**TaCHP: A Wheat Zinc Finger Protein Gene Down-Regulated by Abscisic Acid and Salinity Stress Plays a Positive Role in Stress Tolerance**

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The plant response to abiotic stresses involves both abscisic acid (ABA)-dependent and ABA-independent signaling pathways. Here we describe TaCHP, a CHP-rich (for cysteine, histidine, and proline rich) zinc finger protein family gene extracted from bread wheat (*Triticum aestivum*), is differentially expressed during abiotic stress between the salinity-sensitive cultivar Jinan 177 and its tolerant somatic hybrid introgression cultivar Shanrong No.3. TaCHP expressed in the roots of seedlings at the three-leaf stage, and the transcript localized within the cells of the root tip cortex and meristem. TaCHP transcript abundance was higher in Shanrong No.3 than in Jinan 177, but was reduced by the imposition of salinity or drought stress, as well as by the exogenous supply of ABA. When JN17, a salinity hypersensitive wheat cultivar, was engineered to overexpress TaCHP, its performance in the face of salinity stress was improved, and the ectopic expression of TaCHP in Arabidopsis (*Arabidopsis thaliana*) also improved the ability of salt tolerance. The expression level of a number of stress reporter genes (AtCBF3, AtDREB2A, AtABI2, and AtAB11) was raised in the transgenic lines in the presence of salinity stress, while that of AtMYB15, AtABA2, and AtAAO3 was reduced in its absence. The presence in the upstream region of the TaCHP open reading frame of the cis-elements ABRE, MYBRS, and MYCRS suggests that it is a component of the ABA-dependent and -independent signaling pathways involved in the plant response to abiotic stress. We suggest that TaCHP enhances stress tolerance via the promotion of CBF3 and DREB2A expression.

Abiotic stresses such as salinity, drought, or cold can adversely affect crop quality and/or yield, so improved stress tolerance is an important breeding trait. Bread wheat (*Triticum aestivum*) is a moderately salinity-tolerant species (Maas and Hoffman, 1977), but some of its related species have a much higher level of tolerance (Munns and Tester, 2008). One of these is tall wheatgrass (*Thinopyrum ponticum*), which is among the most salinity tolerant of all monocotyledonous species (Yuan et al., 1999). Somatic hybridization has been successfully employed to transfer blocks of wheatgrass chromatin into bread wheat genome (Xia et al., 2003), and this approach has resulted in the release of an introgression cultivar Shanrong No.3 (SR3), which has inherited enhanced salinity tolerance from tall wheatgrass in a genetic background of the salinity-sensitive wheat cultivar Jinan 177 (JN177; Shan et al., 2008).

The elucidation of the mechanism in stress response is an important area of research in the context of improving stress tolerance. Tolerance to salinity is achieved in plants via one or more of an improved tolerance of osmotic stress, a better ability to selectively exclude toxic ions, and the development of better tissue tolerance to elevated ionic concentrations (Munns and Tester, 2008). The genetic analysis of the stress response of *Arabidopsis (Arabidopsis thaliana)* and other model plants has suggested that all these processes are dependent on concerted action of many genes. Two specific pathways have been identified, one of which relies on the phytohormone abscisic acid (ABA), and the other is ABA independent (Ergen et al., 2009). The latter is mediated mainly through an IP3-Ca2+-DREB cascade (Zhu, 2001), while the former involves AREB and other modules. Both pathways require participation of many regulatory factors (Chinnusamy et al., 2004), and there is a degree of cross talk between the two pathways (Haake et al., 2002; Narusaka et al., 2003). However, the detailed characteristic of the cross talk is still not well dissected.

The zinc finger proteins belong to a prominent family of regulatory proteins. Some wheat zinc finger encoding genes have been identified in silico, and several are responsive to both drought (Kam et al., 2008) and salinity stress (Houde et al., 2006). One of 1

