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Isolation and characterization of *ERECTA* genes and their expression patterns in common wheat (*Triticum aestivum* L.)

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Abstract

The orthologue of Arabidopsis *ERECTA* gene (*ER*) in wheat, *TaER*, is considered to be a promising candidate gene for the genetic improvement of water use efficiency (WUE) and drought tolerance in breeding programs. In this study, we isolated two distinct homologues (*TaER1* and *TaER2*) of *TaER* genes in common wheat through in silico screening and PCR-based homologous cloning. Sequence analysis revealed that these two genes had a similar intron/exon structure with 27 exons and 26 introns, and each of them encode a putative Leucine-rich repeat receptor-like serine/threonine protein kinase in common wheat. The coding sequence (CDS) is 2928 bp for *TaER1*, encoding a protein with 975 amino acid residues, and 2931 bp for *TaER2* deduced to encode 976 amino acid residues, and their corresponding genomic DNA sequences are 6858 bp and 7114 bp, respectively. Cloning and sequencing of 55 *TaER* cDNA clones revealed five transcript variants of *TaER1* and nine spliced isoforms of *TaER2*, designated as *TaER1.1* to *TaER1.5* and *TaER2.1* to *TaER2.9* respectively. Genome specific primers were designed based on the sequence divergence of the promoter regions between the two homologous genes. PCR amplification from genomic DNA of Chinese Spring (CS) nullisomic-tetrasomic lines revealed that *TaER1* and *TaER2* were located on chromosomes 7D and 7B in common wheat, respectively. *TaER1* and *TaER2* have therefore been renamed as *TaER-D1* (GeneBank accession: JQ599260.2) and *TaER-B1* (GeneBank accession: JQ599261.2) respectively. Real-time quantitative RT-PCR analysis showed that the *TaER* genes were strongly expressed in young immature tissues and organs and could be up-regulated by numerous environmental stresses, such as drought, salinity, cold stress and heat stress. This indicated that it may play significant roles in wheat growth and development and be a regulator in the response to environmental stress.

Key words: Abiotic stresses; Expression pattern; *TaER* genes; Wheat.
Abbreviations: CDS- Coding sequence; CID- Carbon isotope discrimination; CS- Chinese spring; CTAB- Cetyltrimethyl ammonium bromide; LRR- Leucine-rich repeat; ORF- Open reading frame; QTL- Quantitative trait loci; RACE- Rapid amplification of cDNA ends; S_TKc- Serine/Threonine protein kinase catalytic domain ; TE- Transpiration efficiency; WUE- Water use efficiency.

Introduction

As one of the most important temperate cereals, common bread wheat feeds more than 40% of the total world population and provides 20% of total food calories and protein for human nutrition (Gupta et al., 2008). In China, wheat is mainly grown in the northern areas that are classified as arid and semi-arid regions. With projected climate change and increasing population, water deficit has become the primary limiting factor for grain production, especially for wheat production in China. The application of cultivation measures has been effective in improving the water use efficiency (WUE) of crops under rainfed and limited irrigation conditions, but WUE is clearly also dependent on the properties of the plant itself. Therefore, breeders have focused their attention on breeding varieties with higher WUE for coping with drought tolerance. Carbon isotope discrimination (CID), Δ, is a reliable and sensitive indicator negatively related to plant transpiration efficiency (Farquhar et al., 1982; Farquhar and Richards, 1984; Hubick et al., 1986), which had been considered to be the physiological WUE. As a result, Δ has been proposed as a valuable proxy measure of WUE to be used in breeding programs for wheat and other C_3 crops (Ehdaie et al., 1991; Hall et al., 1996; Kumar and Singh, 2009; Martin et al., 1999; Rebetzk et al., 2002). Direct measurement of Δ in breeding programs on large numbers of individuals is slow and costly. Therefore, to enhance the selection efficiency of Δ in breeding practice, quantitative trait loci (QTL) analysis has been conducted to elucidate its genetic control and identify markers that are predictive of Δ, and considerable progress has been made in various plants. QTLs for Δ have been identified in soybean (Mian et al., 1996, 1998), cotton (Saranga et al., 2001), rice (Price et al., 2002; Takai et al., 2006, 2009), barley (Teulat et al., 2002), Arabidopsis (Juenger et al., 2005; Masle et al., 2005; Mckay et al., 2008), *Brassica oleracea* (Hall et al., 2005) and tomato (Xu et al., 2008). In *Brassica*, Arabidopsis and tomato, a few major QTLs could explain more than 20% of the total phenotypic variation. The genetic control of Δ is typically complex in wheat and was associated with many loci each with small contributions to phenotypic variance, predominantly under additive genetic control (Rebetzke et al., 2006). Both additive and dominant gene effects were reported for progeny from different wheat crosses (Ehdaie and Waines, 1994; Rebetzke et al., 2006). Shahram et al. (2005) reported that

genes controlling the variation of Δ between wheat varieties under drought condition were located on chromosome 1D. Other QTLs for Δ have been located on chromosomes of 1A, 2B, 3B, 5A, 7A, and 7B in wheat (Wu et al., 2011). It was reported that QTLs for Δ are reproducible among different environments and different wheat mapping populations (Rebetzke et al., 2008). Compared with the application of Δ for selection in wheat breeding and work on the genetic control of Δ, progress on the molecular mechanisms that underlie Δ is less advanced. To date, just one locus has been identified as a major gene controlling Δ in higher plants. The *ERECTA* gene (*ER*) has been shown to be a major contributor to Δ in Arabidopsis, contributing to improved transpiration efficiency under both limited irrigation and well watered condition (Malse et al., 2005). Homologues of *ERECTA* have been isolated from diverse plant species, including maize, soybean, rice and sorghum (Guo et al., 2011). Transgenic maize plants over-expressing *ZmERECTA* showed positive effects on biomass accumulation, growth rate, organ size, and drought tolerance (Guo et al., 2011). Over-expression of *PdERECTA*, an *ERECTA* homolog from poplar, in Arabidopsis resulted in more rapid seedling establishment, longer primary roots and increased leaf area, as well as enhanced long-term WUE and dry-weight biomass of the transgenic lines compared with the wild-type (Xing et al., 2011). As the first major gene shown to modify transpiration efficiency in plants, *ERECTA* is a promising candidate gene in selection for WUE in wheat breeding programs. However, traditional selection for WUE through Δ relies on the measurement of CID to estimate Δ values of many different lines, a costly procedure which is unaffordable in the context of wheat breeding. Furthermore, the absence of reliable candidate genes or molecular markers for Δ has made it difficult to improve either the selection efficiency or the cost. Although homologues of *AtERECTA* have been isolated from many crops (Guo et al., 2011), there are neither published nucleotide nucleic acid sequences nor reliable molecular markers for this gene in common wheat to date. This limits direct selection for Δ and related traits in practical breeding. As an initial step to utilize this gene in improving WUE by either marker assistant selection or genetic engineering in wheat breeding, the objectives of this study were to (1) isolate the coding and promoter region of the wheat *ERECTA* gene (*TaER*); (2) Characterize the expression patterns of *TaER* in different tissues and under different abiotic stresses; (3) Confirm its chromosome location for developing functional markers.

