RESEARCH PAPER

TaHsfA6f is a transcriptional activator that regulates a suite of heat stress protection genes in wheat (*Triticum aestivum* L.) including previously unknown Hsf targets

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Received 19 June 2014; Revised 20 October 2014; Accepted 22 October 2014

Abstract

Heat stress is a significant environmental factor adversely affecting crop yield. Crop adaptation to high-temperature environments requires transcriptional reprogramming of a suite of genes involved in heat stress protection. This study investigated the role of TaHsfA6f, a member of the A6 subclass of heat shock transcription factors, in the regulation of heat stress protection genes in *Triticum aestivum* (bread wheat), a poorly understood phenomenon in this crop species. Expression analysis showed that TaHsfA6f was expressed constitutively in green organs but was markedly up-regulated during heat stress. Overexpression of TaHsfA6f in transgenic wheat using a drought-inducible promoter resulted in up-regulation of heat shock proteins (HSPs) and a number of other heat stress protection genes that included some previously unknown Hsf target genes such as Golgi anti-apoptotic protein (GAAP) and the large isoform of Rubisco activase. Transgenic wheat plants overexpressing TaHsfA6f showed improved thermotolerance. Transactivation assays showed that TaHsfA6f activated the expression of reporter genes driven by the promoters of several HSP genes (*TaHSP16.8*, *TaHSP17*, *TaHSP17.3*, and *TaHSP90.1-A1*) as well as *TaGAAP* and *TaRof1* (a co-chaperone) under non-stress conditions. DNA binding analysis revealed the presence of high-affinity TaHsfA6f-binding heat shock element-like motifs in the promoters of these six genes. Promoter truncation and mutagenesis analyses identified TaHsfA6f-binding elements that were responsible for transactivation of *TaHSP90.1-A1* and *TaGAAP* by TaHsfA6f. These data suggest that TaHsfA6f is a transcriptional activator that directly regulates *TaHSP*, *TaGAAP*, and *TaRof1* genes in wheat and its gene regulatory network has a positive impact on thermotolerance.

Key words: Gene regulation, Golgi anti-apoptotic protein, heat shock factor, Rubisco activase, transcriptional activator, wheat.

Introduction

Heat stress is one of the major environmental factors that have a negative impact on crop yields. Heat stress causes inactivation of many thermo-labile proteins, accumulation of harmful reactive oxygen species in plant cells, and in severe cases induces programmed cell death (Xue and McIntyre, 2011; Mittler et al., 2012; Grover et al., 2013). Heat stress also rapidly induces a suite of heat stress protection genes, such as those encoding heat shock proteins (HSPs), to very high levels (Kotak et al., 2007a; Mittler et al., 2012; Sarkar et al., 2014; Xue et al., 2014). Genotypic variation in thermotolerance in wheat is linked to the levels of HSP transcripts and proteins (Vierling and Nguyen, 1992; Joshi et al., 1997; Skylas et al., 2002). Many HSP proteins are known to act as molecular chaperones for the protection of thermo-labile proteins against heat-induced denaturation in plant cells (Wang et al., 2004; Basha et al., 2010; Waters, 2013).

Heat shock factors (Hsfs) are transcription factors and are present in all eukaryotic organisms. In plants, Hsf proteins...
form a large family, with 21 members in *Arabidopsis thaliana*, 25 members in rice (*Oryza sativa* L), and at least 56 members in wheat (*Scharf et al., 2012; Xue et al., 2014*). Hsf proteins in plants are divided into three classes: HsfA, HsfB, and HsfC (*Scharf et al., 2012*). Several HsfA subclasses (A1, A2, A3, A4, and A9) have been shown to serve as transcriptional activators for HSP genes (*Mishra et al., 2002; Charron et al., 2007; Schramm et al., 2008; Pérez-Salómó et al., 2014; Kotak et al., 2007b; Xue et al., 2014*), whereas HsfA5 acts as a specific repressor for HsfA4 (*Baniwal et al., 2007*). Hsf proteins contain a DNA-binding domain and bind to heat shock elements (HSEs) with a consensus sequence of GAAnnTTTcnGAA (*Santoro et al., 1998; Nover et al., 2001; Guo et al., 2008; Mittal et al., 2011; Xue et al., 2014*). Although many HsfA proteins are capable of binding to this typical HSE sequence, each subclass of HsfA proteins is known to regulate a subset of heat-stress-responsive genes (*Busch et al., 2005; Nishizawa et al., 2006; Yokotani et al., 2008; Liu and Charron, 2013*). HSEs are present in the promoters of many HSP genes (*Nover et al., 2001; Guo et al., 2008; Mittal et al., 2011; Xue et al., 2014*). Hsf proteins have been proposed to play a key role in regulating the expression of HSP genes, which have a significant impact on thermotolerance (*Kotak et al., 2007a; Scharf et al., 2012*).

