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Characterization of a wheat C2 domain protein encoding gene regulated by stripe rust and abiotic stresses

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Abstract

Plant C2 domain proteins play important roles in diverse cellular processes including growth, development, and membrane targeting, as well as in abiotic and biotic stress adaptations by sensing intracellular Ca^{2+} signals. In this study, we isolated a novel C2 domain protein gene, *TaERG3*, from wheat infected by *Puccinia striiformis* f. sp. *tritici*. *TaERG3* was predicted to encode a 144 amino acid protein with molecular mass of 15.68 kD and isoelectric point of 3.93. Analysis of the deduced amino acid sequence of *TaERG3* using *InterProScan* revealed the presence of an N-terminal calcium-dependent phospholipid-binding module (C2 domain, 5 to 103). Transient expression analysis showed that the TaERG3 protein was predominately and uniformly localized in the plasmalemma and nucleus of onion epidermal cells. Quantitative real-time PCR analyses indicated that *TaERG3* transcript was differentially induced in both incompatible and compatible interactions, as well as by applied abscisic acid (ABA) and $CaCl_2$. However, the significant transcript changes induced by methyl jasmonate, ethylene, and salicylic acid treatments were not as dramatic as those induced by ABA. *TaERG3* was also up-regulated by environmental stimuli including low temperature and high salinity. These results imply that *TaERG3* might be involved in wheat defence responses against stripe rust and abiotic stresses in an ABA-dependent signalling pathway.

Additional key words: abscisic acid, ethylene, expression pattern, low temperature, methyl jasmonate, *Puccinia striiformis,* salicylic acid, salinity, *Triticum aestivum*.

Introduction

Cold, drought, salinity, and pathogen infection are the major abiotic and biotic stresses that influence plant growth and development. To overcome detrimental effects, plants have evolved multiple signalling pathways to establish arrays of physiological and biochemical processes. The transient increase of cytosolic calcium

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Abbreviations: ABA - abscisic acid; APR - adult plant resistance; *BLAST* - basic local alignment search tool; C2 domain - C2 calcium-dependent membrane targeting module; CT - threshold value; ERG - elicitor responsive gene; ET - ethylene; HIR - hypersensitive induced response; hpi - hour post inoculation; hpt - hour post treatment; HR - hypersensitive response; MeJA - methyl jasmonate; ORF - open reading frame; PCD - programmed cell death; *PST* - *Puccinia striiformis* f. sp. *tritici*; qPCR - quantitative real-time polymerase chain reaction; RT-PCR - reverse transcription-PCR; SA - salicylic acid; TDF - transcribed derived fragment.

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concentration is a primary event in response to various external stimuli (Hetherington and Brownlee 2004). Higher plants express a variety of proteins with different Ca^{2+} signatures, including EF-hand domains, C2 domains, *etc*., to sense intracellular Ca^{2+} signals and guarantee the specificity of the ensuing physiological responses in the downstream pathways (Nalefski and Falke 1996).

Of the Ca^{2+} sensor proteins, the majority contain a conserved calcium-dependent membrane targeting module (C2 domain). The C2 domain identified from protein kinase C could bind phospholipids in a Ca^{2+}-dependent manner (Nalefski and Falke 1996). The presence of five aspartic acid residues, forming a Ca^{2+}-binding site, in most of the C2 domain proteins is highly conserved in plants and animals (Kim *et al*. 2003). In plants, C2 domain proteins are implicated in growth (Xoconostle-Cazares *et al*. 1999), development (Yang *et al*. 2008), senescence (Ouelhadj *et al*. 2005), membrane targeting (Kim *et al*. 2008, Schapire *et al*. 2008, Wang *et al*. 2009, Yamazaki *et al*. 2010), as well as abiotic and biotic stress responses (Kim *et al*. 2000, 2003, 2004, Yang *et al*. 2006a,b, 2007, Wang *et al*. 2009).

The first reported plant C2 domain protein was Cmpp16-1 from pumpkin involving RNA delivery (Xoconostle-Cazares *et al*. 1999). *OsERG1*, a C2 domain gene identified in rice suspension-cultured cells treated with fungal elicitors, was involved in defence responses and membrane translocation in a Ca^{2+}- dependent manner (Kim *et al*. 2000, 2003). Three C2 domain proteins including barley HvC2d1 (Ouelhadj *et al*. 2005), mung bean VrPLC3 (Kim *et al*. 2004), and *Arabidopsis* SYT1 (Schapire *et al*. 2008, Yamazaki *et al*. 2010), were all implicated in stress response and plant development through a Ca^{2+}-dependent signalling pathway. In addition, *CaRSC2-1* was targeted to the plasma membrane in a Ca^{2+}-dependent manner and played a vital role in the abiotic stresses and defence responses (Kim *et al*. 2008). Overexpression of *OsSMCP1* increased tolerance to abiotic and biotic stresses in transgenic *Arabidopsis* (Yokotani *et al*. 2009). However, negative regulation of C2 proteins in plant responses has also been reported. The *Arabidopsis* BAP and BON proteins negatively regulated defence responses mediated by disease resistance genes and also inhibited a general programmed cell death (PCD) (Yang *et al*. 2006a,b, 2007). Canola IPG-1, on the other hand, was involved in the host PCD in a Ca^{2+}-dependent manner (Wang *et al*. 2009). Thus, C2 domain proteins play many important roles in diverse cellular processes by coordinately transmitting messages coded by Ca^{2+} transients to intracellular interactive proteins. However, their roles and signalling networks in plant defence responses are unclear and need further investigation.