Results

Cloning and characterization of TaER genes

Using the cDNA sequence of the rice *ER* gene as a probe for a BLAST search against the wheat EST database in GenBank, six wheat ESTs were identified. These ESTs were assembled to form a contig of 1193 bp. A further BLASTx search against *Brachypodium distachyon* proteome with this contig identified Bradi1g46450.1, a putative ERECTA-like serine/threonine protein kinase gene. A forward primer B1F (Table 1), designed on the cDNA sequence of Bradi1g46450.1, and a reverse primer B6 (Table 1), which was located on the assembled contig were used to amplify from cDNA of Chinese Spring (CS) and yielded a 2083-bp band. Cloning and sequence analysis revealed that the PCR product contained two very similar sequences, which showed a high sequence identity (97.36%) and included some single nucleotide polymorphisms.

Two 3' end fragments of 658 bp and 592 bp were obtained through 3' RACE with primers located on the conserved region, and a 973-bp fragment was obtained through 5' RACE. Based on the sequence identities of overlapped regions between different primer pairs, those fragments that shared 100% sequence identities in the overlapped region were assembled into two cDNA sequences of 2580 bp and 3204 bp, designated as *TaER1* and *TaER2*, respectively. Subsequent sequence analysis suggested that the putative full-length sequences amplified by I9/I8 (Table 1) were lacking the final exon when compared with Brachypodium *ERECTA*-like gene Bradi1g46450.1. BLAST searches with the final exon of Bradi1g46450.1 against a 5x coverage genomic survey of Chinese Spring (http://www.cerealsdb.uk.net) were assembled into two distinct sequences using Geneious Basic v5.4 (Drummond et al., 2011) and confirmed using RNAseq reads from wheat (cv. Mercia) peduncle (Gallová and Phillips, unpublished). Primer pairs P3/M2 (Table 1) and D3/M5 (Table 1) were used to amplify the missing 3' regions of *TaER1* and *TaER2*, respectively. The predicted coding sequences were 2928 bp for *TaER1* and 2931 bp for *TaER2*, encoding proteins with 975 and 976 amino acid residues, respectively. Comparing the ORF of *TaER2* with that of *TaER1* revealed two indels, a deletion of 6 bp nucleotides in *TaER2* from nucleotide 14 and a 9-bp insertion after nucleotide 50.. In addition, there were 74 SNPs in the coding regions. This variation between the two cDNA sequences resulted in a two-amino acid residues deletion (A6 and P7) and three amino acid residues insertion (SLL) and 9 amino acid residues substitutions of TaER2 compared with TaER1. SMART analysis of the deduced amino acid sequences showed that both of them had a extracellular domain consist of leucine-rich repeat (LRR) elements, a Serine/Threonine protein kinase (S_TKc) domain at the C-terminus, and a transmembrane region (amino acids 579 to 598) (Fig. 1), and shared the same conserved domains with Arabidopsis ER and rice ER. Thus, TaERs were receptor-like kinase proteins, falling into the Serine/threonine-specific kinase subfamily with the diagnostic subdomain VIb and VIII of this subfamily (Hanks and Quinn, 1991). They can be further classified to the RD kinase class for the presence of conserved arginine (R) and invariant catalytic aspartate (D) in the subdomain VI of receptor-like kinase (Afzal et al., 2008; Krupa et al., 2004). Multiple sequence alignment showed that the kinase domain of ER was highly conserved across plant species (Fig. 2). The genomic DNA sequences corresponding to ORFs of *TaER1* and *TaER2* were 6858 bp (accession number: JQ599260.2) and 7114 bp (GeneBank accession: JQ599261.2), respectively. Both *TaER1* and *TaER2* contained 27 exons and 26 introns, and additional indels and SNPs were identified in introns by sequence alignment. Cloning and sequencing of 55 *TaER* cDNA clones with universal primer set I9/I8 revealed two groups of distinct cDNA sequences, which were identical to *TaER1* or *TaER2*, respectively. Among those, five transcript variants of *TaER1* groups (20 clones), and nine spliced isoforms of *TaER2* groups (35 clones) were observed, and designated as *TaER1.1* to *TaER1.5* (Fig. 3a), and *TaER2.1* to *TaER2*.9 (Fig. 3b), respectively. Only the properly spliced isoforms *TaER1.1* and *TaER2.1* could produce putative full-length TaER proteins based on the predicted ORFs whereas the others contained premature stop codons (Fig. 3a and Fig. 3b).

Isolation of promoter region and chromosome location of TaER

After two nested rounds of PCR, two bands were isolated from the *Pvu*II digested Genome Walker library with sizes of 1071 bp and 2915 bp. Sequencing confirmed that the 1071-bp fragment was the upstream sequence of *TaER2*, with a 145-bp overlapped region which was identical with the first exon. To

Table 1. Primers used in this study.

