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A WHEAT CALRETICULIN GENE (*TACRT1*) CONTRIBUTES TO DROUGHT TOLERANCE IN TRANSGENIC *ARABIDOPSIS*

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Abstract

The *TaCRT1* gene is a member of calreticulin (CRT) family in wheat. In our previous study, we showed that transgenic tobacco lines over expressing wheat *TaCRT1* showed enhanced tolerance to salt stress. This study aimed to determine whether *TaCRT1* over expression would increase drought tolerance in transgenic *Arabidopsis*. Over expression of *TaCRT1* in *Arabidopsis* plants enhances tolerance to drought stress. However, the transgenic line was found to retard the growth. Moreover, the transgenic line showed decreased water loss but higher sensitivity to exogenous abscisic acid (ABA) compared with the wild type (Col-0). Meanwhile, the transgenic line had the elevated endogenous ABA level. The semi-quantitative RT-PCR (sqRT-PCR) analysis showed that transcription levels of ABA-biosynthesizing gene (*NCED3*) and ABA-responsive gene (*ABF3*) were higher in the transgenic line than that in the Col-0 under normal condition. The above results implied that the *TaCRT1* might be able to used as a potential target to improve the drought tolerance in crops.

Key words: Calreticulin, *TaCRT1*, *Arabidopsis*, Drought tolerance, ABA sensitivity.

Introduction

Calreticulins (CRTs) are important proteins in both the animal and the plant, which hold many functions on the process of the development and various stress responses. In 1974, the CRT was isolated for the first time, and it was found to be a Ca^{2+}-binding protein with the high affinity and to locate at the endoplasmic reticulum (ER) in the rabbit skeletal muscle cells (Ostwald & MacLennan, 1974). After that, more CRTs were characterized and found to be involved in more than 40 cellular functions of the animal (Fliegel *et al.*, 1989). Currently, the 2-D structure of the CRT proteins were clarified: their N-terminal hold a cleavable signal sequence, which functions as the signal peptide directing the whole protein to migrate to ER and was conservative among species; a P-domain inside the protein sequence confers the high affinity and low binding capacity for the Ca^{2+} binding; a C-terminal domain confers the low affinity and high binding capacity for the Ca^{2+} binding and holds a (K/H)DEL ER retrieval signal (Michalak *et al.*, 1999; Michalak *et al.*, 2009).

In 1993, the CRT was reported in the plant for the first time, which was identified in the spinach (Menegazzi *et al.*, 1993) and after that, more plant CRTs were characterized so that until now, the CRTs have been found in 15 plant species (Chen *et al.*, 1994; Denecke *et al.*, 1995; Kwiatkowski *et al.*,1995; Napier *et al.*, 1995; Navazio *et al.*, 1995; Dresselhaus *et al.*, 1996; Lim *et al.*, 1996; Opas *et al.*, 1996; Coughlan *et al.*, 1997; Nelson *et al.*, 1997; Li & Komatsu, 2000; Lenartowska *et al.*, 2002; Nardi *et al.*, 2006; Jia *et al.*, 2008). The CRTs from the plants hold the similar 2-D structures as the CRTs from the animals, with the N-terminal signal peptide towards ER, P-domain and similar C-terminal. Moreover, the CRTs from the plants have similar basic functions as those from the animals such as the storage of the intracellular Ca^{2+}, the regulation of the Ca^{2+} homeostasis in ER (Coughlan *et al.*, 1997; Li & Komatsu, 2000; Persson *et al.*, 2001; Wyatt *et al.*, 2002; Christensen *et al.*, 2008) and the molecular chaperone function in ER (Nigam *et al.*, 1994; Nauseef *et al.*, 1995; Jin *et al.*, 2009; Jiang *et al.*, 2014; Sakono *et al.*, 2014).

The plant CRTs exist in most of the plant cells and the plant tissues (Crofts *et al.*, 1998) and hold multiple specific functions besides the basic functions mentioned above. The *BrCRT1* gene, found in the Chinese cabbage, was involved in the processes of the shoot and root regeneration through the up-regulation of the response competency of vegetative tissues to the hormonal signals (Jin *et al.*, 2005). Moreover, a CRT protein from the rice was also found to be involved in the regulation of the processes of the root and the shoot regeneration (Li & Komatsu, 2000). Additionally, CRT proteins were found to play essential roles during the reproduction process of the plants, for example, the CRTs has been found to express abundantly in the floral tissues and the germinating seeds (Denecke *et al.*, 1995; Nardi *et al.*, 2006; Borisjuk *et al.*, 1998). In many plant species, the expression of CRT would be up-regulated shortly after the fertilization during the early embryogenesis (Chen *et al.*, 1994; Denecke *et al.*, 1995; Dresselhaus *et al.*, 1996; Nelson *et al.*, 1997; Nardi *et al.*, 2006; Borisjuk *et al.*, 1998), for example, the expression of the CRT in *Petunia hybrid* would be elevated during the processes of the pistil transmitting tract maturation, the pollen germination, the tube outgrowth on the stigma, the gamete fusion, and the early embryogenesis (Lenartowski *et al.*, 2015).