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is one of the most damaging diseases of wheat worldwide. Adult plant resistance (APR) provides sustainable control of the disease in field (Chen 2005). To dissect the molecular mechanisms of APR, we carried out transcriptomic analysis of wheat APR to stripe rust using the cDNA-AFLP technique and obtained a large number of differentially expressed transcribed derived fragments (TDFs) (Zhang *et al*. 2010). Among the TDFs, aTaPST_225 (GenBank acc. No. FL645977) showed high homology (80 %) with a rice C2 domain protein OsERG3 (acc. No. Q25AG5). Here, a C2 domain protein encoding gene *TaERG3* in wheat was identified using *in silico* cloning and RT-PCR. The molecular characteristics of *TaERG3* were analyzed, and expression profiles in response to infection by the stripe rust pathogen and treatments with $CaCl_2$ and defence-related hormones along with environmental stresses were examined using quantitative real time PCR (qPCR). The results of our analysis will be useful for further characterization of *TaERG3* in regulatory networks during plant defence responses to biotic and abiotic stresses.

Materials and methods

Wheat (*Triticum aestivum* L.) cultivar Xingzi 9104 and *Puccinia striiformis* Westend. f. sp. *tritici* Eriks (*PST*) pathotype CYR32 were the biological materials. Adult plants of Xingzi 9104 are resistant to CYR32 (Zhang *et al*. 2010). Wheat plants were grown following the procedure described by Wang *et al*. (2007). For pathogen challenge, inoculation was carried out with freshly collected urediniospores (Zhang *et al*. 2010). Parallel mock inoculations were made with sterile water. Three-week-old wheat plants were subjected to different concentrations of $CaCl_2$, ethylene (ET), salicylic acid (SA), abscisic acid (ABA), and methyl jasmonate (MeJA), and environmental stresses including high salinity (200 mM NaCl), low-temperature (4 °C), and wounding (Xia *et al*. 2010a). Leaves were harvested at different time points as indicated and immediately frozen in liquid nitrogen for total RNA extraction. Three independent biological replications were performed for each experiment.

Total RNA from each sample was extracted using *Trizol*^{TM} reagent (*Invitrogen*, Carlsbad, CA, USA). The quality and integrity of total RNA were determined by running an appropriate amount in a formamide denaturing gel and quantity of total RNA was examined using a *NanoDrop*^{TM} *1000* spectrophotometer (*Thermo Fisher Scientific*, Waltham, MA, USA). The first strand cDNA was synthesized with 2 μg of total RNA using the RT-PCR system (*Promega*, Madison, WI, USA) with a random hexamer $pd(N)_6$ primer.

The TDF aTaPST_225 was used as a query probe to search wheat EST database in GenBank. Homologous sequences were retrieved and assembled. To verify the assembled sequence, a forward primer (5’-AGAAGA TGGCGCAGGGA-3’) and a reverse primer (5’-GTC ACGCACCATAGTTCAGC-3’) were used to amplify the full length cDNA of *TaERG3*. The resulting PCR products were cloned to *pGEM-T Easy Vector* (*Promega*) and sequenced with an *ABI PRISM 3130XL* genetic analyzer (*Applied Biosytems*, Foster City, USA). *DNASTAR v7.10* (*DNASTAR*, Madison, WI, USA) was used for alignment of the deduced protein sequences. A neighbor-joining phylogenetic tree was generated by *MEGA 4.0* (Tamura *et al*. 2007).

For subcellular localization of the TaERG3-GFP fusion protein, *TaERG3* open reading frame (ORF) was first cloned with a forward primer containing a *Hind*III site (5’- CCCAAGCTTATGGCGCAGGGGACG-3’) and a reverse primer 5’-CGCGGATCCAGATGATTGGT TCCACCCACC-3’) containing a *BamH*I site. The 450 bp fragment containing the coding region of the ORF was digested with *BamH*I and *Hind*III followed by cloning into the *pCaMV35S:GFP* to form a *TaERG3-GFP* fusion construct. This construct and a control vector (*pCaMV35S:GFP*) were transformed into onion epidermal cells by particle bombardment using the *PDS-1000/He* system (*Bio-Rad*, Hercules, USA). GFP signals were detected using a *BP 505-550* emission filter set under excitation with the 488 nm argon-ion laser line and visualized by a *LSM 510 META* confocal laser microscope (*Carl Zeiss*, Jena, Germany).