Primer set	Sequence (5'→3')
B1F	AAGCACCTTGAAACCTTGATA
B6	ATGCCAAAGTCCGTAAGGTG
3' RACE Outer Primer	TACCGTCGTTCCACTAGTGATT
P1	ATGGCAGCCTCTGGGATGTTT
3' RACE Inner Primer	CGCGGATCCTCCACTAGTGATTTCACTATA GG
P3	CCTYGACAAAGATTACGAGCCA(Y=C/T)
5' RACE Outer Primer	CATGGCTACATGCTGACAGCCTA
P10	GAAGGAAGCCAATGTTGAAAGG
5' RACE Inner Primer	CGCGGATCCACAGCCTACTGATGATCAGT CGATG
P8	TTCCCAATGGTGTCTGGTATCTC
Ap1	GTAATACGACTCACTATAGGGC
G4	GAAGGTGACGTTGTCGCAGAG
Ap2	ACTATAGGGCACGCGTGGT
G2	TAATGGGGGAGTGAGTTCCTGGT
I9	CACTGCCCGGACGGTGATT
I8	ACGGAACACTGCAACCTACTA
I2	GAAACCAACCAGGAACTCAC
I7	AGGGGAGCAAACCGAAAAGT
J8	AAATATCCATGAGCCCCTAA
G4	GAAGGTGACGTTGTCGCAGAG
15.W2R	CTCTTTGTGGTGCAAGAGTC
I3	GGACGGAACACTGCAACCTA
41.W4F	CTACTCCGCATTTGATTAGG
G6	CGGACGGGAAAGGAACGAA
J8	AAATATCCATGAGCCCCTAA
G3	GAGGAGGGAGGCAATGAGC
M2	CCACCGTTTCAGAGTCCCAT
D3	CCTCGCTTACCTTCACCAT
M5	TTCTACAGTCAGCGGGAGCA
TF	TGTTTGCAGGCCGCATAGT
B4	TTTGCGGGTACTGGGCATACAG
k12	ACCCTGGAGGTGGAGTCAT
k16	CGAGACTTGTGAACCCAGAG

confirm the relationship between the 2915-bp fragment and *TaER1* genomic sequence obtained previously, the gene specific forward primer 41.W4F (Table 1) designed on the 2915-bp fragment was combined with the reverse primer G6 (Table 1) on the first intron of *TaER1*. This primer set yielded a 951-bp band as expected, and was confirmed to be the correct 5' flanking sequence of *TaER1* genomic sequence by subsequent sequencing. Compared with coding sequences, more insertions/deletions and sequence divergences were observed in the promoter regions between *TaER1* and *TaER2*, this formed the basis for designing gene specific primers for chromosome location of *TaER* genes. Thus, gene specific primer sets 41.W4F/G6 (for *TaER1*) (Table 1) and J8/G3 (for *TaER2*) (Table 1) were used to amplify genomic DNA from CS nullisomic-tetrasomic lines of homologous group 7. No PCR products were yielded in N7D-T7B for *TaER1* and in N7B-T7D for *TaER2*, indicating that *TaER1* was located on chromosome 7D and *TaER2* was located on chromosome 7B in common wheat (Fig. 4). Therefore *TaER1* and *TaER2* were renamed as *TaER-D1* and *TaER-B1* respectively.

Phylogenetic analysis

To compare with candidate homologues of wheat ER in grass species, a Phytozome BLASTp search was implemented within

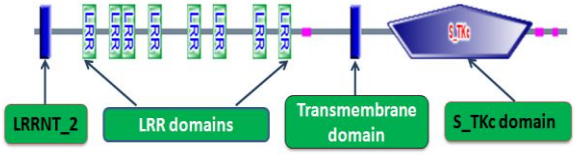

Fig 1. Schematic diagram of the conserved domains of TaER proteins. Domains abbreviations: LRRNT_2 (Leucine rich repeat N-terminal domain); LRR (Leucine rich repeats); S_TKc (Serine/Threonine protein kinases, catalytic domain). The conserve domains of TaER were predicted by SMART (http://smart.embl- heidelberg.de/) online software.

the Grasses node, and four highly similar gene families were identified. In total, 12 gene family members showed high similarity to *TaER*, and the distribution among grass species were as follows: three were identified in sorghum (*Sorghum bicolor*), three in Setaria (*Setaria italica*), three in maize (*Zea mays*), and two in rice (*Oryza sativa*), as well as two in *Brachypodium distachyon*. The amino acid sequences of these characterized or putative gene members, together with the identified ER proteins were used to build a phylogenetic tree (Fig. 5). The wheat ER proteins are most related to the predicted Brachypodium distachyon ER (Bradi1g 46450.1, accession: XP_003564133.1), then OsER (NP_001057087.1) as shown in figure 5.

Expression pattern of TaER genes in different tissues and under abiotic stresses

Expression of *TaERs* (both *TaER-B1* and *TaER-D1*) was detected in all tissue and organs except for roots (Fig. 6). The results indicated that *TaERs* were highly expressed in immature organs originated from shoot apical meristem such as young stem (25 weeks), young spike (27 weeks), and ovary (29 weeks), but weakly expressed in mature organs such as anther (29 weeks) and developing seeds (ovary, one weeks after pollination), corresponding to ESTs identified from sheath, flower and inflorescence deposited in Gene Bank. Two-months-old wheat plants were used to analysis the *TaERs* transcription levels in leaves of different age, and the result is shown in Figure 7. We found that the expression of *TaERs* decreased with increasing age of the leaves. *TaERs* were expressed at the highest level in newly developed leaves (not fully emerged from the sheath), but dramatically decreased in the fully expanded fourth leaf. Almost no transcripts were detected in the third, second and first fully expanded leaves compared with the youngest one. We investigated the effects of various abiotic stresses on *TaER* expression, including drought (simulated with PEG solution), salt and high/low temperatures (Fig. 8). *TaERs* transcripts showed a strong but transient increase in abundance after application of each stress, although the kinetics of induction was different in each case. *TaERs* transcripts started to accumulate after one hour treatment of PEG-simulated drought stress, and continually increased to a peak at the time point of 9 hours. The expression level decreased in the following 36 hours, though there was a small range of fluctuation when the time of treatment was longer than 24 hours; Under salt stress, the expression of *TaERs* was up-regulated rapidly in the first 6 hours, and then decreased rapidly in the next 3 hours; The transcription of *TaERs* could be regulated by high temperature (Fig. 8). The highest amounts of mRNA appeared at 6 hours under 37°C treatment， then decreased gradually until 12 hours, and increased to the highest

levels at 24 hours, and then decreased with some fluctuations; Under low temperature (4°C) treatment, increase in transcript levels could only be observed after long term treatment of 36 hours, and the highest levels were observed at 48 hours after treatment, but an intense decline was then observed at 24 hours after the highest expression appeared.