In addition to the importance of HSPs in the protection of plant cells during heat stress, other classes of proteins are also known to be important for the adaptation of plants to heat stress. These include co-chaperones [e.g. Rof1 (FKBP62) (*Meiri and Breiman, 2001*), enzymes involved in the synthetic pathways of raffinose family oligosaccharides [e.g. galactinol synthase (*Panikulangara et al., 2004*)], and enzymes for the protection of cells from damage by reactive oxygen species [e.g. ascorbate peroxidase (*Panchuk et al., 2002*)]. Heat stress is also known to adversely affect carbon assimilation through inactivation of some thermo-labile proteins involved in photosynthesis. One well-known example of these proteins is Rubisco activase, which is required for maintaining Rubisco in active form (*Portis, 2003*). Rubisco activity and CO₂ exchange rate in wheat leaves decrease during heat stress (*Law and Crafts-Brandner, 1999*).

Severe heat stress can also lead to programmed cell death (*Mittler et al., 2012*). A number of anti-apoptotic proteins are known to have an important role in suppressing programmed cell death in eukaryotes (*Watanabe and Lam, 2009; Ishikawa et al., 2011*). These proteins include Bax (pro-apoptotic protein) inhibitor I (BI-1) proteins, Golgi anti-apoptotic proteins (GAAP), and inhibitor of apoptosis proteins (IAPs) (*Carrara et al., 2012; Marivin et al., 2012*). Both BI-1 and GAAP proteins are highly hydrophobic and contain the transmembrane Bax inhibitor-containing motif. IAP family proteins contain a baculoviral IAP repeat domain. BI-1 in *Arabidopsis* is known to play a positive role in suppressing the programmed cell death induced by biotic and abiotic stress including heat shock (*Watanabe and Lam, 2006*). Loss-of-function mutants of BI-1 in *Arabidopsis* exhibit increased sensitivity to heat shock-induced cell death (*Watanabe and Lam, 2006*). These authors have also shown that expression of *AtBBI* mRNA was up-regulated in *Arabidopsis* leaves by heat in early hours of heat treatment and before the activation of cell death. Transgenic tomato plant overexpressing an anti-apoptotic gene from the IAP family enhances thermotolerance (*Li et al., 2010*). However, transcription factors that directly regulate expression of anti-apoptotic proteins in plants during heat stress have not been reported to date.

Some Hsf genes are constitutively expressed in plants. In particular, constitutively expressed subclass HsfA1 genes serve as master regulators for triggering heat response (*Mishra et al., 2002; Liu et al., 2011; Liu and Charron, 2012*). In wheat, most HsfA genes are expressed at moderately high levels under normal conditions (*Xue et al., 2014*). It is generally considered that constitutively expressed HsfA1 proteins are in inactive forms through their interaction with some repressors under normal conditions (*Scharf et al., 2012*). HsfA1a in tomato has been shown to maintain its inactive monomer state by association with HSP90/HSP70 under non-heat stress conditions (*Hahn et al., 2011*). However, a number of transgenic studies have shown that constitutive overexpression of some HsfA members can up-regulate a subset of heat inducible genes and enhance thermotolerance in plants (*Prändl et al., 1998; Li et al., 2005; Nishizawa et al., 2006; Ogawa et al., 2007; Yokotani et al., 2008; Liu and Charron, 2013*), as overexpression of a Hsf is likely to make Hsf in excess of its repressor.