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these is TaCHP, which is more highly expressed in SR3 than in its parent cultivar JN177, and is down-regulated in the presence of both salinity and drought stress. The TaCHP product is a CHP-rich (for Cys, His, and Pro rich) zinc finger protein with three divergent C1 (DC1) domains and the capacity to bind two zinc ions. DC1 domain-containing proteins have been implicated in plant response to abiotic stress, as for example the tobacco (Nicotiana tabacum) genes NtDC1A and NtDC1B that responded rapidly and strongly to Aa-Glucan, an elicitor of some stress-related genes (Shinya et al., 2007). DC1 domain, which is able to bind diacylglycerol (DAG) and phorbol esters (an analog of DAG) in a phospholipid and zinc-dependent fashion, presents in the N-terminal region of protein kinase C (PKC), a family of Ser/Thr protein kinases (Ono et al., 1989). DAG activates PKC (Azzi et al., 1992) and acts as a signal transducer in a Ca2+-dependent manner. However, in plants, there is little evidence for the existence of either PKC or any IP3 receptors (Munnik and Testerink, 2008). As a result, the function of DC1 domain-containing proteins in plants remains unclear.

Here, we show that the expression of TaCHP in SR3 is associated with an improvement in salinity tolerance, achieved via antagonizing an ABA signaling pathway. The ectopic expression of TaCHP was also shown to increase the accumulation of DREB gene products. The indications are that TaCHP may represent an important component of the cross talk between the ABA-dependent and the ABA-independent signaling pathways involved in plant stress response.

**RESULTS**

**The Differential Expression of TaCHP in SR3 and JN177**

Microarray analysis had indicated that the expression pattern of a CHP family gene, named TaCHP, differed markedly between SR3 and JN177. Consistent with the microarray assay, TaCHP mRNA was present in higher abundance in SR3 roots rather than in JN177 roots, in both stressed and nonstressed conditions, but was hardly detectable in leaves (Fig. 1, A and B). Even during salinity stress, the gene was expressed in SR3 at a higher level than in nonstressed JN177 (Fig. 1, A and B). Both reverse transcription (RT)-PCR and real-time PCR analysis confirmed that this gene was down-regulated in the roots of stressed SR3 and JN177 plants (Fig. 1, A–C). In SR3 roots, TaCHP was down-regulated at the early stages (0.5 h) of imposed salinity stress, and later stayed at a constant level (Fig. 1C). In contrast, in the roots of JN177, TaCHP was expressed at a much lower level than SR3 at every sampling time point, but a decreased transcript level at the 0.5 h is observed as well (Fig. 1C).

**The Structure and Upstream Sequence of TaCHP and Its Chromosomal Location**

RACE PCR was successfully used in obtaining a full-length sequence of TaCHP cDNA from both SR3 and JN177, comprising 955 nucleotides and including a 645-bp open reading frame, a 63-bp 5' untranslated region (UTR), and a 238-bp 3' UTR (Supplemental Fig. S1, A and B; Fig. 2A). The deduced TaCHP polypeptide consisted of 214 residues, with a predicted M, of 23.7 kD and a pI of 6.54. A Blastp search indicated that the peptide is a CHP-rich zinc finger protein-like protein, with three DC1 domains (Fig. 2B). Its first DC1 domain contains five conserved Cys and one conserved His capable of binding zinc (Fig. 2C). A phylogenetic analysis indicated that its most closely related protein

![Figure 1. The differential expression of TaCHP in SR3 and JN177. A, Transcript levels in the root compared by RT-PCR. Salt, 12 h exposure to 200 mM NaCl. Control, no imposed stress. B, Real-time PCR analysis of root and leaf expression. TaCHP transcript in root of NaCl-stressed SR3 was used as a standard (100%). Black column indicates control and white column shown as NaCl treatment. C, Real-time PCR analysis of roots at different time points of stress. The vertical coordinate indicates relative transcript levels compared to TaActin. All data represented as means ± se of three independent experiments.](https://www.plantphysiol.org/doi/fig/10.1104/pp.109.151948)
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is a putative CHP-rich zinc finger protein-like protein from rice (*Oryza sativa*; GI:14192864; Fig. 2D). The 1.3-kb upstream genomic sequence in SR3 contains several cis-elements, including ABRE, MYC, and MYB, whereas the binding sites in JN177 were relatively diverse. The frequency of CAAT and TATA boxes in the JN177 sequence was lower than in the SR3 one (Supplemental Table S3). A PCR based on SR3 and JN177 genomic DNA template amplified a 790-bp sequence comprising the open reading frame together with a 144-bp intron at its 3′ end (Supplemental Fig. S1, A and C). Analysis of aneuploid stocks cultivar Chinese Spring (CS) showed that this product was not amplified from a template of Dt7DS, a stock that lacks long arm of 7D. Thus, we concluded that TaCHP must map to chromosome arm 7DL (Fig. 2E).

