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TaERECTA Responses to Phytohormones, Mg^{2+} Stress and Dehydration and Its Correlation with Stomatal Density in Bread Wheat

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ERECTA is an ancient family of leucine-rich repeat receptor-like kinases (RLKs) that coordinate growth and development of plant. *TaERECTA*, one copy of the ERECTA homologs in wheat, was isolated from bread wheat Chinese Spring. The Ser/Thr kinase of *TaERECTA* was expressed in *E. coli* after IPTG induction and confirmed by immunoblot. *TaERECTA* showed higher expression in younger organs with rapid development, as well as great expression in younger spikes at booting stage. Under exogenous application of gibberellin (GA_3) and abscisic acid (ABA), and Mg^{2+} stress, the expression of *TaERECTA* was largely suppressed, whereas under exogenous application of indole acetic acid (IAA) and brassinolide (BR), and dehydration stress, its expression was initially suppressed and then up-regulated. Natural variation was apparent in the relative expression of *TaERECTA* among 9 different bread wheat lines, and its expression level was negatively correlated with the stomatal density. These results suggested that *TaERECTA* could be exploitable for manipulating agronomical traits important through regulating stomata density, with potential implication for bread wheat improvement.

Keywords: *TaERECTA*, immunoblot, phytohormone, Mg^{2+} stress, dehydration, stomatal density

Introduction

ERECTA (ER) family genes, encoding leucine-rich repeat receptor-like kinases (RLKs) in *Arabidopsis thaliana*, play a key part in cell–cell signal communication based on hormonal regulation, for example, ER partly suppresses the dwarfing effect of *shi* and *spy* gain-of function related to gibberellin (GA_3) pathway (Swain et al. 2001); ER promotes inflorescence growth via largely independent pathway with auxin synergism in their ultimately downstream process (Woodward et al. 2005); the *erecta* mutation assists the short hypocotyl of *Arabidopsis* due to brassinolide (BR) effect on its signal (Borevitz et al. 2002). In *Arabidopsis*, the variation of organs regulated by ER led to the accumulation of

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different mineral element (Ghandilyan et al. 2009). The promoters of *Pythium irregulare* – responsive genes mediated by ER RLKs were revealed with an unexpected overrepresentation of abscisic acid (ABA) response elements (Adie-Bruce et al. 2007).

Compelling evidence suggests that *ER* affects inflorescence architecture, stomatal formation and patterning, ovule development, cell proliferation and pathogen resistance. The transgenic tomato plants with truncated ER protein (AtΔKinase) exhibited increased tolerance to water deficit stress due to reduced stomatal density and their diminished surface area (Villagarcia et al. 2012); Over-expression of *PdERECTA* gene (from *Populus nigra* L.) had a great impact on transgenic *Arabidopsis* (35S:*PdERECTA*), with low transpiration rate and increased water use efficiency (WUEi) (Xing et al. 2011). *ER* gene was a major QTL of rosette carbon isotopic discrimination in *Arabidopsis* and contributed to 21–64% of its total phenotypic variation (Masle et al. 2005). Two sequences homologous to Arabidopsis *ERECTA* were isolated from bread wheat, and which responded to various abiotic stress factors (Huang et al. 2013). The *WELP1* gene of wheat ERECTA member was rapidly induced by several environment stress and localized in the plasma membrane of epidermal onion cells (Zheng et al. 2012). Therefore, ERECTA has been validated to perform numerous functions in drought resistance, the ability to manipulate *ERECTA* genes genetically may be opening great promise for crops improvement.

So far, the evidence of ERECTA was mainly provided by study in *Arabidopsis thaliana*, and the progress in crops was rarely reported. Wheat (*Triticum aestivum* L.) is the third largest crop worldwide and is mainly distributed in arid and semi-arid regions, where water deficit and other environmental fluctuations are the major factors affecting wheat yield. Here, *TaERECTA*, a member of *ERECTA* family, was isolated from wheat variety Chinese Spring, its kinase domain was expressed in prokaryotic system and confirmed by immunoblot. Moreover, the specific expression of *TaERECTA* in different organs, and its response to exogenous phytohormone, Mg^{2+} stress and dehydration were investigated. Correlation between the *TaERECTA* expression level and stomatal density in a few wheat cultivars were conducted to provide the foundation for using *TaERECTA* as a candidate gene on the improvement of transpiration efficiency in bread wheat.