In this study, the role of a subclass HsfA6 member of the wheat Hsf family (TaHsfA6f) in the adaptation of wheat to heat stress was investigated. Functional studies on the role of subclass HsfA6 members in plant adaptation to heat stress have not been reported in any plant species. To understand the biological function of TaHsfA6f, this study focused on the identification of target genes regulated by TaHsfA6f. Transgenic wheat lines overexpressing TaHsfA6f driven by a drought-inducible promoter were generated. Affymetrix array analysis revealed that a large number of HSP genes and other classes of heat stress protection genes were up-regulated in the TaHsfA6f overexpressing lines. In particular, an anti-apoptotic gene (TuGAAP) and a Rubisco activase large isoform (TuRCA-L) were also identified as TaHsfA6f target genes. TaHsfA6f target genes also include a C4-type zinc-finger transcription factor (TaC4ZFP). These three genes are previously unknown Hsf targets. Analysis of selected up-regulated genes by quantitative RT-PCR revealed that all of these genes were markedly induced in wheat leaves during heat stress. High affinity TaHsfA6f-binding HSEs were found in the promoters of many TaHsfA6f up-regulated genes examined. Transactivation analysis showed that TaHsfA6f served as a transcriptional activator capable of activating the expression of reporter genes driven by the promoters of TuGAAP and TaRof1 in addition to HSP genes. These observations demonstrate that a HsfA6 transcription factor plays a role in regulation of several classes of heat stress protection genes.

**Materials and methods**

**Plant materials and growth conditions**

*T. aestivum* (cv. Bobwhite) plants were grown in a controlled-environment growth room in 1.5-l pots, containing a 3:1 mix of sand:soil:peat under night/day conditions of 16-h light (500 µmol m⁻² s⁻¹),
16/20 °C, and 80/60% relative humidity. Heat treatment of one-month-old plants at 36 °C commenced at 2 h after lights on and the leaves and roots of heat-treated plants were harvested after 1.5 h and 5 h of heat treatment. Some plants also went through a 3-day heat treatment regime with 5 h at 36 °C per day. Control plants grown at 20 °C were harvested at the same time as for the 1.5-h heat treated samples.

Isolation of total RNA and TaHsfA6f cDNA
Total RNA was isolated from wheat samples using Plant RNA Reagent (Invitrogen, California, USA), according to the manufacturer’s instructions. RNA was further purified through a Qiagen RNAasy column (Qiagen, Australia) after pre-treatment with RNase-free DNase I (Xue and Loveridge, 2004).

TaHsfA6f cDNA was isolated from the leaves of heat-treated wheat plants using 3′-RACE with primers designed from the partial TaHsfA6f cDNA containing the N-terminal sequence. The PCR-amplified product was cloned into pGEM-T Easy vector (Promega) and sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, USA). The TaHsfA6f cDNA sequence was deposited in GenBank (KJ774108).

Plasmid construction
The HV A1s promoter-driven TaHsfA6f construct (barley-HV A1s:TaHsfA6f:rice-rbcS3′) was made by inserting the coding region of TaHsfA6f after the barley HV A1s promoter, using a pHV A1s/GUSR plasmid as reported by Xiao and Xue (2001). pTaHsfA6f–C ELD was constructed by translational fusion of the coding region sequence of TaHsfA6f to the N-terminus of the 6×His-tagged CELD reporter (Xue, 2005). CELD encodes a 1,4-β-glucanase (cellulase) (Xue et al., 1992). The CELD-positive clones containing in-frame fusion of TaHsfA6f–CELD were identified using a CM-cellulose plate (Xue et al., 2005). Maize Ubil promoter-driven TaHsfA6f, TaHsfA1b, and TaHsfA4e effector constructs (maize-Ubil:TaHsfA:rice-rbcS3′) were constructed by replacing xylanase in pUbiSXR (Vickers et al., 2003) with the coding region of a TaHsfA cDNA. TaHSPl6.8, TaHSPl7, TaHSPl7.3 TaHSPl9.1-A1, TaGAAP, TaRofi, and TaRCA-L promoter-driven GFP reporters were constructed by replacing the HV A1s promoter in a HAV A1s:GFP construct (Xue and Loveridge, 2004) with the PCR-amplified fragment of the promoters of interest (Xue et al., 2014; see the HSP promoter isolation section below). TaHSPl9.1-A1 promoter mutants (psHSPl90.0f, pHSPl90.0g, and pHSPl90.0h) were constructed previously (Xue et al., 2014). HSE90-miniDHn6gfp construct was made by adding three repeats of TaHSPl9.1-E1 to the upstream of a minimal promoter (PminiDHn6), which was derived from a drought-inducible barley Dhn6 gene and was inactive by itself (Xue, 2003). A TaGAAP promoter-driven xylanase (xynA) reporter gene was constructed by replacing the Ubil promoter in pUbiSXR (Vickers et al., 2003) with the PCR-amplified fragment of the TaGAAP promoter. Truncated and HSE mutant constructs of TaGAAP promoter were made using PCR-based chromosomal site-directed mutagenesis.