Expression Pattern of TaCHP

In nonstressed plants, in situ hybridization assay detected the presence of TaCHP mRNA in the cells of the root tip cortex and meristem with more messages present in SR3 than in JN177 (Fig. 3A). There was more signal present in the roots of salinity-stressed SR3 plants than in those of either stressed or nonstressed JN177 plants (Fig. 3A), consistent with the RT-PCR and real-time PCR results discussed above. The RT-PCR analysis showed that TaCHP transcript was present throughout the plant’s life cycle, peaking at the seedling three-leaf stage (Fig. 3B). The reaction to dehydration was a gradual decline in TaCHP abundance over the first 24 h (Fig. 4A). The presence of 200 μM ABA also substantially reduced transcript abundance (Fig. 4B), while the down-regulation of TaCHP expression induced by salinity stress was relieved by the provision of norflurazon, an inhibitor of ABA biosynthesis (Fig. 4C). Ethephon treatment led to an increase in expression after 24 h (Fig. 4D). Neither exogenously provided jasmonic acid (JA) nor salicylic acid (SA) had any noticeable effect on TaCHP transcript level (Fig. 4, E and F).

Figure 2. Structure, phylogeny, and chromosomal location of TaCHP. A, The TaCHP gene. Exons shown as black boxes, and the 5′ UTR, introns, and 3′ UTR as black bars. B, Peptide domain analysis of TaCHP. The DC1 domains are underlined. C, Amino acid sequence alignment of the DC1 domain with other CHP-rich zinc finger proteins. Conserved residues are shaded. D, Phylogeny of TaCHP and related proteins. The tree was derived from the DC1 domain peptide sequence. Branch length numbers are shown. G15224369, 15224373, 15239894, 15218093, 4836889, 15226210, and 15224971 are from Arabidopsis; GI14192864, 24059846, 12559145, 24059841, 12559137 34394191, 125601034, and 24059843 from rice. E, Chromosomal location of TaCHP. Using the ditelosomic lines of CS, the gene was located to the long arm of chromosome 7D. In the CS ditelosomic lines Dt1AS, Dt1AL, Dt7DS, and Dt7DL, the deleted chromosome arms are 1AL, 1AS, 7DL, and 7DS, respectively.
The Stress Response of Wheat and Arabidopsis Lines Expressing TaCHP

Of the 12 independent ubiquitin::TaCHP transgenic wheat lines recovered from a salinity hypersensitive cultivar JN17, two transgenic homozygous T3 selections (TaOE1 and TaOE2) were analyzed for their biochemical and morphological responses to stress. Under nonstressed conditions, both lines accumulated more TaCHP transcript in the root than control plants carrying an empty vector (Fig. 5A). The two OE lines showed larger leaves (Fig. 5B) and 17% to 21% higher relative root growth (Fig. 5C) in the presence of 200 mM NaCl, their leaf malondialdehyde (MDA) content was less under both stressed and nonstressed conditions (Fig. 5D), their leaf peroxidase (POD) activity was 50% higher in the presence of NaCl (Fig. 5E), and their leaf Pro concentration was lower under both stressed and nonstressed conditions (Fig. 5F).

Two homozygous TaCHP-expressing transgenic Arabidopsis lines (B5 and A5) along with one empty vector control line (C13) were used similarly to assess the effect of the TaCHP transgene (Fig. 6A). There was no noticeable difference between the phenotype of BS, A5, and C13 either in the presence or absence of 50 mM NaCl (Fig. 6B). However, when the NaCl concentration was raised to 100 mM and 150 mM, BS and A5 were able to grow more freely than C13, and the former's roots were longer (Fig. 6, B and C). Similarly, the MDA content in the BS and A5 seedlings was 50% below that in C13 (Fig. 6D), while POD activity was increased by 20% to 30% (Fig. 6E) and Pro content was reduced, particularly in the stressed seedlings of BS (Fig. 6F).