Discussion

A feasible and convenient way to isolate genes in common wheat

Common wheat is allohexaploid with a very large genome (16000 Mb), of which at least 80% is repetitive sequence (Gupta et al., 2008). The genome size and the abundant repetitive elements make positional cloning in wheat not a trivial exercise (Yan et al., 2003), especially in the current situation where a complete, assembled whole genome sequence is not yet available (Su et al., 2011). Comparative mapping has revealed that the linkage blocks of individual chromosomes of the genomes of six major grass species (Rice, Wheat, Maize, Foxtail millet, Sugar cane, Sorghum) can be rearranged into highly similar structures (Devos and Gale, 1997; Moore et al., 1995). These formed the basis for homology cloning in the target species based on the gene information located in any grass. In the present study, a PCR based homologous cloning method combined with *in silico* screening was used as the strategy to isolate wheat orthologs of the *ERECTA* gene. As in Arabidopsis, maize and poplar, the *TaER* genes also encode putative Leucine-rich repeat receptor-like serine/threonine protein kinase and shared the same gene structure with the Arabidopsis *ERECTA* gene (Torii et al., 1996) and *ZmERECTA* of maize (Guo et al., 2011). Multiple sequence alignment showed that the peptide homologs of TaER proteins were highly conserved among different species in higher plant, especially the protein kinase domains (Fig. 2). This implied significant roles for the *ER* gene not only in Arabidopsis (Malse et al., 2005; Shpak et al., 2003, 2004) and poplar (Xing et al., 2011) but also in other plants. The most similar homologues of *TaER* genes in the sequenced grasses were located on chromosome 1 of *Brachypodium* [Bradi1g46450, (XP_003564133.1)], chromosome 6 in rice [*OsER* (NP_ 001057087.1)], scaffold 4 of millet (Si005775m.g), chromosome 6 in maize (GRMZM5G809695) and chromosome 10 in sorghum [Sb10g006670 (XP_002438023)]. Comparative genetics has demonstrated that orthologous genes mapped on these chromosomes showed extensive synteny with wheat chromosomes of group 7 (Gale et al., 1998a, b; Devos, 2005; The International Brachypodium Initiative, 2010). Therefore, nullisomic-tetrasomic lines of homologous group 7 were used for a rapid confirmation of potential location of *TaERs* (Fig. 4), and the results also confirmed the good colinearity of gene order across the homologous chromosomes between grass subfamilies. Although the chromosome location of *TaER* genes is different from previously QTL analysis for Δ in common wheat (Shahram et al., 2005), further studies is essential to confirm whether it is involved in the determination of Δ in common wheat as has been shown in Arabidopsis (Malse et al., 2005). To date, PCR based homology cloning is still the most powerful and widely used approach for gene cloning in common wheat. This strategy has become more feasible with the availability of the *Brachypodium* genome sequences, which shares 77% to 84% gene families with rice and sorghum, and is much more closely related to wheat than to rice (The International *Brachypodium* Initiative, 2010). This landmark will have a far-reaching impact on the functional genomics of wheat, and characterization of homologous genes in common wheat becomes more convenient and facile as well.

A modulator of plant growth and response to environment

In this study we cloned two highly similar orthologues of the Arabidopsis *ERECTA* gene from common wheat. They were predominantly expressed in immature tissues and organs originating from the shoot apical meristem which was similar to Arabidopsis (Yokoyama et al., 1998), such as young developing leaves leaf, young stem and young spike etc. These findings suggest that *TaER* genes may regulate plant development beginning in early stage of seedling establishment, and persisted throughout the whole development process. Similar spatially and temporally expression manner with Arabidopsis *ERECTA* (Yokoyama et al., 1998) and *PdERECTA* (Xing et al., 2011), including strong expression in younger organs originated from shoot apical meristem which processed a powerful cell division and differentiation capacity and lower transcripts detected in the older tissues, implied that the *TaERs* might play similar roles in the regulation of wheat growth and development as Arabidopsis *ERECTA* and *PdERECTA*. We also investigated the expression responses of *TaERs* to environmental stresses. The results indicated that the transcription of *TaERs* could be up-regulated by various stress treatments, such as drought stress simulated with PEG, salinity, high temperature and low temperature. The expression patterns revealed by quantitative real time RT-PCR were supported by the identification of ESTs derived from multiple tissues and different libraries under abiotic stress conditions, such as wheat pre-anthesis spike library (BF484583.1, BQ171292.1, BG263694.1), and the salt-stressed sheath cDNA library (BG313266.1). Although the mechanism of *TaERs* mediated signal transduction is unclear, our research has indicated that *TaER* genes could respond to various environmental stresses at the seedling stage. In Arabidopsis, the function of ER is not limited to the regulation of plant development and transpiration efficiency. It plays a role in the resistance to bacterial wilt infected by bacterium *Ralstonia solanacearum* (Godiard et al., 2003). In addition, it is essential for resistance to necrotrophic fungus Plectosphaerella cucumerina in Arabidopsis (Llorente et al., 2005), and mediates the recognition and signaling of defense activation in the defense response to *Pythium irregular* (Adie et al., 2007). Interestingly, ESTs of *TaERs* were also found in the wheat *Fusarium graminearum* infected spike cDNA library, such as BQ162058.1, CN012959.1 etc. It suggests that *ER* gene may be involved in the response to pathogen in common wheat as well. Although little is known about the role of ER in the response to heat stress, it can reduce damage to leaves under high temperature in Arabidopsis (Qi et al., 2004). In common wheat, *TaER* genes showed the up-regulated expression when confronted with high temperature, and this implied a role similar to the response of ER to heat stress in Arabidopsis. In addition, *ER* gene is responsible for microenvironment canalization of rosette leaf number in the long-day environment (Hall et al., 2007) and seed mineral accumulation under low-light conditions (Waters and Grusak, 2008) in Arabidopsis . Clearly, further work is required to characterize the various signaling pathways mediated by ERECTA, these examples along with results from this study are sufficient to draw the conclusion that ERECTA is heavily involved as a modulator of signal transduction pathways in response to phonological changes as well as environmental changes in plants (Zanten et al., 2009).