Production of transgenic wheat overexpressing TaHsfA6f
The HV A1s promoter-driven TaHsfA6f construct (barley-HV A1s:TaHsfA6f:rice-rbcS3′) and the selectable marker cassette containing rice-act1:bar:nos:3′ (Xue et al., 2011b) were used to co-transform Bobwhite wheat plants using the particle bombardment as described by Pellegrineschi et al. (2002). Transgenic plants were selected with the herbicide phosphinothricin and grown in a controlled environment growth room as described above. The presence of the barley-HV A1s:TaHsfA6f:rice-rbcS3′ cassette was verified by real-time PCR using genomic DNA (Kooiker et al., 2013).

Expression analysis using Affymetrix wheat genome array
The Affymetrix wheat genome array contains 61 127 probe sets representing 55 052 transcripts from genes distributed across all 42 chromosomes in the wheat genome. One-week-old seedlings of TaHsfA6f transgenic and wild-type Bobwhite plants were treated with 15% PEG (MW 8000) in a controlled environment growth room at 16 °C/20 °C (night/day) for 3 d to induce the expression of the HV A1s promoter-driven TaHsfA6f transgene. RNA from whole seedlings was extracted and processed as described above. Affymetrix wheat genome array expression profiling was performed as described by Xue et al. (2013). The Affymetrix array data were normalized using GeneChip robust multiarray average (Wu et al., 2004). Normalized values were converted to non-log values for comparative analysis of gene expression between transgenic and wild-type plants.

Expression analysis using quantitative RT-PCR
The mRNA levels of the genes of interest were quantified from cDNA samples synthesized from DNase I-treated total RNA using real-time PCR with a ViiA™ 7 system (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The gene-specific primers were designed at the C-terminal coding or 3′-untranslated region. The sequences of real-time PCR primer pairs are listed in Supplementary Table S1.

Two wheat housekeeping genes (TaRP1536 and TaRP15) were selected as internal reference genes for the calculation of relative transcript levels of the genes of interest (Xue et al., 2006, 2008a). The mRNA levels of these internal reference genes were similar in the control and heat–treated samples and checked by the use of an external reference mRNA synthesized from a bovine cDNA (Xue et al., 2011a). The PCR efficiency of each primer pair was determined by a dilution series of samples. The specificity of real-time PCR amplification was confirmed by a single peak in melting temperature curve analysis of real-time PCR-amplified products. Relative quantitation of mRNA levels was as described by Shaw et al. (2009). The apparent expression level of each gene relative to an internal reference gene (TaRP15) was calculated as described by Stephenson et al. (2007).

DNA-binding activity assays
TaHsfA6f–CELD fusion protein tagged with 6×His was purified using Ni-NTA magnetic agarose beads (Xue, 2005). Biotin-labelled double-stranded oligonucleotide probes containing HSE-like sequences were synthesized by filling in partially double-stranded oligonucleotides using Taq polymerase reaction as described previously (Xue et al., 2006). HSE-like sequences were derived from the promoters of the following TaHsfA6f-up-regulated genes: TaGAAP (GenBank accession number: KJ685918), TaRCA-L (KJ685916), TaRofi (KJ685917), TaHSPl6.8 (KJ685920), TaHSPl6.9b (Supplementary Figure S1), TaHSPl7 (KF208539), TaHSPl7.3 (KJ685919), TaHSPl6.24 (Supplementary Figure S1), TaHSPl9.1-A1 (KF208540), and TaHSPl101 (Supplementary Figure S1).