There are two different *CRT* groups in the higher plants: the *CRT1*/*CRT2* group and the *CRT3* group (Persson *et al.*, 2003). It was found that the *CRT* genes in both groups participate in the responses to different biotic and abiotic stresses, such as the low temperature, the drought, the salt stress, the gravi-stimulation, the pathogen attack and the exogenous phytohormones (Denecke *et al.*, 1995; Li & Komatsu, 2000; Li *et al.*, 2009; Saijo *et al.*, 2009; Persson *et al.*, 2003; Li *et al.*, 2003; Komatsu *et al.*, 2009; Heilmann *et al.*, 2001). For

instance, *AtCRT2* can, as a self-modulator, fine-regulate the SA-dependent immunity initiated by the Ca^{2+}-buffering activity of *AtCRT2* in the process of the pathogen invasion (Qiu *et al.*, 2012); *AtCRT3*, a *CRT* gene in *Arabidopsis*, takes part in the PAMP-associated responses; *TaCRT3* (EF452301), the *CRT3* isoform transcript, was elevated in the wheat seedlings during the drought stress induced by PEG, and in tobacco, it was found that the over expression of *TaCRT3* will lead to the enhanced drought resistance (Jia *et al.*, 2008). Our previously research reported the stress-inducible expression patterns of 3 stress-related *CRT* genes in the wheat and revealed that one calreticulin gene (*TaCRT1*, AY836753) of them could enhance the salinity tolerance in the tobacco (Xiang *et al.*, 2015). In the current study, the functional analysis of *TaCRT1* in details was conducted and it was shown that the overexpression of the *TaCRT1* in transgenic *Arabidopsis* enhanced the plant tolerance to drought stress. These results suggested that *TaCRT1* may function as a stress-responsive gene towards the abiotic stresses. Additionally, it was found that the over expression of *TaCRT1* in *Arabidopsis* influenced various aspects of the plant development. In conclusion, the results from this study suggested that *TaCRT1* was not only important in the abiotic stresses but also participating in diverse processes in the plant development.

Materials and Methods

The plant materials and the growth conditions: *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) and the *TaCRT1*-overexpression *Arabidopsis* plant were used as the plant materials. Both of them grew in a controlled environment chamber (150 μmol photons m^{-2} s^{-1}, 14 h light/10 h dark per day at 20 ± 2°C). The plants were planted in the soil mixture (3 vermiculite: 1 part perlite) and regularly watered with 1/10 volume of the MS salts solution.

The generation of the transgenic *Arabidopsis* plants: To generate the CaMV 35S driven DNA constructs, the open reading frame (ORF) of *TaCRT1* was amplified using the primers 5'-CAGCTCTAGAATGTTCTTCCA GGAGAAGTTC-3' and 5'-GCTCGGATCCCTTCTCG TCATCAGAC-3' and the resultant fragment was then cloned into a modified pBI121 expression vector. The underlined sequences of these two above primers indicated the inserted *Xba*I and *Bam*HI sites, respectively. The recombinant vector was then transduced into *Agrobacterium tumefaciens* strain LBA4404 by the electroporation. *Arabidopsis* transformation was performed using the floral-dip method (Clough & Bent, 1998). The transgenic plants overexpressing *TaCRT1* were screened on the agar solidified with Murashige and Skoog salts and 1% sucrose (MS medium) containing 50 mg/L of the kanamycin (Murashige & Skooj, 1962).

The transgenic plants were verified with the PCR amplification for the introduced wheat DNA fragment and the expression driven by the 35S promoter was examined by the RT-PCR with the primers listed in Table S1. The homozygous T_2 transgenic plants were identified by analyzing the germination segregation of T_3 seeds on MS medium with 50 mg/L of the kanamycin.

DNA and RNA extraction, cDNA synthesis and sqRT-PCR: The genomic DNA was extracted following to the method introduced by Ma and Sorrells (1995). The total RNA was extracted with Trizol reagent (Invitrogene, USA) and then digested with to the RNase-free DNase I (Promega, USA) and finally purified. The first-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, USA), and the oligo (dT15) primers following to the standard protocol. For sqRT-PCR, the thermal cycle parameters included 94°C for 3 min; 21 or 25 cycles of 94°C for 15 s, 58°C for 25 s, 72°C for 30 s and a final extension of 72°C for 5 min. The PCR products were resolved on 1.5% agarose gels and visualized under UV light after being stained with ethidium bromide. The amount of the template cDNA was normalized with the house-keeping reference gene, the *Arabidopsis Actin* gene. The information for the primers was listed in Table S1.

Table S1. Primer sequences used in this study.