Salinity Stress-Induced Gene Expression in Arabidopsis TaCHP Expression Lines

An in vivo analysis of a set of putative genes taking part in the TaCHP signaling pathway was undertaken to study the stress response of the Arabidopsis TaCHP expression lines. A comparison between the transcript abundance in BS and A5 with that in C13 showed that AtHSP101, AtSOS1, AtSOS2, AtSOS3, AtMYB2, AtABF3, and AtP5CS1 shared a similar salt-responsive expression (Fig. 7A), while AtRAB18, AtRD29A, AtCBF3, AtDREB2A, AtABF1, and AtABF2 were all induced by salinity stress (Fig. 7, C–E). The expression of AtCBF1, AtCBF2, AtCBF3, AtDREB2A, and AtDREB2B in BS/A5
and C13 was similar under nonstressed conditions (Fig. 7B). None of AtICE1, AtHOS1, AtHOS2, AtZAT12, AtFRY2, or AtZAT10 was differentially expressed in the absence of salinity stress (Fig. 7B). However, AtMYB15 appeared to be down-regulated in BS and A5 in the absence of salinity stress (Fig. 7B).

The Interaction between the Transgenic Expression of TaCHP and the Presence of ABA

Since TaCHP was down-regulated by ABA treatment, it was of interest to study the effect of this treatment on the Arabidopsis lines expressing TaCHP. Varying the concentration of exogenous ABA had no differential effect on either their germination rate (Fig. 8A), or their growth (Fig. 8B). However, among the genes involved in ABA synthesis, there was some evidence for differential expression in lines A5 and BS under salinity stress—for example, AtAAO3 and AtABA2 were strongly down-regulated, and AtNCED3 and AtABA1 noticeably so (Fig. 8C).

DISCUSSION

Zinc finger proteins play various roles in plant development and their response to the environment. This large protein family includes many members whose function remains unknown. The major observations here regarding the DC1 domain-containing zinc finger protein gene TaCHP were that its abundance was notably higher in the salinity-resistant introgression line SR3 than in its parent JN177, whether or not the plants were subjected to salinity stress, and that its overexpression as a transgene increased the salinity resistance of both wheat and Arabidopsis, suggesting that Arabidopsis can be used as a heterogonous system for the functional study of this gene.
Gene Family Encoding DC1 Proteins of Arabidopsis in Relation to Stress Resistance

TaCHP transcript level was higher in SR3 than in JN177, but was reduced at the early stage under salinity or drought stress, as well as exogenous ABA (Figs. 1 and 4B). However, it is interesting that when TaCHP was overexpressed in wheat and Arabidopsis, it increased salt tolerance of transgenic plants (Figs. 5B and 6B). We hypothesize that this inconsistency may be the result of different expression pattern of DC1 protein genes. To verify this, we conducted a bioinformatic analysis on this gene family in both wheat and Arabidopsis, but we could not find TaCHP homologs in wheat genome in current public accessible databases. There are 16 DC1 genes in Arabidopsis genome (http://www.arabidopsis.org/). The expression pattern of two DC1 genes in Arabidopsis (At2g42060 and At5g46670) is similar to that of TaCHP, they are down-regulated when plant exposed to abiotic stresses and ABA treatment, whereas other three DC1 genes (At1g61830, At1g55410, and At5g46660) are up-regulated. The different expression pattern of the DC1 genes may indicate that DC1 gene family play complicated roles in plant stress tolerance improvement, and partially explain such inconsistency of TaCHP. These results suggest that the higher expression of TaCHP in SR3 is responsible for its salt tolerance.

TaCHP Enhances Salinity Tolerance by Acting on Antioxidation

Elevated intracellular Na⁺ concentrations inhibit the activity of many essential enzymes and reduce the cell’s ability to divide or expand. Prolonged exposure to salinity results in membrane disorganization and osmotic imbalance, and the cessation of growth (Tuteja, 2007). These events are typically accompanied by the accumulation of reactive oxygen species (ROS), which interact with a variety of molecules to cause irreversible cell damage, necrosis, and death (Girotti, 2001). Salinity-sensitive barley (Hordeum vulgare) cultivars appear to be particularly susceptible to ROS-induced damage (Chen et al., 2007), and the ability to neutralize ROS has therefore been proposed as an important component of stress tolerance (Xue et al., 2009). It was reported that higher POD activity can alleviate more rapid and severe ROS changes (Laloi et al., 2004). In the presence of salinity stress, the overexpression of TaCHP in wheat promoted the elongation of the root, while simultaneously increasing POD content (Fig. 5, B, C, and E). The parallel fall in MDA content probably reflected this increased POD activity (Fig. 5D). In Arabidopsis, the accumulation of the zinc finger protein ZAT10 promotes the expression of ascorbate POD (APX2; Mittler et al., 2006), while ZAT12 is required for APX1 expression (Rizhsky et al., 2004). APX is one of the most important ROS-scavenging enzymes in plants (Pitzschke et al., 2006). Proaccumulation is a common plant response to a wide range of biotic and abiotic stresses (Verbruggen and Hermans, 2008). The TaCHP expressors tended to accumulate less Pro than the control lines (Fig. 5F). Meanwhile, the expression of TaCHP in transgenic Arabidopsis also increased salt tolerance (Fig. 6, B and C). The indications are therefore that the greater stress tolerance induced by the overexpression of TaCHP is achieved by an enhancement of the antioxidant pathway (Figs. 5, D and E and 6, D and E), rather than by an increased capacity for Pro accumulation (Figs. 5F and 6F).