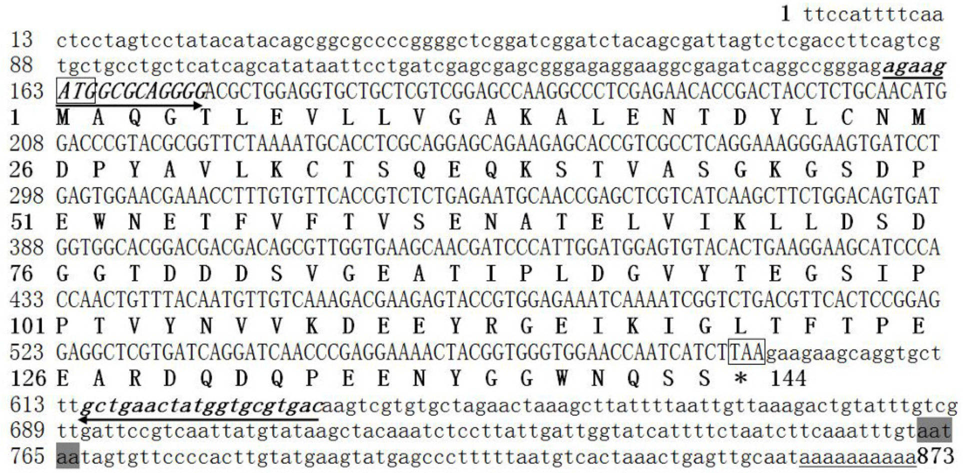
Tissue-specific expression (leaf, stem, and root) and expression profiles of *TaERG3* after various treatments were determined using quantitative real time PCR (qPCR). The forward (5’-ACCTTTGTGTTCACCG TCTCTG-3’ and reverse (5’-TGGTTCCACCCACCGTA GTT-3’) primers amplified a 263 bp product. Prior to qPCR, a regular PCR was performed to test primer specificity and the product was confirmed by sequencing analysis. Wheat 18S rRNA (acc. No. AY049040) was used as the internal reference (Yu *et al*. 2008). Templates were the 10× diluted cDNAs from each time-point sample. The qPCR reaction mixtures contained 12.5 mm^3 2× *SYBRGreen PCR Master Mix* (*Applied Biosystems*), 10 pM of each primer, 2 mm^3 template, and sterile distilled water added to a total volume of 25 mm^3. Thermal conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCRs reactions, including non-template controls, were carried out three times using an *ABI 7500* real-time PCR system (*Applied Biosystems*). Dissociation curves were generated for each reaction to ensure specific amplification. Threshold values (CT) were generated from the *ABI PRISM 7500* software (*Applied Biosystems*). The relative expression ratios were calculated by the comparative ΔΔCT method of relative gene quantification (Pfaffl 2001). A $P \leq 0.05$ was used to determine the significance of differences between time points, or that relative quantity of RNA in treated plants was at least two fold higher or lower than in leaves from mock-inoculated plants.

Results

Using *in silico* cloning, we extended aTaPST_225 to an 873 bp sequence containing the longest ORF (163 to 598 bp) encoding a protein highly similar to the rice C2 domain protein OsERG3. The putative initiation ATG codon at 163 bp is in the context of AAGATGG satisfying the Kozak (1987) consensus A/GXXATGG. A polyA-tail was also observed at the 3’ end of the sequence (Fig. 1). It was thus considered to be a candidate of full length cDNA. Then, a pair of primers spanning the ORF was used for verification. A 477 bp fragment was obtained after cloning and sequencing. *BLASTX* analysis confirmed that the cDNA was highly homologous (81 %) to OsERG3. We therefore designated the gene as *TaERG3* (GenBank acc. No. GU082332).

TaERG3 was predicted to encode a 144 amino acid (aa) protein. The deduced TaERG3 protein was analyzed using a series of programs in the online *Expasy* Tools. The *InterProScan* analysis revealed the presence of an N-terminal C2 calcium-dependent membrane targeting module, *i.e*. a C2 domain (aa position 5 - 102). TaERG3 also contained the C2 domain superfamily calcium/lipid-binding domain (C2_CaLB, aa 1 - 130). No signal peptide or trans-membrane region were observed. The *Compute pI/MW* analysis showed that TaERG3 had molecular mass of 15.68 kD and an isoelectric point (pI) of 3.93. In addition, the *PROSITE Scan* analysis showed that TaERG3 contained five types of motifs, including a protein kinase C phosphorylation site (44 - 46), a tyrosine kinase phosphorylation site (14 - 21), an N-myristoylation site (15 - 20), three N-glycosylation sites (3 - 56, 63 - 66, and 141 - 144), and six casein kinase II phosphorylation sites (34 - 37, 48 - 51, 59 - 62, 78 - 81, 82 - 85, and 123 - 126).

The amino acid sequence of TaERG3 was compared with 12 homologues (Fig. 2*A*) and a number of C2 domain proteins from a variety of plants and animals (Fig. 2B) using the *DNASTAR* program. As presented in Fig. 2*A*, TaERG3 was highly similar to the homologues and all proteins analyzed contained a conserved C2 domain of 98 aa at the N-terminus, as well as five aspartic acid (Asp) residues. The comparison between TaERG3 with 25 C2 domain proteins from various plants and animals (upper and lower alignments in Fig. 2*B*, respectively) also suggested the presence of five Asp residues among these proteins except a few, like CaSRC2-1 and CaSRC2-2 (Kim *et al*. 2008). A