Gene	Primer sequences (5'-3')	PCR reaction
TaCRT1 fragment	TTCTTCCAGGAGAAGTTC	RT-PCR
	CTTCTCGTCATCAGAC	RT-PCR
Genome walking	CGAGGAGCATCGTGGAAAAAG	First round PCR
	GTAATACGACTCACTATAGGGC	First round PCR
Genome walkin	CGAGGAGCATCGTGGAAAAAG	Second round PCR
	TGGTCGACGGCCCGGGCTGC	Second round PCR
AtABI1	TCAAGATTCCGAGAACGGAGATC	sqRT-PCR
	GAGGATCAAACCGACCATCTAAC	sqRT-PCR
AtNCED3	CGTGAAATCCGTACGGAACC	sqRT-PCR
	CCGGAATCCGGTGAACTCTT	sqRT-PCR
AtABF3	CATCTTTGTCCCCTGTTCCTTAC	sqRT-PCR
	ACCCGTCAATGTCCTTCGC	sqRT-PCR
AtActin	TGGGATGACATGGAGAAGAT	sqRT-PCR
	ATACCAATCATAGATGGCTGG	sqRT-PCR

The genome walking PCR: The genome walking PCR was used to verify the *TaCRT1*-overexpression *Arabidopsis* plant and was performed according to the procedures described by O'Malley *et al.* (2007). In brief, the genomic DNAs from the wild type and the *TaCRT1*-overexpression *Arabidopsis* plant were respectively digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Nco*RI and *Pst*I. Then, the corresponding adaptors were added to the products of the above digestions and the resultant products were used as the DNA templates for the following PCR (Table S1); in the first round PCR, the steps were: 94ºC 3 min, 94ºC 30 s, 55ºC 30 s, 72ºC 3 min, 30 cycles; the last step was 72ºC, 7 min; in the second round PCR, the nested PCR was performed as the following: the product from the first round PCR was diluted by 50 times and 1 μL of the dilution was used as the template; the PCR steps were (gradient PCR): 94ºC 3 min, 94ºC 30 s, 68ºC 30 s, 72ºC 3 min, 3 cycles; 94ºC 30 s, 65ºC 30 s, 72ºC 3 min, 3 cycles; 94ºC 30 s, 62ºC 30 s, 72ºC 3 min, 3 cycles; 94ºC 30 s, 60ºC 30 s, 72ºC 3 min, 21 cycles; the last step was extension at 72ºC for 7 min.

Morphological analysis and the exogenous ABA treatment: After vernalization treatment at 4ºC for 3 days, the seeds of T_3 transgenic and Col-0 plants were sown in the soil (3 vermiculite mixture, 1 perlite), and regularly watered with 1/10 volume of the MS salts solution. From the 16th day after the sowing, the leaf number was recorded every 4 days. Accordingly, the numbers of the plants with ≥6, ≥8, ≥9, ≥10 and ≥12 rosette leaves were respectively and successively recorded. From the 26th day after the sowing, the numbers of the flowering plants were recorded every 4 days. From the 51th day after the sowing, the height of the plants was recorded every 3 days.

The seeds from the T_3 transgenic and the Col-0 *Arabidopsis* plants were sown on MS agar plates with 5% sucrose (pH 5.8) with or without 1 μM ABA; after the stratification at 4°C for 2 d, the plates were moved to room temperature with 12 h of light per day; then the cotyledon opening and greening were scored daily.

Germination assay, drought-stress treatment and water-loss analysis: For the germination assay, the seeds from the T_3 transgenic and Col-0 *Arabidopsis* plants were sown on the filter papers in the Petri dishes soaked with distilled water and 0.5 μM ABA. For each plant line, 150 seeds were sown on three plates; after the stratification at 4°C for 2 d, the plates were moved to the room temperature with 14 h of light per day; finally, the germination (indicated by the emergence of radicals) was scored daily until the 6th day after the sowing.

For the drought-stress treatment, the seeds of the *TaCRT1*-overexpression *Arabidopsis* plant and the wild type plant were germinated as normal; the seedlings with 3-4 rosette leaves were transferred to the soil in the same tray and grew under normal conditions in a greenhouse (14 h day/10 h night, 22°C). The soil drying treatments were imposed with the water holding for 3 weeks from the time when the plants beheld 8-10 leaves.

For the water-loss analysis, the water loss of the detached leaves from the plants was detected by measuring the leaf weight at the specified time. In brief, 50 fully expanded leaves were collected and placed on the open petri dishes under strictly controlled conditions and the leaf weights were measured at every 20 minutes.

The endogenous ABA measurement: The total amount of the endogenous ABA was measured by the indirect enzyme-linked immunosorbent assay (ELISA). In brief, the tissues of the fresh flag leaf (0.5 g of each sample) were collected from the plants with 8-10 rosette leaves and frozen in the liquid nitrogen; the extraction, purification and ELISA assay for the endogenous ABA followed the standard procedures; typically, the optical density values (ODs) at 490 nm for the ABA from the above processed leaf samples were measured; the standard curve for ABA was then constructed: the concentrations of the standard samples used were selected at 100 ng/ml, 300 ng/ml and 500 ng/ml; each sample was measured for 3 times, and the experiment was repeated for 3 times.