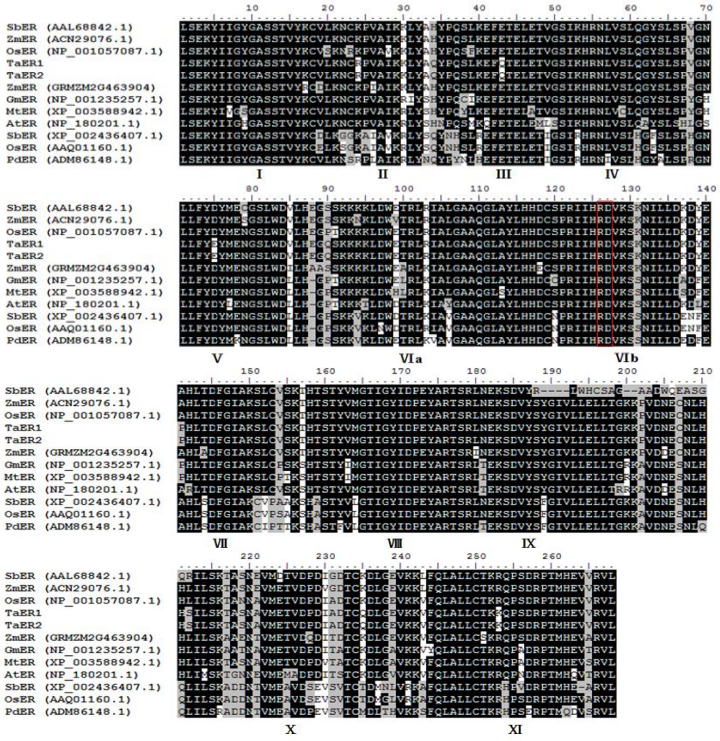

Fig 2. CLUSTAL X alignment of the kinase domains of different ER proteins. The 11 diagnostic subdomains of a typical protein kinase are indicated by Roman numerals. The highly conserved arginine (R) and aspartate (D) are highlighted with red rectangle. Threshold for shading with black boxes is 60%. The Genbank accession numbers for *TaER-D1* (*TaER1*) and *TaER-B1* (*TaER2*) are JQ599260.2 and JQ599261.2 respectively. Species abbreviations: Ta= *Triticum aestivum*, OS=*Oryza sativa*, Sb=*Sorghum bicolor*, Zm=*Zea mays*, At=*Arabidopsis thaliana*, Gm=*Glycine max*, Mt=*Medicago truncatula*, Pd=*Populus nigra* x (*Populus deltoides* x *Populus nigra*)

A promising candidate gene for molecular improvement of wheat

The identical gene structure, highly conserved protein functional sites and domains and similar expression profile of *TaER* to its homologues indicate a similar role and mechanism in regulating plant growth and development in common wheat. As a pleiotropic gene, potential uses of *TaER* include overexpression in transgenic wheat to increase biomass accumulation, resistance to pathogens and improve tolerance to drought, salt, heat and other abiotic stresses. Tissue-specific overexpression driven by specific promoters may increase the sizes of spike, spikelet and potential of increased protein, starch and mineral content. At this stage, the most promising aspect is the utilization of *TaER* genes in improvement of wheat WUE by regulating transpiration efficiency, since the *ERECTA* gene has been characterized as a major gene controlling plant transpiration efficiency (Masle et al., 2005) and enhancing WUE of Arabidopsis through overexpression of *PdERECTA*, a orthologue of *ERECTA* (Xing et al., 2011). The isolation and chromosome location of *TaER* genes thus forms the basis for characterization of allelic variation and functional marker development in common wheat and can be practical in directional selection for Δ and related traits in wheat breeding programs.

Materials and methods

Plant materials

The common wheat line Chinese Spring (CS) was used for isolating the full length cDNA and genomic DNA sequences of *TaER* genes and analysis of their expression patterns. The Chinese Spring (CS) nullisomic-tetrasomic lines of homoeologous group 7 were used for the chromosome localization. These materials were sown at No.1 farm of Northwest A&F University, Yangling, China, in October, 2010.

Samples for expression analysis of TaER* genes *in tissues

Tissue samples of shoot (2 weeks), root (2 weeks), sheath (23 weeks), young stem (25 weeks), young spike (27 weeks), anther (29 weeks), ovary (29 weeks), and seeds (7 days after flowering) of CS were collected from plants grown in the field under natural conditions and were used for analysis of tissue specific expression.

Stress treatments for expression analysis of TaER genes

Well-filled seeds of CS were germinated in a growth chamber at 20°C for 5 days under a 12-h light/12-h dark photoperiod. The most uniform seedlings were transplanted into a 96-well culture plate from which the well bottoms had been removed. Plates were placed on the inverted lids of Petri dishes with a diameter of 120 mm such that the roots could be immersed in deionized water without injury. The seedlings were transferred to different solutions or temperatures for stress treatments at the stage when the second leaves were emerged but not fully expanded. For drought stress treatment, the plate was transferred to a 16.1% PEG6000 (-0.5Mpa) solution. A solution of 250mM NaCl was used to simulate salt stress. For high temperature and low temperature treatments, plates were directly moved to 37°C and 4°C, respectively. The growth temperature for drought and salt stress treatment was 20°C and all the treatments shared the same photoperiod of 12-h light/12-h dark.