The measurement of DNA-binding activity of TaHsfA6f–CELD was performed as described by Xue (2002) using StreptaWell High Bind (streptavidin-coated 96-well plates from Roche) and binding/washing buffer (25 mM HEPES/KOH, pH 7.0, 50 mM KCl, 2 mM MgCl2, and 0.5 mM DTT) containing 0.15 μg μl−1 sharing sperm DNA, 0.3 mg ml−1 bovine serum albumin, 10% glycerol, and 0.025% Nonidet P-40. About 20 000 fluorescent units h−1 of the CELD activity of TaHsfA6f–CELD fusion protein and 0.4 pmol of biotinylated probes were used per assay. The cellulase activity of the CELD fusion protein bound to immobilized biotinylated probes was measured by incubation in 100 μl of the CELD substrate solution (1 mM methylumbelliferyl β-D-cellobioside in 50 mM Na-citrate buffer, pH 6.0) at 40 °C for 4 h. A biotin-labelled double-stranded oligonucleotide without a HSE was used as a control of background activity in DNA-binding assays.
Transactivation assays

Transactivation of reporter genes by a maize Ubi1 promoter-driven TaHsfA effector construct was analysed as described previously (Xue, 2003). Constructs were introduced into the seedlings of wheat (cv. Bobwhite) by particle bombardment (Xue et al., 2003). An effector gene was co-introduced with a GFP or xynA reporter gene driven by the promoter of interest to determine the transactivation activity. The reporter genes without a TaHsfA effector construct were used as a control. A maize Ubi1 promoter-driven β-glucuronidase (Ubi1:GUS+) construct (pUbiGUS+, Vickers et al., 2003) was also co-bombarded for validation of transformation events among assays. The bombarded seedlings were kept at room temperature (22 °C) or a heat stress temperature in dark until examination of GFP foci (usually for about 20 h except where they are indicated). GFP expression was visualized as green GFP foci under a fluorescence microscope. Tissue sections that had GFP foci were subsequently stained for histochemical detection of GUS activity (Jefferson, 1987). When xynA was used as a reporter gene, the reporter expression was quantitatively determined by measurement of xylanase activity and Ubi1:GUS+ construct activity (Ubi1:GUS+ used for normalisation of transformation efficiency between assays) (Xue et al., 2011a).

Isolation of promoter sequences

Promoter sequences were isolated using PCR-amplification of genomic DNA of T. aestivum genotype SB169 (Xue et al., 2008b). PCR primers designed for isolation of the following five promoters were based on assembled sequences through extension of EST or cDNA sequences using the wheat genome sequence database in CerealDB (Wilkinson et al., 2012). The PCR-amplified DNA fragments were cloned and sequenced. The isolated promoter sequences were deposited in GenBank [TaRCA-L (1184bp upstream of the translation start codon), KJ685916; TaRof1 (815bp), KJ685917; TaGAAP (1045bp), KJ685918; TaHSP17.3 (1652bp), KJ685919; TaHSP16.8 (1253bp), KJ685920].

Phylogenetic analysis

Phylogenetic analysis was conducted to identify the TaHsfA6f subclass position among TaHsfA members that were reported previously (Xue et al., 2014). Hsf DNA-binding domain and heptad repeat region (HR-A/B) sequences of Hsf proteins were used for generation of a phylogenetic tree by ClustalW alignment and the unrooted phylogenetic analysis using the neighbor-joining method using MEGA 6.0 (Machiyama et al., 2014). The region (HR-A/B) sequences of Hsf proteins were used for generation of a phylogenetic tree by ClustalW alignment and the unrooted phylogenetic analysis using the neighbor-joining method using MEGA 6.0 (Machiyama et al., 2014).