Data statistics: All data were given with means and standard errors (mean ± SE). The statistical analyses were performed with the software SPSS 19.0. The comparison was performed with the independent sample *t*-test method. $p<0.05$ and $p<0.01$ were recognized as the existence of the significant and the highly significant difference, respectively.

Results

The generation of the transgenic *Arabidopsis* plants: The transgenic *Arabidopsis* plants over expressing the *TaCRT1* gene were produced through the *Agrobacterium*-mediated transformation and the gene *TaCRT1* was under the control of a cauliflower mosaic virus (CaMV) 35S promoter. Five resultant transgenic lines (T-1, T-19, T-43, T-48 and T-51) were examined by the PCR targeting the transduced DNA from the *TaCRT1* gene (Fig. 1A) and the examination successfully verified the transgenic lines. Then, the transcriptional expression levels of *TaCRT1* in these five T_3 homozygous lines were examined: the bands for the *TaCRT1* expression were obvious after 25 cycles' amplification on the gel for all the five independent T_3 homozygous *TaCRT1*-overexpressing lines (Fig. 1B up). In the following, the No.43 transgenic plant (T-43) was selected and the genome walking operation was performed starting from 35S promoter and the obtained sequence information was compared with the *Arabidopsis* genome sequence. The above genome walking operation demonstrated that in T-43, the *TaCRT1* gene was inserted in the transposable element gene AT4G01515.1 on chromosome 4 (Fig. S1). For the T-43 line, *TaCRT1* was transcriptionally expressed in all the important plant organs including the root, the stem, the leaf and the flower (Fig. 1B down). The T-43 line was then used for the following morphological and drought stress analyses.

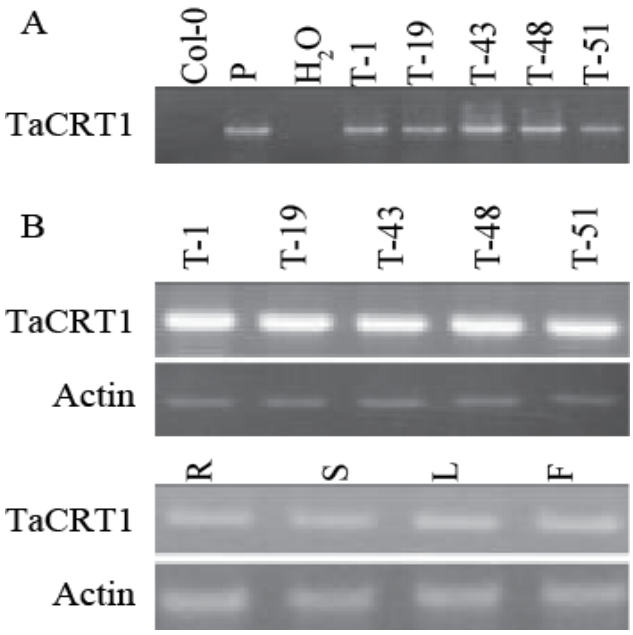

Fig. 1. Detection and expression analysis of *TaCRT1* in transgenic *Arabidopsis* plants by RT–PCR. (A) the genomic PCR of *TaCRT1* gene. (B) top, RT-PCR with leave tissues; down, RT-PCR with leave tissues of T-43 line. T-1, T-19, T-43, T-48 and T-5: transgenic plants; Col-0: non-transgenic plant; P: the positive control; H_2O: the water control; R: toot; S: stem; L: leave; F: flower.

The growth of the *TaCRT1* over expression line was retarded: The phenotype of the T_3 transgenic *Arabidopsis* (T-43) and the wild type (WT) seedlings at different developmental stages under the normal conditions were compared. It was shown that for the transgenic plant, the flowering and leaf development were obviously delayed, and the growth in the aerial parts was also obviously retarded (Fig. 2A, 2B). Moreover, during the vegetative growth period, at the time for about ~50% of Col-0 to beholding ≥ 6 rosette leaves, only ~20% of T-43 beheld ≥ 6 rosette leaves; at the time for about 51.1% of Col-0 to beholding ≥ 8 rosette leaves, only 11.3% of T-43 beheld ≥ 8 rosette leaves; for the percentages of the plants with ≥ 9, ≥ 10 and ≥ 12 rosette leaves at the 25th day after the sowing, the Col-0 were 67.2%, 70% and 60.3%, while these percentages for the T-43 were 38.7%, 38.4% and 34.3%, respectively (Fig. 2C). At the 35th day after the sowing, 28.8% of Col-0 plants began bolting and flowering, while for the T-43, only 4.4%; at the 47th day after the sowing, 90.5% of Col-0 plants began bolting and flowering, while for T-43, only 50%. In addition, from the 51th day after the sowing, the plant height was recorded every 3 days and the height of Col-0 plants was successively 10.5, 14.5, 17.9 and 21.6 cm, while for the T-43 plants, the height was successively 6.4, 9.0, 11.4 and 15.0 cm (Fig. 2E). In summary, the development of the *TaCRT1*-overexpression *Arabidopsis* plant was slower than that of the WT under the normal environment.