TaCHP Is Involved in an ABA-Dependent Pathway

A boost in ABA biosynthesis is a frequently observed plant response to abiotic stresses (Ingram and
Bartels, 1996), and this has the effect of triggering a number of ABA-dependent genes involved in the stress response. However, the regulation of ABA biosynthesis under stressful conditions is not well researched (Chinnusamy et al., 2004). Here, we have shown that salinity stress reduced the expression of TaCHP (Fig. 1, A and C), as did the exogenous supply of ABA (Fig. 4B), while the ABA biosynthesis inhibitor norflurazon overcame this repression (Fig. 4C). In contrast, the overexpression of TaCHP significantly down-regulated several ABA biosynthesis pathway genes (Fig. 8C). Furthermore, the TaCHP promoter contains the cis-elements ABRE, MYCRS, and MYBRS (Supplemental Table S2). Thus, it appears that TaCHP can act as a negative regulator of ABA biosynthesis, so that its down-regulation in response to salinity stress may partially account for the accumulation of ABA. Some MYB transcription factors are known to be required for ABA biosynthesis (Zhu et al., 2005), and the abundance of AtABA1 transcript was increased when AtMYB15 was overexpressed in nonstressed Arabidopsis seedlings (Ding et al., 2009). Here, the overexpression of TaCHP significantly reduced AtMYB15 transcription (Fig. 7B), indicating that the down-regulation of AAO3 and ABA2 by TaCHP (Fig. 8C) is likely mediated through its action on AtMYB15.

The abundance of AtAB11 and AtAB12 transcript was higher in the Arabidopsis TaCHP expressors than in the control line under stressful conditions (Fig. 7E). ABI1 and ABI2 are both key negative regulators of the ABA signaling pathway (Leung et al., 1994, 1997; Meyer et al., 1994). Thus, in addition to its involvement in the ABA biosynthesis pathway, TaCHP appears also to negatively modulate the ABA signaling pathway, and such modulation is activated by salinity stress. The observation that TaCHP expression did not alter the sensitivity of Arabidopsis to ABA during germination and early seedling growth (Fig. 8, A and B) provides further evidence that the gene plays a specific role in the ABA biosynthesis and signaling pathway during salinity response.

TaCHP Expression Promotes the ABA-Independent Pathway

DREB transcription factors are a well-known component of the ABA-independent response to abiotic stress (Liu et al., 1998; Medina et al., 1999; Haake et al., 2002; Dubouzet et al., 2003; Sakuma et al., 2006). The overexpression in wheat of TaCHP was associated with an increased accumulation of AtDREB2A, AtCBF3, AtRD29A, and AtRAB18 under salinity conditions (Fig. 7, C and D). The overexpression of these genes in Arabidopsis tends to affect plant development (Liu et al., 1998), but there was no such effect noted here, which possibly owes to that their dramatic accumulation in TaCHP overexpression lines is salinity stress dependent (Fig. 6A). A complex regulatory network controls the accumulation of DREB gene products (Gilmour et al., 1998; Zarka et al., 2003; Knight et al., 2004; Fowler et al., 2005). In Arabidopsis, ICE1 and MYB15 bind directly to the CBF promoter region (Zhu et al., 2007), and ICE1 interacts physically with MYB15. ICE1 activates CBF transcription, while MYB15 is...
negative regulator for the accumulation of CBF transcript (Chinnusamy et al., 2003; Agarwal et al., 2006). Here, AtICE1 was not differentially expressed, although the level of AtMYB15 expression was reduced in the TaCHP-expressing Arabidopsis lines (Fig. 7B). AtCBF3 and AtDREB2A transcript levels were not affected by the presence of the transgene under non-stressed condition (Fig. 7B), so TaCHP appears not to be able to directly activate the transcription of DREB genes. It is therefore possible that the enhanced accumulation of AtCBF3 and AtDREB2A (Fig. 7C) shown by the salinity-stressed transgenic lines may be also mediated by a decrease in the expression of AtMYB15.