Thermotolerance test

Five-day-old seedlings of TaHsfA6f transgenic lines and Bobwhite were treated with a nutrient solution [0.08% Aquasol (Yates, Australia), 2.5 mM CaCl₂, and 1 mM MgCl₂] containing 15% PEG for 2 d, followed by heat treatment at 45 °C for 2 h, and then recovered at 16 °C ± 2°C (night/day) in a controlled-environment growth room (normal plant growth conditions were used) for 3 weeks. In the first two days of recovery 15% PEG was included in the nutrient solution for maintaining TaHsfA6f transgene expression. The shoot length was measured after the end of the recovery phase. Conditions for other control experiments are specified in the figure legends.

Results

TaHsfA6f is constitutively expressed in various organs and up-regulated by heat stress

A full-length Hsf cDNA was isolated based on the EST sequence that contains the N-terminal half of the Hsf DNA-binding domain. Phylogenetic analysis based on conserved DNA-binding domain and HR region sequences showed that this cDNA encoded a Hsf protein that clustered with subclass HsfA6 members and was therefore designated as TaHsfA6f (Supplementary Fig. S2a). A full-length sequence alignment of TaHsfA6f with other TaHsfA members is shown in Supplementary Figure S2b. The TaHsfA6f transcript was constitutively expressed in various wheat organs with the highest expression in the mature flag leaf (Fig. 1A). Upon heat stress, the TaHsfA6f mRNA level was markedly up-regulated in both wheat leaves and roots (Fig. 1B). The heat up-regulation of TaHsfA6f was attenuated in response to prolonged heat treatment (Fig. 1B), in a similar pattern with those of other TaHsfA members (Xue et al., 2014).

Overexpression of TaHsfA6f up-regulates expression of HSP and other classes of heat stress protection genes including Golgi anti-apoptotic protein, Rof1, and Rubisco activase

To elucidate its regulatory role in modulating the expression of genes involved in wheat adaptation to heat stress, transgenic lines overexpressing TaHsfA6f driven by a barley HVA1s promoter were investigated. The barley HVA1s promoter is drought-inducible (Xiao and Xue, 2001). A drought-inducible promoter, instead of a heat-inducible one, was used to assist in the identification of TaHsfA6f target genes in the transgenic lines, as most heat-inducible downstream genes are not, or are little, affected by drought stress based on our analysis of publicly available Affymetrix wheat array data set (accession # TA23 at http://www.plexdb.org, Aprile et al., 2009). A total of eight transgenic lines carrying HVA1s promoter-driven TaHsfA6f were generated. Preliminary quantitative RT-PCR analysis in the seedlings of T1 transgenic lines showed that four transgenic lines had TaHsfA6f expression level >5-fold higher than wild-type Bobwhite plants under polyethylene glycol (PEG)-induced dehydration conditions. In addition, a heat-inducible HSP gene, TaHSP17 (Xue et al., 2014), in these four T1 transgenic lines with PEG induction of the TaHsfA6f transgene was 2.5–16 times higher than Bobwhite, indicating that TaHsfA6f is a positive regulator of HSP genes. At the T2 stage, two TaHsfA6f lines (A6f-9 and A6f-17) showed >15 times higher level of TaHsfA6f expression than Bobwhite under PEG-induced dehydration conditions (Fig. 2A). These two lines together with the wild-type Bobwhite were then used for identification of potential TaHsfA6f target genes using Affymetrix Wheat Genome Array.

This study focused on TaHsfA6f up-regulated genes. This is based on that TaHsfA6f overexpression up-regulated TaHSP17 expression and that an initial transactivation analysis using a TaHSP90.1-A1 promoter-driven reporter gene showed TaHsfA6f acting as a transcriptional activator (also see the section below). Affymetrix array expression profiling revealed that a total of 50 Affymetrix probesets were significantly up-regulated in the TaHsfA6f lines (A6f-9 and A6f-17), at least 1.5-fold higher than Bobwhite (Supplementary Table S2). These up-regulated probesets are also heat-inducible based on the analysis of a publicly available Affymetrix dataset.
TaHsfA6f regulates heat stress protection genes

C4 zinc finger transcription factor (C4ZFP), calmodulin-like protein, and peptidase genes (peptidase M50 and FTSH6). The rest of the genes up-regulated by TaHsfA6f are shown in Supplementary Table S2.