The over expression of *TaCRT1* improved the drought stress tolerance: The T_3 transgenic *Arabidopsis* (T-43) and the WT with 3-4 rosette leaves was examined for the tolerance to the drought stress (Fig. 3). Under the normal conditions, the early growth statuses of the T-43 plant and the wild type plant were similar (Fig. 3A left). From the time when the plants beheld 8-10 leaves, the soil drying treatments were imposed by the water holding for 3 weeks: the leaf wilting was observed in the wild type plant at the 8th day from the start of the drought stress, but it was not seen in any of the T-43 plant at this time; at the 15th day from the start of the drought stress, the wild type plants exhibited the severe leaf wilting, the chlorosis, and the stunted growth, but the transgenic plants did not exhibited these phenotypes except for the just start of wilting; at the 21th day, the leaves of Col-0 and T-43 both wilted severely and the chlorophyll of both was obviously destroyed; however, the T-43 plant displayed better growth status compared to the wild type (Fig. 3A right). Furthermore, compared with the wild type plants, the T-43 plants maintained significantly more green leaves than the wild type plant under the drought stress (Fig. 3B). For the water-loss analysis, during the 180 min of water-deficit stress, a higher water loss rate was observed in the leaves of the wild-type plants than that in the leaves of the T-43 plant (Fig. 3C): at the 20th minute after the detachment from the plant, the water loss rate of the detached leaves from Col-0 was 9.1% and that of the T-43 was 6.7%; at the 120th minute after the detachment from the plant, the water loss rate of the detached leaves of Col-0 was 22.0% and that of the T-43 was 16.2%; at the 180th minute after the detachment from the plants, the water loss rate of the detached leaves of Col-0 was 42.1% and that of the T-43 was 34.2%. Taken together, the above results indicated that *TaCRT1* improves the plant tolerance to drought stress.

The *TaCRT1* over expressing plants were hypersensitive to ABA: The germination of the seeds from the T_3 transgenic (T-43) *Arabidopsis* and the WT was examined in the presence of ABA. At first, the germination was examined following the treatment of seeds with 1.0 μM ABA on the MS medium and it was demonstrated that the over expression of *TaCRT1* significantly affected the plant's responses to ABA: the cotyledon opening and the greening of T-43 seedlings were delayed more seriously by 1 μM ABA treatment than that of WT (Fig. 4A). Almost all cotyledons of T-43 seedlings failed to open and turn green (Fig. 4A). To determine the effects of the ABA treatment on the seed germination of transgenic lines, the germination assay was conducted on filter papers soaked with 0.5 μM ABA. Compared with the control, the ABA treatment delayed the pace of germination and reduced the germinating rate (Fig. 4B). Taken together, in the presence of ABA, the seed germination rate of transgenic lines was lower than that of the wild type.

To study if the *TaCRT1* affects the ABA level, the endogenous ABA contents in the leaves of the T_3 transgenic *Arabidopsis* (T-43) and the WT were measured. The ABA concentration in WT plant was 65.8 mg/g (fresh weight), while that in the T-43 plant was 79.5 mg/g (fresh weight) (Fig. 5), which showed that the contents of ABA in the transgenic plants were higher than that in the untransformed plants.

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                                                                    Score     E
Sequences producing significant alignments:                         (bits) Value

AT4G01515.1| Symbols: | transposable element gene |chr4:...   1237    0.0
AT1G23990.1| Symbols: | transposable element gene |chr1:...    133    3e-30
AT5G39862.1| Symbols: | transposable element gene |chr5:...     76    7e-13
AT5G35725.1| Symbols: | transposable element gene |chr5:...     74    3e-12
AT1G37057.1| Symbols: | transposable element gene |chr1:...     72    1e-11

>AT4G01515.1 | Symbols:  | transposable element gene | chr4:647692-649794
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         Length = 2103

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 Strand = Plus / Plus

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Fig. S1. *Arabidopsis* sequence of genome walking.