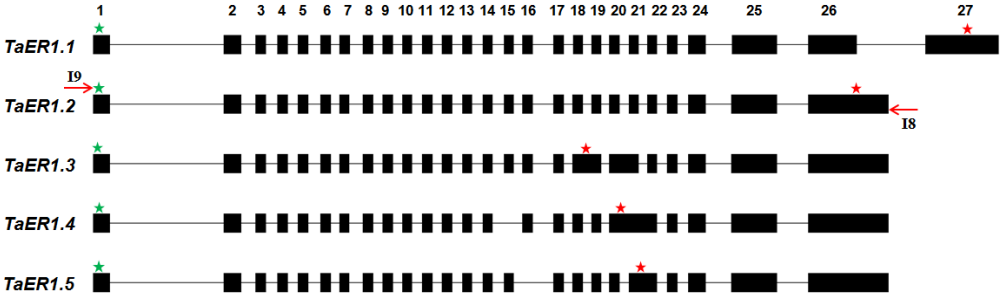

Fig 3a. Schematic diagram of transcript variants of *TaER1* (*TaER-D1*). *TaER1.1* is the only transcript with 27 exons coding a complete TaER1 protein; *TaER1.2* transcripts continue beyond the GT splicing site after exon 26 until a stop codon 19–bp after the splicing site; *TaER1.3* transcripts retain introns 18 and 20 in the mature mRNA, and thus introduce a premature stop codon in intron 18 (1-bp after exon 18); *TaER1.4* transcripts lack exon 15 and retain intron 20 and 21, these changes in open reading frame generate a stop codon 1-bp after exon 20; *TaER1.5* transcripts do not include exon 16 and continue through intron 21, which generates a change in reading frame and a stop codon 3-bp after exon 21. Stars indicate start codon (green) and stop codon (red). Lengths of the sequences from *TaER1.2* to *TaER1.5* amplified with I9/I8 are 2861bp, 3016bp, 2946bp, 2864bp, respectively. Numbers of exons are indicated above. Sequence alignment and picture preparation was prepared by Geneious Basic v5.4 software.

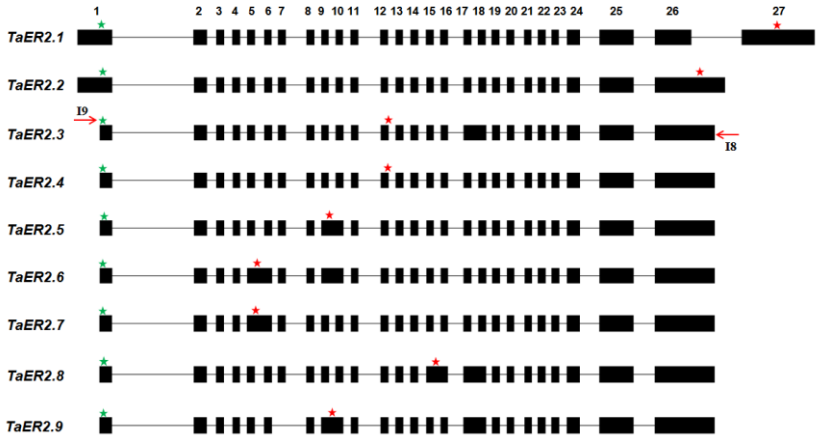

Fig 3b. Schematic diagram of alternative transcript variants of *TaER2* (*TaER-B1*). *TaER2.1* is the only transcript with 27 exons encoding the complete TaER2 protein with 976 amino acid residues; *TaER2.2* transcripts continue beyond the GT splicing site after exon 26 until a stop codon 19-bp after this splicing site; *TaER2.3* transcripts have an alternative GT splicing site located 3-bp before intron 9 and retain the 17th intron in mature mRNA, resulting in a stop codon located 6-bp before exon 13; *TaER2.8* transcripts continue beyond the GT splicing site after exon 15 and exon 17, these changes in ORF generate a stop codon located in intron 15 (1-bp after exon 15); *TaER2.4* transcripts have an alternative GT splicing site located on exon 9 (3-bp before the conserved GT splice site), resulted in a premature stop codon the same as *TaER2.2*; the retention of intron 9 in *TaER2.5* generates a stop codon 23-bp after the GT splicing site; *TaER2.6* transcripts have a premature stop codon 1-bp after exon 5, result from the retention of intron 5 and intron 9 in mature mRNA; *TaER2.7* transcripts continue through the intron 5 and generate a stop codon as with *TaER2.6*; *TaER2.9* transcripts do not include the seventh exon, but conserve the intron 9 and intron 17, these differences generate a stop codon 23-bp after the conserved GT splicing site of intron 9. Start codon and stop codon are indicated by green stars and red ones. Numbers of exons are indicated above, and lengths of the transcript variants from *TaER2.2* to *TaER2.9* are 3204 bp, 2941 bp, 2859 bp, 2946 bp, 3053 bp, 2971 bp, 3025bp and 2956 bp, respectively. Sequence alignment and picture preparation was prepared by Geneious Basic v5.4 software.

DNA extraction, RNA isolation and synthesis of the first strand cDNA

Genomic DNA was extracted from young leaves using a CTAB method (Murray and Thompson, 1980). Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. Gel electrophoresis and a Gene Quant Pro spectrophotometer (Amersham Biosciences, USA) were used to confirm the quality and estimate the concentration of genomic DNA and total RNA. The Reverse Transcription System (Promega, USA) was adopted to synthesize the first-strand cDNA.

Cloning of the full-length cDNA of TaER genes

In silico cloning was employed to accelerate the cloning of *ERECTA* in wheat. The cDNA sequence of rice *ER* (GenBank accession: NM_001063622) was used as a probe for a BLAST search against the wheat EST database in GenBank (http://www.ncbi.nlm.nih.gov/). All ESTs identified by the query sequence with an E value less than e-100 and with a nucleotide sequence identity of more than 80% were downloaded for contig assembly using the CAP3 Sequence Assembly Program (Huang and Madan, 1999). The assembled contig was used to search against the *Brachypodium distachyon* genome on Phytozome v7.0 (http://www. phytozome. net/) to

identify homologous sequences. Primers B1F/B6 (Table 1) were designed based on the most conserved regions of the cDNA sequence of *Brachypodium distachyon* and the wheat contig using Premier Primer 5 software (http://www.premierbiosoft.com) and synthesized by Shanghai Sunny Biological Technology Co., Ltd (http://www. sunnybio.cn/). The 5'-Full RACE Kit (TaKaRa, Dalian, China) and the 3′-Full RACE Core Set Ver.2.0 Kit (TaKaRa, Dalian, China) were used for isolating the 5' end and 3' end cDNA sequences of *TaER* genes, respectively. The target fragments obtained were purified by Agarose Gel DNA Purification Kit (GenClean column, Generay, Shanghai, China), and cloned into PMD18-T vector (TaKaRa, Dalian, China) and transformed into competent cells of *E. coli* DH5a by heat shock. Positive clones were sequenced by Shanghai Sunny Biological Technology Co., Ltd (http://www. sunnybio.cn/). At least three times independent PCR reactions and sequencing were performed to guarantee the accuracy of sequencing. The putative full-length open reading frames (ORF) of these two *TaER* cDNA sequences were then amplified with primer set I9/I8 (Table 1).Methods for Gel Purification, cloning, transformation, and sequencing were as detailed above.