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                                                                          1 ttccattttcaa
13  ctcctagtcctatacatacagcggcgccccggggctcggatcggatctacagcgattagtctcgaccttcagtcg
88  tgctgcctgctcatcagcatataattcctgatcgagcgagcgggagaggaaggcgagatcaggccgggagagaag
163 ATGGCGCAGGGGACGCTGGAGGTGCTGCTCGTCGGAGCCAAGGCCCTCGAGAACACCGACTACCTCTGCAACATG
1   M  A  Q  G  T  L  E  V  L  L  V  G  A  K  A  L  E  N  T  D  Y  L  C  N  M
208 GACCCGTACGCGGTTCTAAAATGCACCTCGCAGGAGCAGAAGAGCACCGTCGCCTCAGGAAAGGGAAGTGATCCT
26  D  P  Y  A  V  L  K  C  T  S  Q  E  Q  K  S  T  V  A  S  G  K  G  S  D  P
298 GAGTGGAACGAAACCTTTGTGTTCACCGTCTCTGAGAATGCAACCGAGCTCGTCATCAAGCTTCTGGACAGTGAT
51  E  W  N  E  T  F  V  F  T  V  S  E  N  A  T  E  L  V  I  K  L  L  D  S  D
388 GGTGGCACGGACGACGACAGCGTTGGTGAAGCAACGATCCCATTGGATGGAGTGTACACTGAAGGAAGCATCCCA
76  G  G  T  D  D  D  S  V  G  E  A  T  I  P  L  D  G  V  Y  T  E  G  S  I  P
433 CCAACTGTTTACAATGTTGTCAAAGACGAAGAGTACCGTGGAGAAATCAAAATCGGTCTGACGTTCACTCCGGAG
101 P  T  V  Y  N  V  V  K  D  E  E  Y  R  G  E  I  K  I  G  L  T  F  T  P  E
523 GAGGCTCGTGATCAGGATCAACCCGAGGAAAACTACGGTGGGTGGAACCAATCATCTTAAgaagaagcaggtgct
126 E  A  R  D  Q  D  Q  P  E  E  N  Y  G  G  W  N  Q  S  S  *  144
613 ttgctgaactatggtgcgtgacaagtcgtgtgctagaactaaagcttattttaattgttaaagactgtatttgtcg
689 ttgattccgtcaattatgtataagctacaaatctccttattgattggtatcattttctaatcttcaaatttgtaat
765 aatagtgttccccacttgtatgaagtatgagccctttttaatgtcactaaactgagttgcaataaaaaaaaaa873
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Fig. 1. The *in silico* (873 bp) and the near full length cDNA sequence (477 bp) of *TaERG3* amplified using the gene specific primers represented by italicised nucleotides. *Underlined arrows* indicate amplification directions. The predicted amino acid sequence is shown under the *TaERG3* coding region. The *boxed* triple nucleotides are start and stop codons, respectively. The *shadowed* nucleotides show a polyadenylation "aataa" signal. The poly-A tail sequence is *underlined*. Numbers show different positions within the cDNA and coding amino acids of *TaERG3*.

neighbour-joining phylogenetic tree of the 37 proteins mentioned above was constructed using *MEGA 4.0* (Fig. 3). TaERG3 was grouped together with the maize ACG44414 and the rice OsERG3, and had a close relationship to the maize ZmERG3 and the rice BAD25356. This gene cluster, separately from the known small C2 domain gene groups of CmPP16-1 and CmPP16-2, was distantly related to the groups of CaSRC2, BAP1, BAP2, and animal C2 domain proteins.

To examine subcellular localization of *TaERG3*, transient expression using onion epidermal cells was performed. The fusion construct *pCaMV35S:TaERG3-GFP*, as well as the control *pCaMV35S:GFP* were introduced into onion epidermal cells by particle bombardment. Confocal microscopy showed that the TaERG3-GFP fusion protein was predominately localized in the plasmalemma and the nucleus of the transformed cells (Fig. 4).

The spatial expression of *TaERG3* in different tissues was determined using qPCR analysis. The results showed significant variations in expression levels of *TaERG3* in wheat tissues, the highest in the leaves, the lowest in stems and intermediate in roots (Table 1).

Table 1. The expression of *TaERG3* in wheat leaves, stems, and roots. Data were normalized to the wheat 18S rRNA. Means ± SD, *n* = 3.

Organ	*TaERG3*
Leaf	1.00 ± 0.14
Stem	0.35 ± 0.02
Root	0.63 ± 0.11

The expression profiles of *TaERG3* in response to *PST* infection were studied using qPCR analysis (Table 2). *TaERG3* transcript was greatly up-regulated in both incompatible and compatible interactions as early as 12 hpi, and reached a maximum expression by 13.5 fold in the incompatible interaction and 14.4 fold in the compatible interaction, respectively. Although the expression of *TaERG3* had a generally decreasing trend from 12 to 120 hpi, the expression was still significantly higher than at 0 hpi. Firstly, the expression of *TaERG3* was slightly higher in the compatible interaction than in the incompatible interaction but the difference was not statistically significant. However, the expression was significantly higher in the incompatible interaction than in the compatible interaction from 18 to 120 hpi.

To examine whether *TaERG3* responds to changes in Ca^{2+}-concentration, we tested the effects of $CaCl_2$ treatment on *TaERG3* expression using qPCR (Table 3). When the plants were treated with 0.5 mM $CaCl_2$, the *TaERG3* expression increased at 0.5 hpt and decreased significantly at 1.0 hpt but greatly increased from 2 to 6 hpt with the highest level at 2 hpt. In contrast, in plants treated with 2 mM $CaCl_2$, the expression of *TaERG3* was significantly decreased at 0.5 and 1.0 hpt but greatly increased at 2 and 6 hpt with the highest level at 2 hpt. After these treatments, the *TaERG3* expression was higher in the plants treated with 0.5 mM Ca^{2+} than 2.0 mM Ca^{2+} and the differences were significant at both 0.5 and 2.0 hpt.