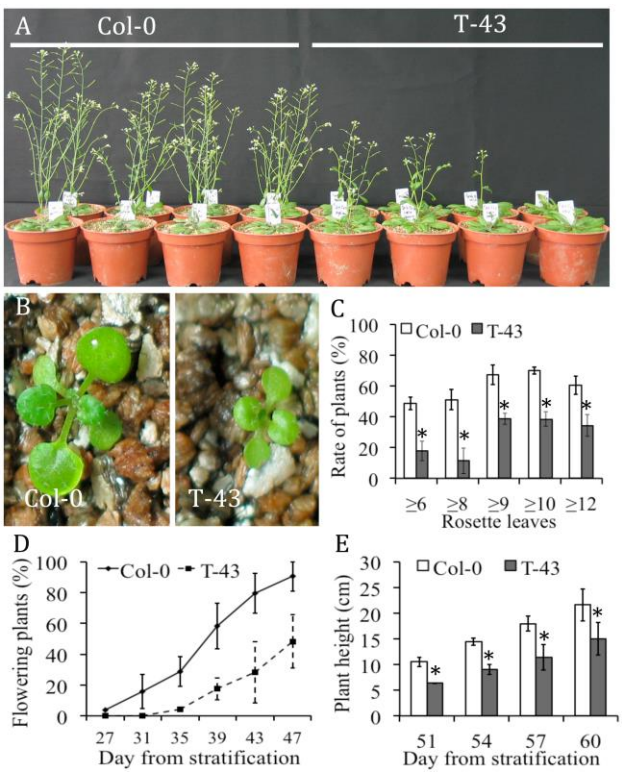

Fig. 2. The comparison of the development between the transgenic line (T-43) and the wild type plant. (A) Five-week-old plants. (B) ten-day-old plants; (C) numbers of the rosette leaves. (D) Comparison of flower development. (E) Comparison of plant height development. Error bars represent the standard deviation of results obtained for three replicate experiments, n ≥ 30; asterisks indicate significant differences from WT plants at $P = 0.05$.

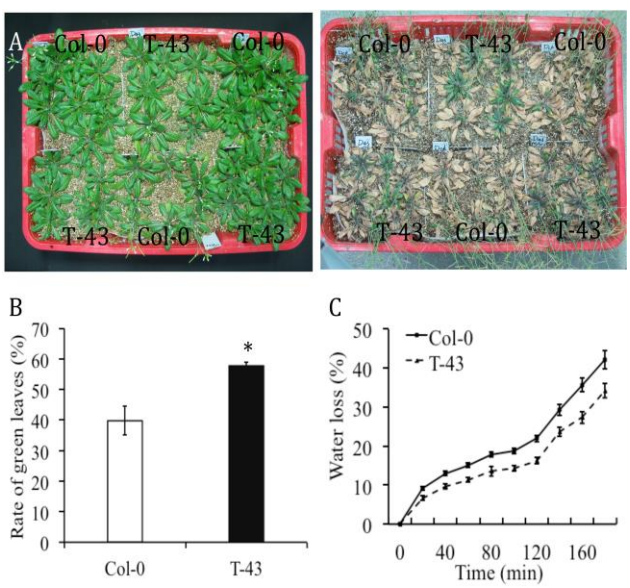

Fig. 3. The tolerance of transgenic plants to drought stress. (A) Left, wild-type and transgenic plants grown under normal conditions; right, wild-type and transgenic plants grown under drought stress. (B) Ratio of green leaf to total leaf (C) Fully expanded leaves were detached from wild-type and *TaCRT1* transgenic plants and exposed to strictly controlled conditions. Water loss rates were measured by weighing the leaf at the specified time. Error bars represent the standard deviation of results obtained for three replicate experiments, n ≥ 30; asterisks indicate significant differences from WT plants at $P = 0.05$.

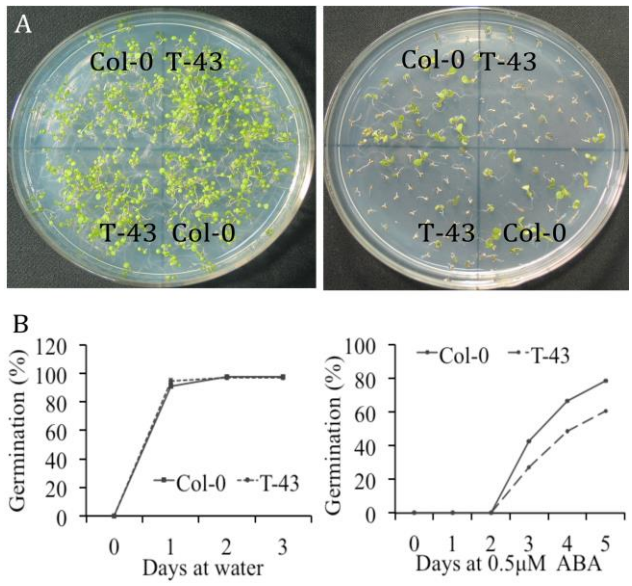

Fig. 4. Effects of exogenous ABA treatments on cotyledon opening, seedling greening and germination. (A) Sterilized and stratified seeds were grown in plates with (right) or without (left) 1.0 μM ABA. Photographs were taken on 10th day. (B) Germination rate from 0 to 6 day after sowing. Col-0 represents non-transgenic *Arabidopsis*; T-43 represents transgenic *Arabidopsis* lines. Error bars represent the standard deviation of results obtained for three replicate experiments, n ≥ 100.

The transcriptional expression analysis of ABA biosynthesizing and ABA-responsive genes: To further investigate the mechanisms behind the phenotypes described above, the transcriptional expression of ABA biosynthesizing and ABA-responsive genes in the *TaCRT1*-overexpresssion plant and the wild type plant were analyzed. As shown in Fig. 6, in the *TaCRT1*-overexpresssion plant, compared with the wild type plant, the transcriptional expression of the important ABA biosynthesizing gene *NCED3* was significantly up-regulated, which could partially explain the higher content of the endogenous ABA in the transgenic plant; at the same time, the transcriptional expression of the ABA responsive-element binding factor gene *ABF3* was significantly up-regulated, which could partially explain why the over expression of *TaCRT1* increased the sensitivity to ABA and improved the tolerance to the drought stress in the transgenic plant.