Genomic DNA sequences isolation of TaER genes and their chromosome location

The upstream genomic DNA sequences of *TaER* were obtained using the Universal Genome Walker^{TM} Kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instruction. Two rounds of PCR reactions were performed with TaKaRa LA Taq® Hot Start Version (TaKaRa, Dalian, China). The primer combination AP1/G4 (Table 1) and AP2/G2 (Table 1) were used for the first and secondary PCR amplification, respectively. The major bands were recovered and sequenced as above. Based on sequence variation in the upstream region, two forward gene-specific primers J8 (Table 1) and 41.W4F (Table 1) were designed. They were combined with the reverse primers G4 and G6 (Table 1), respectively, to form two pairs of gene specific primers. The Chinese Spring (CS) nullisomic-tetrasomic lines of homoeologous group 7 were used for chromosome localization of *TaER* genes with primer combinations of 41.W4F/G6 and J8/G4 (Table 1). The primer pairs I2/I7 and P3/M2 (Table 1) were used to amplify the genomic sequence of *TaER1* in CS nullisomic 7B-tetrasomic 7D line (N7B-T7D). Three sets of primers J8/G4, 15.W2R/I3 and D3/M5 (Table 1) were used to detect the full-length *TaER2* genomic sequence in CS N7D-T7B line. Procedures for gel purification, cloning, transformation, and sequencing were the same as previously described above, except that pGEM-T Easy vector (Promega, USA) was used for cloning fragments larger than 3 kb.

Sequence analysis and phylogenetic analysis

Bioedit (Hall, 1999) and Geneious Basic v5.4 (Drummond et al., 2011) software were used for sequence assembly, multiple sequence alignment, sequence translation, and figure preparation. The intron positions were determined by alignment of the genomic sequences of *TaER* with their corresponding cDNA sequences using Splign (http://www.ncbi.nlm.nih.gov /sutils/splign/splign.cgi). SMART (http://smart.embl-heidelberg.de/) was used for predicting conserved domain and functional sites; The BLASTp program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Phytozome v7.0 (http://www. phytozome.net/) were used to identify peptide homologs among different species of higher plants. A phylogenetic tree was constructed from the CLUSTALX 2.0

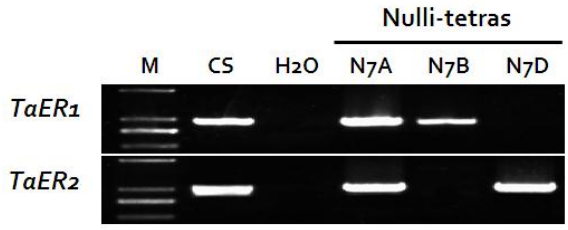

Fig 4. Chromosome location of *TaER1* and *TaER2*. Gene specific primers J8/G3 and 41.W4F/G6 were used to amplify from genomic DNA of the Chinese Spring nullisomic-tetrasomic lines corresponding to homoeologous group 7. The first lane is a lane marker (DL2000), followed by normal Chinese Spring, no template control (deionized water), nulli-tetrasomic lines of N7A-T7B, N7B-T7D and N7D-T7A. *TaER1* and *TaER2* were thus located on chromosome 7D and 7B, respectively. Therefore, they were renamed as *TaER-D1* and *TaER-B1* respcetively.

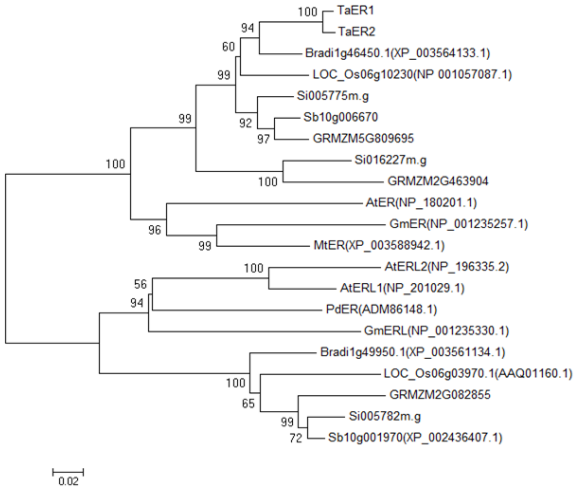

Fig 5. Phylogenetic tree of ER proteins in higher plant. Neighbor-joining tree based on the complete protein sequence showing the relationships between wheat TaER and other higher plant ER proteins. Bootstraps are based on 1000 replications and indicated in their respective nodes. Sequences from BLASTp search target at the grass node in the Phytozome v 7.0 and NCBI databases with the most significant E values. As shown in figure, the ER from the grass species cluster together, and the Bradi1g46450.1 (XP_003564133.1) is the closest peptide ortholog to TaER. The Genbank accession numbers for TaER-D1 protein (TaER1) and TaER-B1 protein (TaER2) are AFJ38186.2 and AFJ38187.2 respectively. Species abbreviations: Ta= *Triticum aestivum*, Bradi=*Brachypodium distachyon*, Os=Oryza sativa, Sb=*Sorghum bicolor*, Si=Setaria italica, GRMZM=Zea mays, At=*Arabidopsis thaliana*, Gm=*Glycine max*, Mt=*Medicago truncatula*, Pd=*Populus nigra x (Populus deltoides x Populus nigra*).

