animals and plants, but not in prokaryote creatures and yeast (Michalak et al., 2009). In human genome, 27 calreticulin genes have been identified, 22 in rat, 32 in rice, 20 in *Arabidopsis*, and 12 in corn, and many other higher plant species have being reported to contain calreticulin homologues. Through structure comparison, it was found that calreticulin proteins both from plants and animals contain N, P and C conserved domains (Chen, 1998). There are three copies of motif Repeat A and Repeat B in the P structural domain, and C domain is rich in low-affinity and high-through capacity Ca^{2+} binding

sites. Moreover, there is an endoplasmic reticulum anchoring motif at its C terminal. Therefore, calreticulin family members are highly conserved during its molecular evolution, even emerging before occurrence of animal and plant kingdoms. According to Arabidopsis AtCRT1, AtCRT2 and AtCRT3 structural features, Nelson et al. (1997) divided plant CRT family into CRT1/2 and CRT3 subfamilies, and proposed that variation of certain amino acid residues among the CRT3 subfamily members may lead to different functions (Nelson et al., 1997). In the cluster analysis of this study, calreticulin proteins from higher plants were divided into group I, group II and group III. The *Arabidopsis* AtCRT1 and AtCRT2 belong to group II, whereas AtCRT3 is in group III. Group I only contains proteins from monocot species, group II members from dicots, and those in group III from both. As biological functions, proteins in group I involved in plant development regulation, defense response and stress resistance. Although corn ZmCRT1 was 97.2% identical to rice Q9SP22, they function differently. Overexpression of ZmCRT1 in tobacco could suppress intercellular transportation of tobacco mosaic virus in tobacco, leading to enhancing tobacco plant resistance to the virus infection (Chen et al., 2005). However, rice Q9SP22 was only related to low temperature, and was upregulated by cold (Komastu et al., 2009). We found Group II members participated in plant defense responses and regulations of growth and development. Chitosan oligosaccharide could inhibit tobacco mosaic virus multiplication and its intercellular transportation in tobacco plants through regulating tobacco calreticulin gene *ACH72686* expression, which adjusted Ca^{2+} channel. This result suggested that tobacco ACH72686 plays a key role in the defense response process upon infection by pathogens (Lu et al., 2010). Another tobacco calreticulin gene *NtRPL60* involved in regulation of plant growth and development by giberrelin acid pathway (Denecke et al., 1995), although the identity between ACH72686 and NtRPL60 was as high as 99.2%. Through function-known proteins in group I and II, it can be concluded that plant calreticulin protein conserved highly in its structure evolution, but with diversity in biological functions, thus promoting plant adaption abilities to environmental stresses. In addition, AtCRT3 and TaCRT in group III were identified to play roles in plant response to salt and drought stresses respectively (Persson et al., 2003; Jia et al., 2008). TaCRT1, a member of group III, is 97.2% identical to TaCRT, and so it may play certain roles in plant defense responses or stress resistance.

Different expression patterns of plant calreticulin genes are found when under diverse stress treatments or in defense responses. Some members are upregulated by stress factors, such as *Arabidopsis AtCRT3* by salt stress, wheat *TaCRT* by drought, rice *OsCRO1* by low temperature, and papaya *CpCRT* by papaya ringspot virus infection. *Arabidopsis AtCRT3* expression was immediately activated after 30 min of salt treatment, then declined to its original expression level (Persson et al., 2003). Similar expression patterns to *AtCRT3* were also found in *TaCRT* (Jia et al., 2008), *OsCRO1* (Arun et al., 2004) and *CpCRT* (Chen et al., 2005) .In this study, the *TaCRT1* was induced by low temperature and infection of CYR32 of *Puccinia striiformis*. Expression pattern of *TaCRT1* after infection by CYR32 was similar to those of the four homologous calreticulin genes mentioned above. But during the low temperature stress process, *TaCRT1* expression was reduced greatly in the initial stage, but jumped to higher level 3 hours later and then gradually declined. As a result, cold and CYR32 infection had different effects on *TaCRT1* expression patterns. In contrast, some calreticulin genes were downregulated by stress treatments. For instance, expression level of tobacco *ACH72686* reduced to the lowest point after 4 h of infection by tobacco mosaic virus, and chitosan oligosaccharide could also induce its expression to decline (Lu et al., 2010). We found *TaCRT1* expression

level decreased slightly at the initial stage of MeJA treatment, and then began to increase. It was similar to that of tobacco calreticulin gene *ACH72686*, implying that wheat *TaCRT1* may function similarly as tobacco *ACH72686.* After 6 or 8 h treated by ABA or drought, it was hardly to detect *TaCRT1* transcripts. Therefore, *TaCRT1* was negatively regulated by ABA, suggesting *TaCRT1* may regulate possibly wheat response to drought through ABA pathway. From what has been discussed above, *TaCRT1* has roles in plant defense response and stress resistance and its biological functions should be further tested on the transgenic plants.

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