Discussion

In the animals the CRTs are involved in the Ca^{2+} storage and the ER quality control (ERQC) of N-glycosylated proteins (Michalak *et al.*, 2009). In the plant, the CRTs are similarly the widespread ER proteins and participate in many cellular processes, such as the protein folding and the calcium homeostasis (Persson *et al.*, 2001; Christensen *et al.*, 2008; Christensen *et al.*, 2010). Earlier reports demonstrated that the CRTs were involved in the growth and the development of the plant (Menegazzi *et al.*, 1993). Nardi *et al.* (2006) suggested that the CRTs were important in the calcium signaling regulation and the protein folding assistance, and the CRT might be a general regulator of the gene expression (Li & Komatsu, 2000). To date, the plant *CRT3* genes have been well studied, however, the *CRT1/CRT2* genes remain poorly understood.

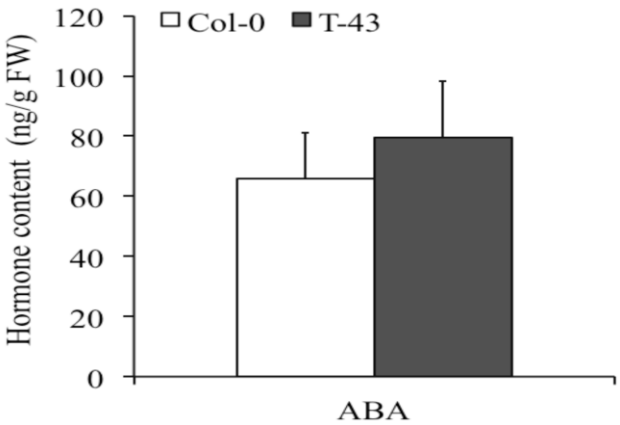

Fig. 5. Endogenous levels of ABA in the leaves of T_3 transgenic *Arabidopsis* (T-43) and WT seedlings. Data are presented as mean ± S.E. Error bars represent the standard deviation of results obtained for three replicate experiments.

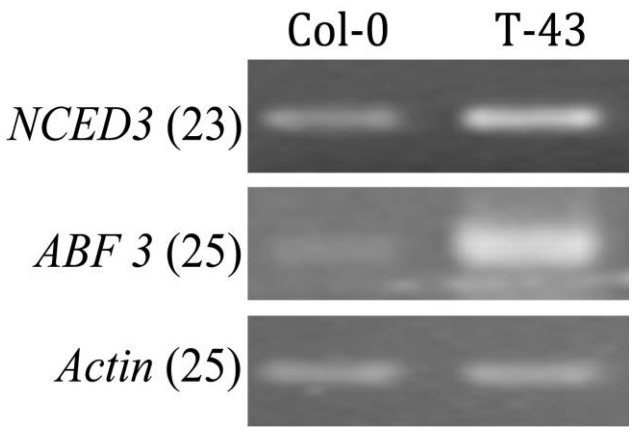

Fig. 6. The semi-quantitative RT-PCR analysis the expression of ABA biosynthesizing and ABA-responsive genes. The analysis of the transcriptional expression of *NCED3* and *ABF3* in the *TaCRT1*-overexpression plants and the wild-type plants by sqRT-PCR.

In this study, by the genome walking experiment, it was found that the *TaCRT1* gene was inserted in the transposable element gene AT4G01515, which is thought to be nonfunctional gene (Fig. S1) and this implied that the disturbance from the insertion was minimal. The transgenic *Arabidopsis* line (T-43) constitutively expressing *TaCRT1* displayed the obvious retardment in the leaf development, the time of flowering, and the plant height (Fig. 2). The above supported that the *TaCRT1* gene participated in the plant growth and the development. In the similar reports, Jin *et al.*, (2005) reported that in the transgenic tobacco, the constitutive over expression of Chinese cabbage calreticulin 1, *BrCRT1*, resulted in the robust shoot production and the root formation, but the plant growth was retarded; Persson *et al.* (2001) demonstrated that the *AtCRT3*-overexpression *Arabidopsis* plant displayed no obvious phenotypic differences in the appearance, the growth rate, the time of flowering. Because the plant *CRT1*/*CRT2* and *CRT3* have obvious difference (Persson *et al.*, 2003; Vitale, 2009), the above reports and the finding in this study suggested that the *CRT1*/*CRT2* may retard the growth of the plant, but this is not the case for *CRT3*.