Materials and Methods

Plant materials and sampling

Bread wheat (*Triticum aestivum* L.) variety Chinese Spring was sown in October of 2013 in the experimental field of Northwest A&F University, Yangling, Shaanxi, China. Leaf samples were collected at eight stages of germination, seedling, jointing, booting, heading, flowering, grain filling and maturity, stem samples were at seven stages except germination, respectively; while root samples were at four stages of germination, seedling, jointing and maturity, and spike samples were at five stages of booting, heading, flowering, grain filling and maturity, respectively. Three biological replicates were taken with three plants at each stage of sample collection.

Natural variation of *TaERECTA* expression levels and its correlation with stomatal density was investigated in 9 wheat parental lines of WISP (http://www.wheatisp.org/) LoLa Mapping Populations grown in a large field trial at Rothamsted Research, in Harpenden, UK, in 2013, by Dr Malcolm Hawkesford and Mr Andrew Riche. These parental lines included the UK spring wheat cultivar Paragon and 8 accessions of the Watkins Collection (Miller et al. 2001): 1190034, 1190141, 1190209, 1190292, 1190352, 1190468, 1190481, and 1190729. For convenience, hereby these accession lines were named W34, W141, W209, W292, W352, W468, W481 and W729. At booting stage, flag leaf samples were taken from three plants, one from each of three replicate plots of each cultivar. Samples were quickly frozen in liquid nitrogen and stored at –80 °C for preparation of total RNA. The total RNA was extracted by following the manufacturer's instruction of RNeasy Plant Mini Kit (QIAGEN, UK) and cDNA was synthesized by referring the instruction of Super Script™ III Reverse Transcriptase (Invitrogen, UK).

Phytohormone, dehydration and Mg^{2+} treatment

Seeds of Chinese Spring were grown on soil mixture (Nutrient soil: vermiculite = 2:1) in a growth chamber with 60% humidity, and 20/23 °C night/day with a 8 h/16 h dark/light (about 125 micromol photons s^{-1} m^{-2}) photoperiod. Seedlings (Z12) (Zadoks et al. 1974) after 10-day growth were cleaned with water and exposed on filter paper for instant airing, then used for different treatments. As for different treatments, the final concentration of the solution was 30 mM/L for GA_3 (Swain et al. 2001), 0.75 μM/L for brassinolide (BR), 10 μM/L for IAA (Woodward et al. 2005), 100 μM/L for ABA (Kanyuka et al. 2003) and 0.2 mM/L for $MgSO_4$ (Waters and Grusak 2008), respectively. GA_3, BR, IAA, ABA and $MgSO_4$ were ordered from AaLaDing biotech, China. Seedlings (Z12) were cultured in the solution of each treatment for the required time periods. For dehydration stress, seedlings were placed on the clean filter paper for the same time periods (Zhang et al. 2009). The control was cultured in water. The time periods of sampling for each treatment and control were at 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h and 24 h. Each treatment contained three biological replicates with three plants. Up to each time period, leaves, stems and roots were mixed together and sampled, quickly frozen in liquid nitrogen and stored at –80 °C for RNA extraction and cDNA synthesis, as mentioned above.

Isolation and sequence analysis of TaERECTA *gene*

Based on the sequence information of *TaERECTA* (Gene Bank accession JQ599261.2), two pairs of primers were designed with the *Nco*I site in the middle of the sequence to separate it into two segments. The two segments were amplified with Phusion High-Fidelity DNA Polymerases (Thermo Scientific, UK) using the peduncle cDNA of Chinese Spring as template with the primers of *TaERECTA-1F*: CACTGCCCGGACGGTGATTC and *TaERECTA-1R*: CCAAGCTGCCGATGGCTGATGG, or *TaERECTA-2F*: TTCCGTCG GAGCTGGGAAAGC and *TaERECTA-2R*: CATCCTCCGCCGCCTGAGTTC,

respectively. The two segments were then ligated together using the *Nco*I site. The full segment was sequenced.

Prokaryotic expression and immunoblot of TaER_Kinase

The Ser/Thr kinase region of TaERECTA (TaER_Kinase) was selected and amplified with primers of *TaERECTA-KF*: CG**GGATCC**CCGCACAGTCCTCCTGTTTTC and *TaERECTA-KR*: CCC**AAGCTT**GGGGTGCACCAGGCAGTC. The amplified 954 bp segment with *BamH*I and *Hind*III site was introduced into pET-32a-c(+), and transformed into *E. coli* (*DH5α*). The single positive clone was selected and cultured in 100 µl LB solution medium (including 100 µg/ml ampicillin) for 12 h at 37 °C, and then were cultured again in 10 ml LB solution at 37 °C until the OD_{600} of 0.6 ~ 0.8. IPTG was added into the culture with a final concentration of 0 mM/L, 0.4 mM/L and 0.8 mM/L, respectively. The cells cultured at 28 °C or 37 °C for 3 h and 5 h were harvested subsequently, and split by repeated freezing and thawing with lysozyme, the supernatant was then run on SDS-PAGE to identify the target protein.