**TaCHP Is a Key Link between the ABA-Dependent and the ABA-Independent Pathways**

Drought and salinity both provoke plants to synthesize ABA (Ingram and Bartels, 1996). In Arabidopsis, both ABA-dependent and ABA-independent response pathways to drought and high salinity have been defined (Tran et al., 2007). The former depend on either MYC/MYB- or bZIP-regulated gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997). Stress-responsive genes, such as AtRD29A, AtRD22, AtCOR15A, AtCOR47, AtP5CS, AtRD19, AtKIN1, and AtADH, are up-regulated via an ABA-dependent pathway (Pitman and Lauchli, 2002; Xiong et al., 2002), and the promoters of these genes commonly contain ABRE, MYCRS, and MYBRS cis-elements (Zhu, 2002), as does the TaCHP promoter. The regulation of DRE elements may be mediated by an ABA-dependent pathway (Haake et al., 2002). The ABA-independent pathways are mediated by DREB genes or other as yet unidentified factors. The ABA-dependent and ABA-independent pathways do converge at several points. Both DRE and AREB cis-elements are present in the

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**Figure 8.** Phenotypic and molecular analysis of TaCHP transgenic Arabidopsis with respect to ABA. A, Germination rate in the presence of various concentrations of ABA (mean of three replicates). B, Relative fresh weight of seedlings. C, Relative expression of ABA biosynthesis genes in TaCHP overexpression Arabidopsis lines in comparison with the vector control line after 3 h exposure to 100 mM NaCl. Amplicons of RT-PCR were quantified using Image J software (http://rsbweb.nih.gov/ij/, v1.21). Amplicon intensities of genes among were normalized using AtTublin as a standard, then their relative expression levels were calculated as the ratio of the normalized amplicon intensity of A5 and BS to those of C13. Data are means ± SD (n = 4). *, Means differ P < 0.05; **, means differ P < 0.01. A5 and BS, Expressing the TaCHP transgene; C13, negative control (empty vector only).

**Figure 9.** A model for the regulatory network involving TaCHP in Arabidopsis. Solid lines indicate support from previously published data, dashed ones derived from this study. T-shaped line: repression; arrow: promotion.
AtRD29A promoter, and the two pathways coactivate the expression of AtRD29A (Narusaka et al., 2003). In Figure 9, we present the model we have elaborated to describe the function of TaCHP. First, salinity and dehydration induce ABA synthesis and the triggering of relevant signaling cascades (Ingram and Bartels, 1996), which serve to inhibit the expression of TaCHP (Fig. 1, A and B). Meanwhile, the expression of ABI1 and ABI2 is also induced, and these have a negative feedback effect on the ABA signaling cascades (Merlot et al., 2001), which alleviates the repression of TaCHP expression as salinity stress continues (Fig. 1C). The overexpression of TaCHP inhibits the expression of both AAO3 and ABA2 in the absence of salinity stress, and promotes the expression of ABI1 and ABI2 under stressed conditions (Figs. 7E and 8C). Thus, TaCHP may also act as a negative modulator or even a cofactor of ABI1/ABA2 regulation in ABA synthesis and the relevant signaling cascades. Second, the overexpression of TaCHP restricts the expression of MYB15 in the absence of stress (Fig. 7B). MYB15 is known to be a negative transcription factor for CBF3/ DREB2A (Chinnusamy et al., 2003; Agarwal et al., 2006). The contribution of TaCHP to salinity tolerance is therefore achieved via the up-regulation of CBF3/ DREB2A transcription by inhibiting MYB15 expression (Fig. 7, B and D). It should be noted that some detected marker genes were either not altered by transcription by inhibiting DREB2A is therefore achieved via the up-regulation of MYB15 gene-specific primers, 0.5 units Easy Taq, and 100 ng DNA, with a cycling regime of 94°C for 2 d followed by 25°C/30 s, 72°C/30 s, 72°C/20 s. A melting curve analysis was performed over the range 80°C to 95°C at 0.5°C intervals. A positive control was provided by a post-amplification analysis based on the wheat actin gene, and three independent replicates were performed per experiment. The specificity of the real-time PCR was confirmed by agarose gel electrophoresis of the amplicon. Relevant primer sequences are listed in Supplemental Table S1.