Next, we tested the effects of hormones (SA, ET, MeJA, and ABA) and various stresses (cold, high salinity, and wounding) on the expression of *TaERG3* using qPCR (Table 4). Although increase and decrease

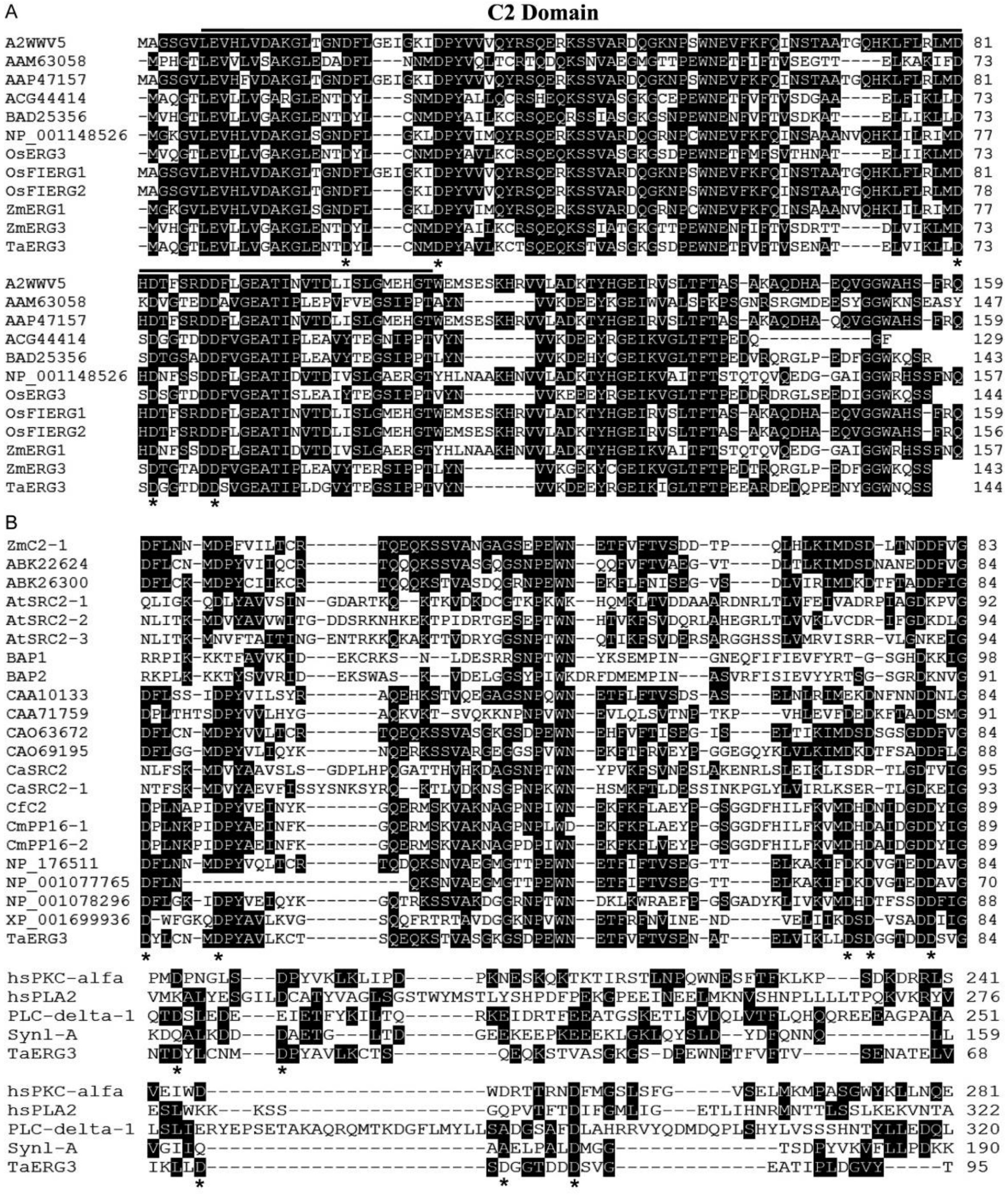

Fig. 2. *A* - Alignment of amino acid sequences of TaERG3 with 12 homologues using the *DNASTAR* program. The genes (GenBank accessions) included in the comparison are: TaERG3 (GU082332) from *Triticum aestivum*; AAP47157, BAD25356, A2WWV5, OsFIERG1 (Q0JHU5), OsFIERG2 (AAC04628), and OsERG3 (Q25AG5) from *Oryza sativa*; NP_001148526, ACG44414, ZmERG1 (ACG31844), and ZmERG3 (ACG48610) from *Zea mays*; AAG52148 and AAM63058 from *Arabidopsis thaliana*. The consensus amino acid sequences are *highlighted in black*. The C2 domains are marked with a *thick line* at the top. The full Ca^{2+}- binding aspartates are shown with *asterisks*. *B* - Comparison of TaERG3 and of 25 C2 domain proteins from various plants (upper alignment) and animals (lower alignment). The genes included are: *T. aestivum* TaERG3 (GU082332), *Z. mays* ZmC2-1 (U64437); *A. thaliana* NP_176511, NP_001077765, NP_001078296, AtSRC2-1 (AJ007586), AtSRC2-2 (NM_112522), AtSRC2-3 (NM_148335), BAP1 (NP_567111), and BAP2 (NP_182100); *Cucurbita maxima* Cmpp16-1 (AAD05496) and Cmpp16-2 (AAD05497); *Capsicum annum* CaSRC2-1 (DQ465394) and CaSRC2 (AB000130); *Cucurbita ficifolia* CfC2 (ABK41003); *Picea sitchensis* (ABK26300 and ABK22624); *Chlamydomonas reinhardtii* (XP_001699936), *Cicer arietinum* (CAA10133); *Sporobolus stapfianus* (CAA71759); *Vitis vinifera* (CAO69195 and CAO63672); human hsPKCα (P17252), hsPLA2 (M72393); bovine PLCδ1 (P10895); and rat Syn1-A (P21707). The consensus amino acid sequences are *highlighted in black*. *Asterisks* represent the putative calcium binding aspartate residues.