A 50 µg supernatant was run on SDS-PAGE, and transformed onto nitrocellulose filter membrane (NC membrane) by wet transblot apparatus (Bio-Rad, USA). Primary and secondary antibodies were Anti-His (mouse monoclonal antibody) and IgG Antibody (horseradish peroxidase IgG), ordered from Tiangen biotech, China. The immune complexes were visualized with scanner system (Canon, Japan).

Tissue-specific expression of TaERECTA *determined by semi-quantitative RT-PCR*

cDNA samples were diluted 10-fold with double distilled water. The expression of *TaERECTA* was investigated by semi-quantitative RT-PCR with primers of *TaERECTAQ-F*: CAACGA GTACGTGAGCCTGCG and *TaERECTAQ-R*: GACTGACTACCTGCTTGCTGCATC, *TaActin* was used as the reference gene with primers of *TaActin-F*: TTGCTGACCGTAT GAGCAAG and *TaActin-R*: ACCCTCCAATCCAGACACTG. The optimal amount of primers was obtained by adjusting the concentration of forward and reverse primer added for *TaERECTA* amplification using the same cDNA, as well as *TaActin*. The optimal amount of cDNA was then obtained by adjusting the cDNA amount added, showing the similar band of *TaActin* expression at different developmental stages. The optimal amount of primers for *TaERECTAQ* and *TaActin* were used at the same time with optimal amount of cDNA added in 20 µl reaction with 25 cycles. PCR products were run on 1.5% agarose gels and visualized with the Gel Doc EQ System (Bio-Rad, USA). The relative expression was estimated based on the light density of the bands.

TaERECTA *expression in response of to phytohormones and stresses, and in flag leaves of 9 wheat lines by qRT-PCR*

The cDNA from the treatments of different phytohormones and stresses was used as template to investigate the response of *TaERECTA* to different treatments with *TaERECTAQ*

primers through the real time PCR system ABI 7300 (Applied Biosystems, USA). Three technical replicates were applied for each sample according to the specifications of the SYBR Premix ExTaq Kit (TaKaRa, China). Two reference genes of *TaActin* (as above) and *TaSand* with primers of *TaSand-F*: TGCCTTGCCCATAAGAAATC and *TaSand-R*: GTGCGGACCAGTTGCTTTAT were used to standardize the background expression. The same procedure was also used to investigate the expression of *TaERECTA* in the flag leaves of the 9 wheat lines at booting stage. The amplification procedure included an initial step of 95 °C for 20 s, followed by 40 cycles of 95 °C for 5 s, 61 °C for 30 s. Data was analyzed using the formula:

$$NE = \frac{(E_X)^{-Ct,X}}{(E_R)^{-Ct,R}} = \frac{RQ_X}{RQ_R}$$

Here E_X and E_R are primer efficiency of target and reference genes, respectively. C_t, X and C_t, R are C_t value of target and reference genes in qRT-PCR process, respectively. The denominator of the formula is consisted of the geometric mean of two reference genes (Ramakers et al. 2003; Rieu and Powers 2009; Olsen et al. 2012).

Measurement of stomatal density

At the late flowering stage (Z69), leaf epidermal samples were taken from the adaxial (top) and abaxial (bottom) surface of the flag leaves of three plants, one from each of three replicate plots of each of the 9 wheat lines, for estimating the stomatal density. The leaf surface was brushed with 1 cm^2 of transparent nail polish for about 20 s, and glued with sellotape avoiding veins, if possible. The sellotape was then removed and placed on a microscope slide. Leaf epidermal samples were observed with a Zeiss Axiophot upright light microscope (Zeiss, Germany). Images were recorded using a QImaging Retiga Exi CCD digital camera (QImaging, Canada) and the MetaMorph Microscopy Automation & Image Analysis software (Molecular Devices, LLC, USA). An epidermal area free of debris was selected and oriented to allow as many stomata as possible inside the area of the image acquired (viewing area). Three images were collected from each slide. The total number of stomata in each image was counted and the average stomatal density of each flag leaf was estimated using the formula:

$$SD\left(No.mm^{-2}\right) = \frac{Number\ of\ stomata\,(No.)}{View\ area\,(mm^2)}$$

Data analysis

Analysis of variance was used to assess variation of *TaERECTA* expression. Correlation analysis between *TaERECTA* expression and stomatal density was performed by using the Pearson Product Moment Correlation test in nine wheat lines. All of the analyses were conducted with the SPSS Statistics Software version 19.0 (IBM SPSS Statistics, USA) and GenStat 16th edition (VSN International Ltd., UK).