(Larkin et al., 2007) multiple sequence alignment using MEGA 5 (Tamura et al., 2011).

Expression analysis of TaER genes by real-time quantitative RT-PCR

Total RNA extracted from organs of shoot (2 weeks), root (2 weeks), sheath (23 weeks), young stem (25 weeks), young

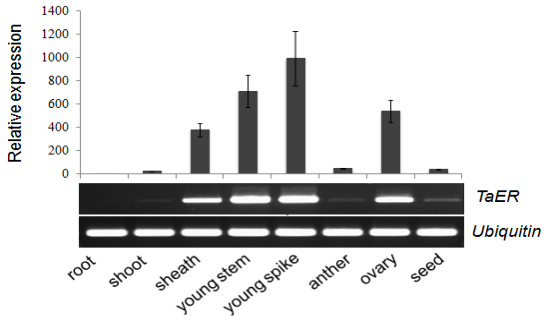

Fig 6. Tissue specific expression profile of *TaERs*. Transcripts levels of *TaERs* in different tissues by qRT-PCR. Time points for sampling from left to right were 2 weeks (root), 2 weeks (shoot), 23 weeks (sheath), 25 weeks (young stem), 27 weeks (young spike), 29 weeks (anther), 29 weeks (ovary), 7 days after pollination (seed). Plants were grown under natural condition. *Ubiquitin* was used as endogenous control. Each data point is the average of 3 biological replicates ± SE of the mean.

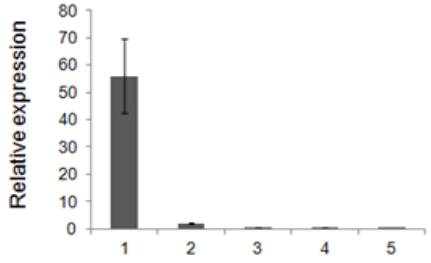

Fig 7. *TaERs* transcription levels in leaves of different age relative to *Ubiquitin*.The five green leaves from main stem of two-month-old CS seedling under field condition were used for preparing RNA for qRT-PCR. Number 1 indicates the heart leaf (the youngest but not full emerged from the sheath), followed by the fourth, the third, the second fully expanded green leaves from left to right in order. Number 5 indicates the oldest green leaf. Each data point is the average of 3 biological replicates ± SE of the mean.

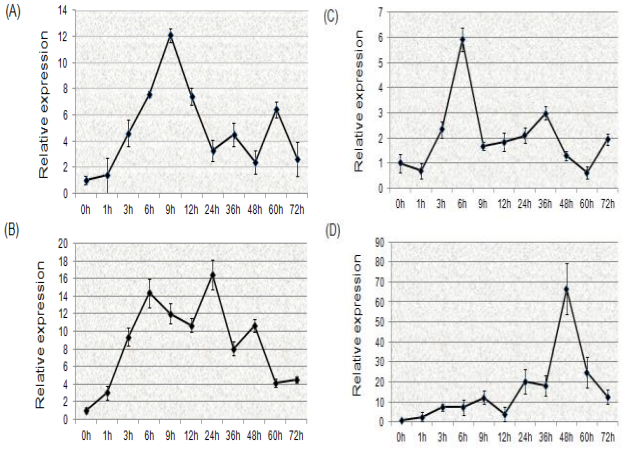

Fig 8. Expression patterns of *TaER* in CS under different stress treatments by qRT-PCR. (A) Treatment with 16.1% PEG6000 (-0.5Mpa). (B) Salt stress simulated by 250mM NaCl. (C) & (D) Treatment at high temperature (37°C) and low temperature (4°C) for heat stress and cold stress, respectively. Growth temperature for PEG treatment and salt stress is 20°C. All the treatment shared a photoperiod of 12-h light/12-h dark. Non-treatment plants were used as controls. *Ubiquitin* was used as endogenous control and each data point is the average of 3 biological replicates ± SE of the mean.

spike (27 weeks), anther (29 weeks), ovary (29 weeks), seeds (7 days after flowering) of CS grown in the field under natural condition were used for analysis of tissue specific expression. To quantify transcript levels of *TaER*s (*TaER1* and *TaER2*) in leaves at different age, total RNA was extracted from each of the five green leaves from the main stem of the same individual at two months after seeding; For expression analysis under stress treatments, leaves were collected at the time point of 1 h, 3 h, 6 h, 9 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h after treatment, and the leaves at 0h treatment were used as the control. All samples collected were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation. First strand cDNA for q-PCR was synthesized with PrimeScript® RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer' instruction. The expression analysis was performed with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Dalian, China) on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primer pairs of TF/B4 were used for detecting the expression of *TaERs* based on the shared regions of their cDNA sequences. The amplification procedure included an initial step of 95°C 10s, followed by 40 cycles of 95°C 5s, 60°C 31s. The wheat ubiquitin gene (X56601.1) was used as an endogenous control with primer pairs of k12/k16 (Table 1).Reactions were performed with three biological replications to estimate average and standard deviation. To monitor contamination, an additional no-template control was set up for each sample simultaneously. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to calibrate relative expression level of *TaERs* with the reference gene.

Conclusion

In this study, we isolated two similar homologues of *Aradidopsis ER* gene in common wheat. Each of them encoded a putative Leucine-rich repeat receptor-like serine/threonine protein kinase, and shared high similarity with the previous identified ER proteins. *TaER1* was located on chromosome 7D and *TaER2* was located on chromosome 7B. Thus, they were renamed as *TaER-D1* and *TaER-B1* respectively. *TaER* genes were found to be expressed in a spatially and temporally regulated manner. They were strongly expressed in young immature tissues and organs such as young stem, young spike, but weakly expressed in roots, anther, and developing seeds. Up-regulated expression of *TaER* genes under different stress treatments implied potential roles in the tolerance to abiotic stresses during plant growth and development. Although further studies are required to reveal their exactly functions in plant growth and development, the cloning and charicterization of *TaER-B1* and *TaER-D1* in common wheat from the basis for developing functional markers for this gene towards marker assisted selection in wheat breeding programs for WUE.

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