Table 2. The expression of *TaERG3* in adult plant (incompatible reaction, IR) and seedling leaves inoculated with *PST* CYR32 (compatible reaction, CR). Means ± SD, $n = 3$.

	0 h	12 h	18 h	24 h	48 h	72 h	120 h
IR	1.00 ± 0.10	13.50 ± 0.64	10.86 ± 0.75	8.33 ± 0.13	11.4 ± 0.25	9.22 ± 0.42	6.93 ± 0.63
CR	1.00 ± 0.08	14.48 ± 0.52	8.28 ± 0.97	5.12 ± 0.40	2.84 ± 0.89	2.40 ± 0.16	1.92 ± 0.25

Table 3. The expression of *TaERG3* in wheat leaves in response to different concentrations of $CaCl_2$. Means ± SD, $n = 3$.

$CaCl_2$ [mM]	0 h	0.5 h	1 h	2 h	6 h
0.5	1.00 ± 0.11	1.78 ± 0.34	0.37 ± 0.25	3.12 ± 0.31	1.90 ± 0.20
2.0	1.00 ± 0.02	0.13 ± 0.02	0.06 ± 0.06	2.15 ± 0.15	1.22 ± 0.28

Table 4. The expression of *TaERG3* in wheat leaves sprayed with ethylene, salicylic acid, methyl jasmonate, and abscisic acid, or affected by high salinity (200 mM NaCl), wounding, and low-temperature (4 °C). Means ± SD, $n = 3$.

Treatments	0 h	2 h	6 h	12 h	24 h
ET	1.00 ± 0.05	1.14 ± 0.11	0.95 ± 0.04	0.61 ± 0.09	0.86 ± 0.02
SA	1.00 ± 0.02	0.59 ± 0.12	1.70 ± 0.09	1.50 ± 0.30	1.33 ± 0.04
MeJA	1.00 ± 0.06	1.44 ± 0.02	0.76 ± 0.02	1.33 ± 0.07	0.75 ± 0.09
ABA	1.00 ± 0.08	2.76 ± 0.22	0.65 ± 0.12	0.58 ± 0.04	0.74 ± 0.07
NaCl	1.00 ± 0.08	0.85 ± 0.13	0.28 ± 0.06	1.46 ± 0.30	6.50 ± 0.05
Wounding	1.00 ± 0.11	0.37 ± 0.04	1.24 ± 0.25	0.90 ± 0.04	0.61 ± 0.06
Low temperature	1.00 ± 0.02	4.35 ± 0.22	3.06 ± 0.12	2.83 ± 0.06	1.53 ± 0.30

of the*TaERG3* expression were observed for each of the four hormones and different patterns were found among the hormone treatments, only ABA increased the *TaERG3* expression by more than two fold at 2 hpt. The expression of *TaERG3* was also affected by abiotic stresses (Table 4). Compared to salinity and low-temperature treatments, the effect of wounding on the expression of *TaERG3* was relatively low and only the expression at 2 hpt was significantly lower than at 0 hpt. The salinity (200 mM NaCl) did not induce *TaERG3* expression until 12 hpt but the treatment increased the expression by more than 5 times at 24 hpt, the highest among all abiotic stresses. The low-temperature (4 °C) treatment significantly increased the gene expression in all time-points after the treatment and the highest level (4.35 fold) occurred at 2 hpt.

Discussion

In this study, we identified a C2 domain gene, *TaERG3*, from wheat following *PST* infection. The N-terminal region of the deduced TaERG3 protein has a conserved C2 calcium-dependent membrane targeting module (C2 domain) similar to other C2 domain proteins (Kim *et al.* 2000, 2003, 2004, 2008, Ouelhadj *et al.* 2005, Schapire *et al.* 2008, Yang *et al.* 2008, Wang *et al.* 2009, Yamazaki *et al.* 2010). The five Asp residues predicted to participate in Ca^{2+}-dependent binding (Kim *et al.* 2003) are conserved in the TaERG3 protein. A phylogenetic study demonstrated that though separated from the known C2-domain protein groups and distant to groups of CaSRC2, BAP1, BAP2, and the animal C2 domain proteins, the protein encoded by *TaERG3* may function as a C2 domain protein.