It was well reported that the expression of *CRT* genes could be influenced by the environmental stimuli. For example, the expression of *CRT* genes can be up-regulated by a wide range of the developmental and the environmental stimuli, including the low temperature (Borisjuk *et al.*, 1998; Sharma *et al.*, 2004), the drought and the pathogens (Qiu *et al.*, 2012). In particular, Jia *et al.* (2008) found that the expression of a wheat *CRT3* gene (EF452301) was significantly enhanced by the PEG-induced drought stress in wheat seedlings; An *et al.* (2011) found that the expression of another wheat *CRT3* gene (HM037186) could be induced by the cold treatment, but suppressed by the dehydration in the wheat seedlings. These reports clearly demonstrated that the expression of *CRTs* increases under the stress conditions, which supported that the *CRT*s have potential roles in the regulation of the plant stress responses. In the previous study from the laboratory, the over expression of the wheat *TaCRT1* gene in *Nicotiana tabacum* reduced the damage from the salt stress in the transgenic tobacco plants. In this study, it was found that the over expression of the *TaCRT1* in the transgenic *Arabidopsis* led to the enhanced tolerance to drought stress. The over expression of the wheat *CRT3* gene has been reported to be able to enhance the drought tolerance in the transgenic tobacco plants (Jia *et al.*, 2008). Meanwhile, Tsou *et al.* (2012) demonstrated that the over expression of the maize *CRT1* could improve the tolerance to both the salt and the drought stress in *Arabidopsis*, increase the total Ca^{2+} in the plant by ~25% and raise the expression level of calcineurin B-like protein-interacting protein kinases 6 (*CIPK6*), a member of the *CIPK* gene family. For the *CIPK* gene family, Deng *et al.* (2013) found that the over expression of the wheat *CIPK14* induced the higher catalase activity, while decreased the amount of hydrogen peroxide and malondialdehyde, and ion leakage under the salt stress, which led to the salinity and the coldness tolerance in the tobacco; Deng *et al.* (2013) also showed that the over expression of the wheat *CIPK29* could result in an increased salt tolerance, accompanied by an increase of the expression level and activity of catalase and peroxidase under the salt stress in the tobacco. The above findings suggested that the wheat *TaCRT1* gene is a drought-stress-responsive gene.

ABA is one of the “classical” plant hormones and has been found to regulate many aspects of the growth and the development of the plant, including the embryo maturation, the seed dormancy, the germination, the cell division, the elongation, the floral induction, and the responses to the environmental stresses such as the drought, the salinity, the coldness, the pathogen attack and the UV radiation (Ruth, 2013). In this study, the *TaCRT1*-overexpression *Arabidopsis* plant was found to be hypersensitive to the exogenous ABA. This finding led us to further measure the amount of the endogenous ABA because the hypersensitivity to the exogenous ABA and the higher amount of the endogenous ABA are

usually connected (Ruth, 2013). The further study demonstrated that under the normal growth conditions, the *TaCRT1*-overexpression *Arabidopsis* plant produced more endogenous ABA than the wild type plant, which partially explained the hypersensitivity of the *TaCRT1*-overexpression *Arabidopsis* plant to exogenous ABA because the endogenous ABA is the internal effecter of the exogenous ABA. Next, the further study for the molecular mechanisms behind the above findings showed that in the *TaCRT1*-overexpression *Arabidopsis* plant, the important ABA biosynthesizing gene *NCED3* (Ruth, 2013) was transcriptionally up-regulated, which could explained the elevated amount of the endogenous ABA in the *TaCRT1*-overexpression *Arabidopsis* plant. Meanwhile, the ABA-responsive-element binding factor *ABF3* (Hong *et al.,* 2016; Ruth, 2013) was also transcriptionally up-regulated, which could explain the elevated sensitivity of the *TaCRT1*-overexpression *Arabidopsis* plant to the exogenous ABA. In summary, these results suggested that the *TaCRT1* is involved in the regulation of gene expression and acts as the regulator in the ABA signaling.

In the common sense, under the drought stress, ABA is accumulated and stimulates the expression of the ABA-responsive-elements, which finally induces the tolerance to the drought stress. The physiological mechanisms behind the drought tolerance involve the increased stomatal closure, the accumulation of compatible solutes such as soluble sugars and free proline, and the increased root system size (Ruth, 2013). This study showed that the water loss rate in the leaves of the *TaCRT1*-overexpression *Arabidopsis* plant decreases reduced, when compared with that in the wild type plants. The detailed physiological changes in our *TaCRT1*-overexpression *Arabidopsis* plant would be further explored in the next research.

In this study, the development of the *TaCRT1*-overexpression *Arabidopsis* plant was retarded, when compared with that of the wild type plant. This phenomenon was similar to the research in the over expression of another stress-responsive NAC transcription factor gene *ONAC022* (Hong *et al.,* 2016). The adverse effects of *TaCRT1* and *ONAC022* on the plant growth and on the plant development may be due to the re-allocation of energy between the stress tolerance and the normal growth/development. The detailed and delicate mechanisms behind need to be further investigated.

In conclusion, based on the results here, the over expression of *TaCRT1* could raise the expression levels of the important ABA biosynthesizing and responsive genes, and these up-regulations could increase the endogenous ABA level and further the raised endogenous ABA level could eventually retard the growth of the *TaCRT1*-overexpression *Arabidopsis* plant, and could improve the tolerance to the drought stress in the transgenic plant. This study enriched the understanding of the *TaCRT1* gene and provided new insights into its usage in the improvement of the drought tolerance in crops.

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