Results

Isolation of TaERECTA *gene in bread wheat*

Due to higher *ER* expression in the peduncle of *Arabidopsis thaliana* (Shpak et al. 2004) and wheat (Huang et al. 2013), the cDNA synthesized with total RNA from the peduncle of Chinese Spring was used as template to isolate the *TaERECTA* fragment by PCR amplification. The full segment was obtained by ligation of two segments (1391 bp and 1938 bp) (GeneBank accession No. JQ599261.2) (Fig. S1*). *TaERECTA* had an open reading frame of 2931 bp, which encoded a protein of 976 amino acids with a predicted molecular mass of 106 kDa and a predicted pI of 6.21. Analysis on deduced TaERECTA protein sequence indicated high similarity with other proteins of RLKs family.

Prokaryotic expression and immunoblot of TaER_Kinase

Under constant 37 °C culture either with or without IPTG induction for 3 h, there was no target pET-32a-c(+) accumulated. Small amount of pET-32a-c(+)-TaER_Kinase was observed without IPTG induction. However, after induction with IPTG at the final concentrations of 0.4 mM/L and 0.8 mM/L, pET-32a-c(+)-TaER_Kinase was over accumulated with the molecular mass of nearly 50 Kda (Fig. S2A). After removal of 14 Kda histidine protein, TaER_Kinase was 36 Kda, which was similar to the expected molecular mass. Immunoblot confirmed the massive band of pET-32a-c(+)-TaER_Kinase by IPTG induction (Fig. S2B). These results suggested that the kinase domain of TaERECTA could encode the serine/threonine protein kinase as to ER family in *Arabidopsis*.

Tissue specific expression of TaERECTA *at different developmental stages*

Expression analysis indicated different tissue-specific expression patterns of *TaERECTA* throughout wheat development (Fig. S3A). For leaves, *TaERECTA* transcripts mainly accumulated at germination stage, declining at the following stages. In roots, the highest expression was observed at jointing stage, and relatively higher expression at maturity stage. In stems, the considerably greatest expression was observed at jointing stage. The most accumulation of *TaERECTA* transcripts in the spikes was at booting stage, with relatively higher expression at heading stage. However, at the same stage of booting, highly abundant transcript levels of *TaERECTA* were observed in young spikes, followed by in stems and leaves, and the lowest in roots (Fig. S3B), its expression in young spikes was about 22-fold more than that in young stems and leaves, and was hardly detected in roots.

The response of TaERECTA *to exogenous phytohormone treatments*

Seedlings (Z12) were cultured in different solutions for exogenous phytohormone treatments, leaves, stems and roots were mixed together to investigate the response of *TaERE-*

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

CTA to exogenous phytohormones. Compared with the reference genes, the response of *TaERECTA* to exogenous application of different phytohormones was significantly variable at different treatment time periods (Fig. S4). For ABA treatment, the expression of *TaERECTA* was dramatically reduced within 2 h of treatment, then its expression was lower and stable as to the reference genes, averagely presenting 31% of that at 0 h. While, for exogenous GA_3 treatment, the expression of *TaERECTA* decreased by 49% at 0.5 h, continuously reduced until at 4 h, then was stable at lower level as to reference genes, averagely showing 23% expression of *TaERECTA* at 0 h. However, the expression of *TaERECTA* actively responded to the application of exogenous IAA, its expression was initially reduced and obtained the lowest level at 2 h with 32% expression of *TaERECTA* at 0 h, while after 2 h, which was steadily increased until at 24 h, restored to 76% expression of *TaERECTA* at 0 h. Similarly, under BR treatment, *TaERECTA* transcripts were firstly reduced and reached the lowest level at 2 h with 32% expression of that at 0 h, then increased, up to 24 h, restored to 77% expression of *TaERECTA* at 0 h. *TaERECTA* was more susceptible to GA_3 and IAA than to ABA and BR.