Full-Length cDNA Cloning

RNA harvested from nonstressed or salinity-stressed SR3 and JN17 seedlings was subjected to both 5′ and 3′ RACE PCR to isolate the full-length TaCHP cDNA, using the SMART RACE kit (CLONTECH). The gene-specific primer sequences were based on differentially expressed probes in a microarray experiment (C. Li and G. Xia, unpublished data) and are listed in Supplemental Table S1. The RACE reactions employed a touchdown protocol of five cycles of 94°C/30 s, 72°C/2 min, followed by five cycles of 94°C/30 s, 70°C/30 s, 72°C/60 s, and 30 cycles of 94°C/30 s, 65°C/30 s, 72°C/60 s. PCR products were cloned and sequenced using the pMD18-T vector according to the manual (Takara).

Gene Structure Analysis and Phylogenetic Tree Analysis

Gene structure was predicted using SMART software (Schultz et al., 1998), and sequence alignments and phylogenetic analyses were conducted using DNAMAN software (Version 5.2.2, Lynnon Biosoft). Phylogenetic trees were constructed using the neighbor-joining algorithm method.

Chromosomal Assignment

The chromosomal assignment of TaCHP was achieved by an analysis based on cultivar CS cytogenetic stocks. Plant material for DNA extraction included CS nullisomic-tetrasomic lines of all 42 chromosomes (Sears, 1954) and some ditelomeric lines. The ditelomeric lines included Dt1AS, Dt1AL, Dt7DS, and Dt7DL. For Dt7DS, the long arm of 7D is missing and the short arm of 7D is present. Genomic DNA extracted from these lines was used as template for 20 μL PCRs containing 1× Easy Taq buffer, 0.2 mM deoxyribonucleotide triphosphates, 0.5 μM TaCHP-specific primers, 0.5 units Easy Taq, and 100 ng DNA, with a cycling regime of 94°C/5 s, followed by 35 cycles of 94°C/60 s, 60°C/60 s, 72°C/30 s.

MATeRIALS AND METHODS

Wheat Material and Treatments

Twenty-five-day-old seedlings of SR3 and JN177 grown in half-strength Hoagland liquid medium under a 16-h light/8-h dark regime at 25°C were subject to following treatments for TaCHP stress-responsive expression analysis: salinity stress by adding 200 mM NaCl to the liquid medium; dehydration by placing seedlings on dry filter paper; a variety of hormonal treatments by supplementing one of ABA (200 μM), ethephon (200 μM), JA (200 μM), or SA (200 μM) directly to the medium; inhibition of ABA biosynthesis by the inclusion of 100 μM norflurazon along with 200 mM NaCl. Seven-day-old seedlings of JN17, a salinity-sensitive wheat (Triticum aestivum) cultivar, and a number of its ditelomeric lines. The ditelomeric lines included Dt1AS, Dt1AL, Dt7DS, and Dt7DL. For Dt7DS, the long arm of 7D is missing and the short arm of 7D is present. Genomic DNA extracted from these lines was used as template for 20 μL PCRs containing 1× Easy Taq buffer, 0.2 mM deoxyribonucleotide triphosphates, 0.5 μM TaCHP-specific primers, 0.5 units Easy Taq, and 100 ng DNA, with a cycling regime of 94°C/5 s, followed by 35 cycles of 94°C/60 s, 60°C/60 s, 72°C/30 s.