To confirm the results from the Affymetrix array analysis, 15 representative up-regulated genes, and another TaHSP gene (TaHSP16.9b) that has no probeset in the Affymetrix wheat genome array were selected for further expression analysis using quantitative RT-PCR. All of these genes showed a significant increase in expression levels in the high TaHsfA6f-expressing transgenic lines (A6f-9 and A6f-17) under PEG-induced dehydration conditions (Fig. 2B). The expression levels of TaGAAP, TaRCA-L, TaRof1, and TaGalSyn were more than 2.5 times higher in the highest TaHsfA6f-expressing line (A6f-17) than Bobwhite. More than 10-fold up-regulation of TaHSP16.8, TaHSP17, and TaHSP17.3 expression levels was observed in the A6f-17 line (Fig. 2B).

To investigate the heat-responsive patterns of these TaHsfA6f up-regulated genes, we determined their relative expression levels in the leaves of 1-month-old Bobwhite plants treated at 36 °C for 1.5 h or 5 h in comparison with plants without heat treatment. Many of these genes (TaGAAP, TaRof1, TaHSA32, TaFTSH6, TaHSP16.8, TaHSP16.9b, TaHSP17, TaHSP17.3, TaHSP62.4, TaHSP90.1-A1, and TaHSP101b) showed a 1000-fold or more increase in expression levels after 1.5-h heat treatment (Fig. 3). About 200-fold or more increase in expression levels was observed for TaRCA-L and TaGalSyn, and a more than 45-fold increase was observed for TaGST, TaC4ZFP, and TaHSP101. The heat-responsive patterns of all these genes except TaRCA-L during the period of 5-h heat treatment were similar to that of TaHsfA6f (Fig. 1B); i.e. a rapid increase at 1.5 h followed by a marked attenuation at 5 h (Fig. 3). TaRCA-L expression remained high after 5-h heat treatment (Fig. 3A).

The effect of PEG treatment on the expression of TaHsfA6f and its target genes was also examined in wild-type plants. PEG treatment for 3 d did not significantly affect the expression of TaHsfA6f, TaC4ZFP, TaGST, and TaHSP17 in Bobwhite plants, but resulted in around 2-fold up-regulation of many genes targeted by TaHsfA6f (Supplementary Figure S3). TaFTSH6 was the only gene that showed a marked increase in mRNA level by PEG-induced dehydration and TaRCA-L was down-regulated by PEG. The expression profiles of TaHsfA6f and its target genes indicate that these genes except TaFTSH6 are mainly involved in heat adaptation.

TaHsfA6f is a transcriptional activator involved in regulation of heat-induced expression of TaGAAP, TaRof1, and TaHSP genes

To identify target genes directly regulated by TaHsfA6f, the promoter sequences of seven genes up-regulated by TaHsfA6f were successfully identified through sequence assembly from a partial wheat genome sequence database in CerealDB (Wilkinson et al., 2012) and were subsequently isolated. The isolated promoters were used to drive the expression of the green fluorescence protein (GFP) reporter gene (Fig. 4A). The TaHsfA6f effector construct was driven by a constitutive
maize *Ubi1* promoter. Transactivation analysis was performed by bombarding reporter and effector constructs into wheat seedlings. As shown in Fig. 4B, the expression of the GFP reporter gene driven by the promoter of *TaGAAP* or *TaRof1* was induced by heat stress or by co-introduction with the *TaHsfA6f* effector construct without a heat treatment. In contrast, *TaRCA-L* promoter-driven reporter was inducible by heat, but not by *TaHsfA6f*. The GFP reporter driven by a HSP promoter (*TaHSP16.8, TaHSP17, TaHSP17.3, or TaHSP90.1-A1*) was also strongly induced by heat treatment at 36 °C (Supplementary Fig. S4) or by *TaHsfA6f* (Fig. 4B). These data indicate that all of the analysed genes except *TaRCA-L* are potentially direct targets of *TaHsfA6f*.