The response of TaERECTA *to* Mg^{2+} *stress and dehydration*

For Mg^{2+} stress treatment, *TaERECTA* expression was suppressed as to the level of reference genes before 2 h, showing 28% expression of *TaERECTA* at 0 h. From 2 h to 24 h of Mg^{2+} treatment, *TaERECTA* expression was steadily suppressed in the low level. However, under dehydration treatment, the expression of *TaERECTA* was declined, obtaining the lowest expression at 2 h, then its expression was gradually increased without significant difference among 2 h, 4 h and 6 h, up to 24 h, displaying 57% expression of *TaERECTA* at 0 h (Fig. S5).

Natural variation in TaERECTA *expression and its correlation with stomatal density*

The expression level of *TaERECTA* in the flag leaves was compared among 9 wheat genotypes (Fig. S6). *TaERECTA* transcripts were more abundant in the accessions *W34*, *W209* and *W481*, and less in *W141*, *W292* and *W729*. Although there was large variation within the limited biological replication (three replicates), significant difference was observed between the accessions *W34*, *W209* and *W481* and other genotypes. The determination of stomatal density on the top and bottom of flag leaves indicated that stomata were more abundant in the cultivar Paragon, and the accessions *W141*, *W292* and *W729*, whereas they were relatively more sparsely distributed in *W209*, *W468* and *W481* (Fig. S6). The stomatal density of *W209* and *W481* was significantly lower than that of *Paragon*, *W141*, W292 and *W729*.

Comparison on *TaERECTA* expression and stomatal density of the 9 wheat genotypes showed that the wheat genotype with lower stomatal density displayed the higher expression level of *TaERECTA* in flag leaves. Correlation analysis indicated a negative and linear relationship between the *TaERECTA* expression and stomatal density, with the correlation coefficient of 0.523 in Plot-1, 0.629 in Plot-2 and 0.418 in Plot-3, as an average of –0.678 in general for the 9 wheat genotypes in the three plots (Fig. S7).

Discussion

ERECTA (ER) family belonging to RLKs could transduce extracellular signals into the cells to control a wide range of physiological responses (Shpak et al. 2004). *TaERECTA* gene, one copy of *ER* genes in bread wheat, was isolated from wheat cultivar Chinese Spring, structure analysis suggested its typical domain of ER family with the extracellular LRR region and the intracellular serine/threonine region. Compelling evidence conferred that ER protein truncated with the serine/threonine kinase region (AtΔKinase) dominant-negatively affected the wild-type plants of *Arabidopsis* (Villagarcia et al. 2012), these implied the important role of the kinase domain of *ER* family. Here, under IPTG induction for 3 h, TaER_Kinase was over-accumulated in *E. coli* strain, and confirmed by immunoblot, these indicated that the kinase domain of TaERECTA family in bread wheat could be expressed in prokaryotic system, and TaER_Kinase could be used to synthesize its specific antibody in the future identification of TaER family in wheat.

The expression of *TaERECTA* was variable in spikes, stems, leaves, and roots of bread wheat at different developmental stages, which were largely in accordance with the higher expression of ER family members in younger organs (Shpak et al. 2004; Xing et al. 2011). However, in roots and stems, *TaERECTA* performed the highest expression in some growth stages, these suggested that TaERECTA did not equally regulate the differentiation of each organ during the developmental stages of whole plants, but showed abundant transcripts in the rapidly differentiated tissues.

Previous findings suggested that GA-reliant growth mechanisms were necessary for the ER modulation on the elongation of plant growth in *Arabidopsis* (Ragni et al. 2011). In bread wheat, *TaERECTA* showed the negative response to GA_3 induction, suggesting the repressed effect of GA on the *ER*. *Erecta* mediated an ecotype-specific difference through ABA content with independent pathways (Häffner et al. 2014), the expression of *TaERECTA* under ABA treatment indicated a negative pattern, which further clarify *TaERECTA* interaction with ABA signals. An incubation with IAA resulted in increased activity in wild-type *ER* background and *er-103* seedlings (Woodward et al. 2005), while the response of *TaERECTA* to IAA treatment displayed an initially reduced expression followed by an increased pattern with the duration of treatment, these implied the interaction of ER and auxin was largely involved in downstream processes. ER was proposed to affect the hypocotyl length in response to BR treatment in *Arabidopsis*, the expression of *TaERECTA* under BR treatment presented significant decrease followed by the recuperative increase, although suffering from the merely overdose stress (0.75 μM/L), which supplement the suppressed effect of BRZ on the wild-type *ER* background and *er* mutations in *Arabidopsis* (Borevitz et al. 2002). ER improved not only the transpiration rate and water use efficiency of *Arabidopsis*, but also the active response of *TaERECTA* to the dehydration stress with an initially suppressed expression, followed by the restoring patterns in the responded period, implying that *TaERECTA* might be an useful candidate gene in the improvement of wheat breeding for stress resistance.