Arabidopsis Transgenic Lines and Treatments

A 355:TaCHP construct in vector pCAMBIA1301 or the empty vector alone were transformed into Arabidopsis (Arabidopsis thaliana) Columbia-0 using the floral-dip method (Clough and Bent, 1998). The surface-sterilized seeds were plated on the surface of one-half Murashige and Skoog agar medium, which was first held at 4°C in the dark for 2 d to break dormancy, and subsequently transferred to a 22°C, 16-h light/8-h dark for 2 or 14 days. Two-day-old plants were transferred to fresh one-half Murashige and Skoog agar medium supplemented with various concentrations of NaCl for 3 weeks to assess the phenotypic response to salinity stress, and to the medium supplemented with 0 to approximately 10 μM ABA for 7 d to determine their hormonal response. Fourteen-day-old plants were transferred into one-half Murashige and Skoog liquid medium containing 100 mM NaCl for 3 h, then RNA was extracted for RT-PCR and real-time PCR assay. Germination assay was conducted using approximately 50 surface-sterilized seeds placed on one-half Murashige and Skoog solid medium containing 0 to approximately 30 μM ABA at 4°C for 2 d followed by at 22°C for 3 d to score germination rate. The emergence of radicle was taken as representing a successfully germinated seed. Germination rates were expressed as a percentage of the number of germinated seeds to that of seeds plated. All measurements were carried out with three repeats.

RT and Real-Time PCR Analysis

RNA was isolated using TRizol reagent (Invitrogen), and converted to cDNA using the M-MLV reverse transcriptase kit (Invitrogen). RT-PCR was conducted in 20 μL solution containing 100 ng, 1× Easy Taq buffer (Transgene), 1 unit Easy Taq (Transgene), 100 μM deoxyribonucleotide triphosphates, 0.5 mM forward and 0.5 mM reverse primers, and 1 μL diluted (1:10 v/v) cDNA. The PCR regime consisted of a 5 min denaturation at 94°C, followed by 28 cycles of 94°C/30 s, 55°C/30 s, 72°C/30 s, completed by an extension step of 10 min at 72°C. Amplicons were visualized in ethidium bromide-stained 1% agarose gels. Real-time PCR was performed in 20 μL volumes containing 10 μL 2×SYBR Premix Ex Taq mix (Takara), 0.2 μM forward and 0.2 μM reverse primers, 1 μL diluted (1:10 v/v) first-strand cDNA, with a cycling regime comprising an initial denaturation step (95°C/2 min) followed by 45 cycles of 95°C/10 s, 60°C/20 s, 72°C/20 s. A melting curve analysis was performed over the range 80°C to 95°C at 0.5°C intervals. A positive control was provided by a post-amplification analysis based on the wheat actin gene, and three independent replicates were performed per experiment. The specificity of the real-time PCR was confirmed by agarose gel electrophoresis of the amplicon. Relevant primer sequences are listed in Supplemental Table S1.

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Promoter Sequence Isolation

The promoter sequence was obtained using the sitefinding-PCR method described by Tan et al. (2005). The used primers were listed in Supplemental Table S2. The cis-elements of the promoter sequence were identified using www.dna.afrc.go.jp/PLACE/index.html (Higo et al., 1999).

In Situ Hybridization

The roots of 25-d-old SR3 and JN177 seedlings were used for in situ hybridization, consisting of the most distal 3 to 5 mm root tip of the first lateral root region genes. The full-length TaCHP cDNA was labeled with digoxigenin using SP6 or T7 RNA polymerase (Roche Diagnostics GmbH) following Shan et al. (2008), and were purified and hydrolyzed as described by Hejatko et al. (2006). Signal detection was achieved using an alkaline phosphatase-linked immunoassay (DIG nucleic acid detection kit, Roche).

Biochemical Markers of Stress

Wheat seedlings were exposed to salinity stress by the stepwise twice-introduction of 25 mM NaCl from 50 mM to 200 mM as described above. Arabidopsis seedlings (21-d-old) were cultured in liquid medium supplemented with 100 mM NaCl for 3 d. A POD assay was conducted on the wheat leaves and the whole Arabidopsis seedlings following Sequeira and Mineo (1966), the content of MDA was measured as described by Heath and Packer (1968), and Pro concentration was determined according to Trolld and Lindesly (1955).

Accession number of TaCHP in the National Center for Biotechnology Information is GQ079226.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Isolation of cDNA TaCHP and its corresponding genomic sequence.

Supplemental Table S1. Primers for RACE, real-time PCR, and RT-PCR.

Supplemental Table S2. Primers for SR3 and JN177 TaCHP promoter isolation.

Supplemental Table S3. Binding sites in the upstream genomic sequences of TaCHP of SR3 and JN177.

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LITERATURE CITED


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