Genes of the same family usually share similar sequences, functional domains, and occur in closely related species (Frech and Chen 2010). Wheat, barley, rice, and maize are evolutionarily close monocots. Genes across the four species are highly conserved in both DNA sequence and biological function, for example the hypersensitive induced response (HIR) genes (Nadimpalli *et al.* 2000, Yu *et al.* 2008, Zhang *et al.* 2011) and NAC transcription factors (Kikuchi *et al.* 2000, Takashi *et al.* 2009, Xia *et al.* 2010a,b). *TaERG3* has many homologues with high similarity in various plant species including six in rice, four in maize, and several in *Arabidopsis* and castor bean. Therefore, more homologues of *TaERG3* are

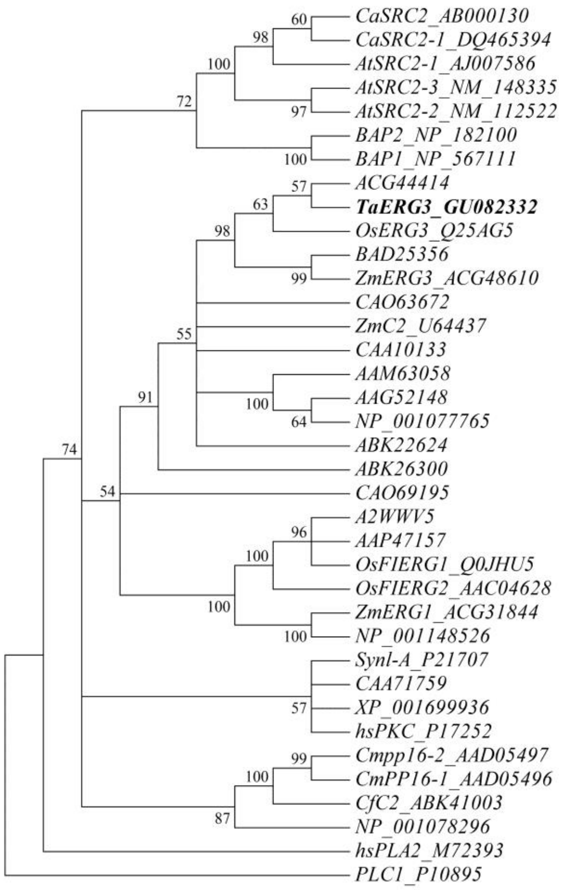

Fig. 3. A neighbour-joining phylogenetic tree of C2 domain proteins from various plants and animals using the *MEGA 4.0*. The whole protein sequences of all 37 genes analyzed in Fig. 2*A*,*B* were used in the comparison. The numbers at branches represent bootstrap values (≥ 50 %) based on 1 000 replications. Genes following GenBank accessions by an underscore sign "_" or the GenBank accession alone (a gene name was not given in GenBank) were included.

undoubtedly present in polyploid wheat. However, this needs further studies.

Localization of proteins to distinct subcellular compartments is important in unravelling cellular pathways. Rice OsERG1 was uniformly located in the cytosol, and was induced in response to fungal elicitor and Ca^{2+} treatment (Kim *et al*. 2003) as part of a defence response. Similar results were also observed for other C2 domain proteins. OsPBP1 was localized mainly in the cytoplasm and nucleus and was required for pollen fertility and germination (Yang *et al*. 2008). The pepper CaSRC2-1 fusion with GFP was localized to the plasma membrane and played an essential role in abiotic stress and defence responses (Kim *et al*. 2008). Interestingly, our results showed that protein TaERG3 was predominantly localized in the cytoplasm membrane and nucleus of transformed epidermal onion cells (Fig. 4) in accordance with those C2 domain proteins previously identified from different plants species (Kim *et al*. 2003, 2008, Ouelhadj *et al*. 2005, Yang *et al*. 2008, Wang *et al*. 2009). Moreover, all above mentioned C2 domain proteins are translocated in a Ca^{2+}-dependent manner, a characteristics of the C2 domain proteins (Nalefski and Falke 1996). The TaERG3 protein has the C2 calcium-dependent membrane targeting domain. It is likely that TaERG3 is associated with Ca^{2+}-signalling. Because there was no biochemical evidence supporting this assumption, we analyzed the expression pattern of *TaERG3* in wheat leaves under $CaCl_2$ treatment (Table 3). The *TaERG3* transcription was regulated by changing the Ca^{2+} concentration in a similar way to *CaSRC2-1* showing repression before a peak was reached (Kim *et al*. 2008). Therefore, we suggest that *TaERG3* might play a role in the Ca^{2+}-signalling pathway.

The expression patterns of several plant C2 domain proteins have been reported to be tissue-specific. The *CaSRC2-1* transcription was high in roots but not in

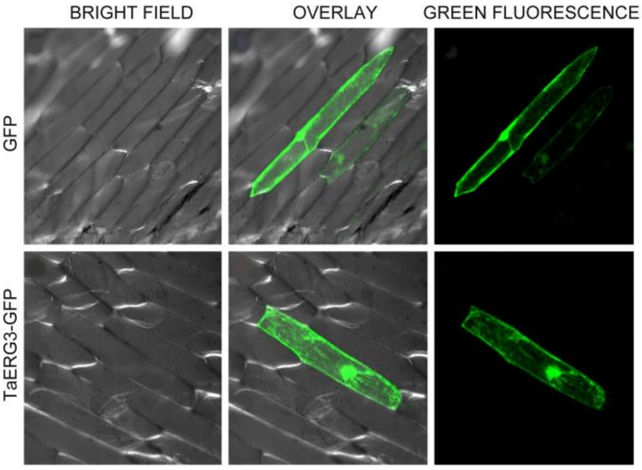

Fig. 4. Subcellular localization of the TaERG3 protein. The TaERG3-GFP fusion and GFP control constructs were transformed into onion epidermal cells by particle bombardment. GFP signals in cells expressing the TaERG3-GFP and GFP control constructs are observed with a confocal microscope. The images are the same fields observed under bright field microscopy.

other tissues (Kim *et al.* 2008). *IPG-1* was strongly expressed in canola flowers but the weakest in stems (Wang *et al.* 2009). *OsPBP1* was strongly expressed in non-pollinated pistils and weakly expressed in rice leaves and roots (Wang *et al.* 2008). We found that the expression of *TaERG3* was the lowest in wheat stems suggesting a different regulatory mechanism of *TaERG3* in different tissues.