The spacing and pattern of stomata distribution limit the efficiency of gas-exchange in plants, affecting CO_2 assimilation and water loss. *ER*-family regulates stomata prolifera-

tion and epidermal patterning during plant growth. Three *ER*-family RLKs were reported to affect stomatal differentiation in overlapping but unique manners; loss-of-function in all three *ER*-family genes (*ER, ERL1* and *ERL2*) led to stomatal clustering and 50% to 200% increase in the stomatal index in *Arabidopsis* (Shpak et al. 2005). In the nine wheat lines, the expression levels of *TaERECTA* were negatively correlated with their stomatal density. However, difference in stomatal density across the different genotypes was not very pronounced except for the Watkins accessions *W209* and *W481* comparing to the cultivar Paragon. These results suggested that stomatal development was likely to be simultaneously regulated by other LRR-RLKs family members. Further insight into the function of different *TaER* genes will be gained by manipulating their expression in wheat. The results obtained to date suggested that *TaER* expression was correlated with stomatal differentiation and therefore variation in this gene family (natural or introduced) may be exploitable to improve agronomic trait of wheat improvement.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Figure 1*. The agarose gel electrophoretogram of *TaERECTA* in bread wheat M: 1500 maker; 1 and 2: the first segment amplified (1391 bp); 3 and 4: the second segment amplified (1938 bp)

Electronic Supplementary *Figure 2*. Detection of TaER_Kinase
(A) SDS-polyacrylamide gel electrophoretogram of TaER_Kinase; (B) Immunoblot of TaER_Kinase. Target protein is indicated by black arrow. 1: pET-32a-c(+) empty vector; 2: pET-32a-c(+)-TaER_Kinase protein (0 mM/L IPTG); 3: pET-32a-c(+)-TaER_Kinase protein (0.4 mM/L IPTG); 4: pET-32a-c(+)-TaER_Kinase protein (0.8 mM/L IPTG)

Electronic Supplementary *Figure 3*. Expression profiles of *TaERECTA* in wheat organs
Panel (A): at different developmental stages of wheat; Panel (B): at booting stage of wheat. The data was as the average of three biological replicates. The reference genes of semi-quantitative RT-PCR was *TaActin*, whereas *TaActin* and *TaSand* in qRT-PCR

Electronic Supplementary *Figure 4*. Expression of *TaERECTA* in response to exogenous ABA, GA_3, IAA and BR
Seedlings (Z12) were cultured in the solution of each treatment for required time periods. The control was cultured in water. The final concentration of the solution was 30 mM/L for GA_3, 0.75 μM/L for brassinolide (BR), 10 μM/L for IAA, 100 μM/L for ABA and 0.2 mM/L for $MgSO_4$, respectively. The leaves, stems and roots of each treated plant were mixed together and sampled for RNA extraction and cDNA synthesis. The reference genes were *TaActin* and *TaSand*. Uppercase letters in the figure represent significant difference between different time periods ($P < 0.01$)

Electronic Supplementary *Figure 5*. Expression of *TaERECTA* in response to Mg^{2+} stress and dehydration
Seedlings (Z12) were cultured in the solution of $MgSO_4$ (0.2 mM/L) to detect the response of *TaERECTA* on the Mg^{2+} stress. The leaves, stems and roots of each treated plant were mixed together and sampled for RNA extraction and cDNA synthesis. The reference genes were *TaActin* and *TaSand*. Uppercase letters in the figure represent significant difference between different time periods ($P < 0.01$)

Electronic Supplementary *Figure 6. TaERECTA* expression at booting stage and the stomatal density in fully developped flag leaves of the 9 wheat lines
Asterisks highlight significant differences in relation to the cultivar Paragon at the $\alpha = 0.05$ level

Electronic Supplementary *Figure 7*. Correlation between the expression of *TaERECTA* and stomatal density
Correlation analysis was processed between *TaERECTA* expression and stomatal density of nine wheat lines in each of the three plots. The mean of correlation coefficient between the relative expression of *TaERECTA* and the stomatal density was –0.678, which was significant at the $\alpha = 0.05$ level