Functional analyses of C2 domain proteins in plant disease resistance have been reported. *Arabidopsis* BAP and BON negatively regulated defence responses (Yang *et al.* 2006a,b, 2007). By interacting with a fungal effector Sspg1d from *Sclerotinia sclerotiorum*, canola IPG-1 was involved in host programmed cell death (Wang *et al.* 2009). Over-expression of rice *OsSMCP1* increased tolerance to biotic stresses in transgenic *Arabidopsis* (Yokotani *et al.* 2009). We conducted expression analyses of *TaERG3* in wheat leaves in response to *PST* infection (Table 2). Significant induction of *TaERG3* transcript was detected as early as 12 hpi in both incompatible and compatible interactions. Plant defence responses against microbial infection are often triggered by signals generated during the initial stages of pathogen infection (Lamb *et al.* 1989). It is believed that the perception of *PST* by wheat occurs from 6 to 12 hpi when fungal penetration into host cells is underway (Kang *et al.* 2002). When an incompatible *PST* pathotype is applied to wheat plants, the hypersensitive response (HR) at infection sites normally becomes evident at 24 hpi (Wang *et al.* 2007). In the plants of Xingzi 9104 expressing adult plant resistance to stripe rust at the boot stage, histological and cytological studies revealed that infection sites established themselves at 12 hpi and HR appeared at 36 hpi (Zhang *et al.* 2012). The data of the present study showed that *TaERG3* responded to *PST* infection as early as 12 hpi suggesting the involvement of *TaERG3* in wheat early defence against fungal attack in a similar way to other C2 domain genes that were induced after pathogen attack, such as *OsERG1* (Kim *et al.* 2003), *CaSRC2-1* (Kim *et al.* 2008), *IPG-1* (Wang *et al.* 2009), and *OsSMCP1* (Yokotani *et al.* 2009). The second increase of the *TaERG3* transcription from 48 to 120 hpi was coincident to HR that was observed from 36 to 120 hpi at the histological level (Zhang *et al.* 2012). Although the gene transcription increase in the compatible interaction was similar to that in the incompatible interaction at 12 hpi, the transcription level became much lower than those in the compatible interaction afterwards and did not have a second increase. Considering that only slight chlorotic blotches were evident on inoculated leaves until 120 hpi in the seedling Xingzi 9104, the expression of *TaERG3* may be suppressed during the late infection process in the compatible interaction. These results indicate the involvement of *TaERG3* in the host HR-associated defence responses. The underlying molecular mechanism may be more complex and further studies are needed to address the issue.

Plant hormones SA, ET, MeJA, and ABA are closely related to defence responses (Mauch-Mani and Mauch 2005). They are important signalling compounds and may act independently or coordinately in disease responses (Fujita *et al.* 2006). Previously, the effects of only limited numbers of hormones on expression of C2 domain genes were studied in different plant species and the results showed that C2 domain genes are not regulated. Kim *et al.* (2003) investigated the effect of SA on *OsERG1* expression and found that the *OsERG1* transcription was not induced. The transcription of *VrPLC3* mRNA was unaffected by treatment with ABA for 24 h (Kim *et al.* 2004). The expression patterns of *CaSRC2-1* treated with above mentioned four hormones revealed no regulation of *CaSRC2-1* transcription by any of them (Kim *et al.* 2008). In the present study, SA, ET, MeJA, and ABA affected the *TaERG3* expression but only ABA induced the two fold increase in *TaERG3* expression.

Increasing evidence has revealed that C2 domain proteins are regulated by diverse biotic and abiotic stresses suggesting their importance in stress responses and signalling. *VrPLC3* was up-regulated by drought and salt stresses (Kim *et al.* 2004). The *HvC2d1* transcription was induced by heavy metal stress and leaf senescence in barley, and thus was implicated in stress and development dependent signalling (Ouelhadj *et al.* 2005). *Arabidopsis* SYT1 was recently shown to be involved in maintenance of plasma membrane integrity under freezing stress (Schapire *et al.* 2008, Yamazaki *et al.* 2010). Comparatively, pepper *CaSRC2-1* was elicited by various abiotic stresses including cold, high salinity, dehydration as well as pathogenic bacteria infection, and the gene was assumed to play important roles in abiotic stress and defence responses (Kim *et al.* 2008). In this study, the expression of *TaERG3* was regulated by abiotic stresses including low temperature and high salinity (Table 4) indicating involvement of *TaERG3* in responses to environmental conditions.

To our knowledge, this is the first report on characterization of a novel C2 domain protein encoding gene in wheat. Our results provide evidence that *TaERG3* might be involved in wheat resistance to stripe rust and abiotic stresses possibly through an ABA-dependent signalling pathway.

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