To examine whether transactivation of these target genes is unique to *TaHsfA6f*, three *TaHsfA6f* target genes (*TaGAAP, TaRof1*, and *TaHSP17.3*) were chosen for analysis of *TaHsfA1b* and *TaHsfA4e* transactivation activity in comparison with *TaHsfA6f*. As shown in Supplementary Figure S5, all three effector genes were able to transactivate these three reporter genes. However, the transactivation strength of *TaHsfA1b* and *TaHsfA4e* for *TaGAAP* and *TaRof1* reporter genes was visibly weaker than that of *TaHsfA6f*, as assessed by relative intensity of GFP foci. In contrast, no visible differences in the intensity of GFP foci of *TaHSP17.3* reporter were observed between *TaHsfA6f* and *TaHsfA1b* or *TaHsfA4e*.

*TaHsfA6f* binds to the elements present in the promoters of *TaGAAP*, *TaRof1*, and *TaHSP* genes

To provide further evidence that *TaGAAP, TaRof1*, and *TaHSP* genes are direct target genes of *TaHsfA6f*, the promoter sequences of 10 genes [above seven genes used in transactivation analysis plus three additional HSP genes (*TaHSP16.9b, TaHSP62.4, and TaHSP101*)] were analysed to identify whether they contain HSE elements. HSE-like motifs were identified in the promoters of all 10 genes (Fig. 5). *TaHsfA6f*-binding HSEs present in these promoters were subsequently identified by *in vitro* DNA-binding assays using a CELD reporter system (Xue, 2002). Although the typical HSE (GAAnnTTCnnGAA or TTCnnGAAnnTCC) motif was found only in the promoter of *TaHSP90.1-A1*, many HSE-like elements (GAAnnnCTCnnGAA or TTTnnnGAAnnTCC) were present in the promoters of *TaHSP90.1-E1, TaHSP90.1-E2, TaHSP90.1-E3, TaHSP90.1-E4, and TaHSP90.1-E1*) present in these promoters had a high binding affinity for *TaHsfA6f* (Fig. 5). However, the *TaHsfA6f* binding affinity to the HSE-like element from the *TaRCA-L* promoter (*TaRCA-E1*) was low. A close examination of high-affinity *TaHsfA6f* motifs in Fig. 5A revealed that *TaHsfA6f* effectively bound to a sequence of GAAAnnnCTCnnGAA (e.g. *TaRof1E1* and *TaHSP90.1-E1*). These DNA-binding data together with transactivation and expression data suggest that *TaGAAP, TaRof1, TaHSP16.8, TaHSP17, TaHSP17.3, and TaHSP90.1-A1* are direct targets of *TaHsfA6f*, whereas *TaRCA-L* is likely to be an indirect target.

Transactivation of *TaHSP90.1-A1* and *TaGAAP* reporter genes by *TaHsfA6f* relies on the presence of a *TaHsfA6f*-binding element in their promoters

To assess whether transactivation of the reporter genes by *TaHsfA6f* occurs through its binding to HSE present in the promoters of the reporter genes, promoter truncation and mutation analyses of *TaHSP90.1-A1* and *TaGAAP* were performed. A truncated *TaHSP90.1-A1* promoter (a 328-bp fragment, named as *sHSP90*) containing a *TaHSP90.1-E1* HSE was still functional for *TaHsfA6f*-mediated transactivation of the
TaHsfA6f regulates heat stress protection genes

Fig. 3. Heat-responsive expression patterns of genes up-regulated by TaHsfA6f (A and B). Expression responses were examined in the leaves of 1-month-old Bobwhite plants. Values are means±SD of three biological replicates. Statistical significance (P<0.05) of differences between control and heat-treated groups (36 °C for 1.5h or 5h) is indicated by an asterisk.

reporter gene (Fig. 6). A further deletion of a 64-bp fragment with the removal of TaHSP90.1E1 (ΔHSE90) abolished the TaHsfA6f-mediated transactivation of the GFP reporter gene (Fig. 6B) or the heat-inducible promoter activity as shown previously (Xue et al., 2014). Addition of the TaHSP90.1E1 element to the ΔHSE90 promoter-driven GFP reporter gene restored transactivation by TaHsfA6f. TaHsfA6f was also able to transactivate a reporter gene driven by a minimal promoter (MiniDhn6) from a barley drought-inducible promoter (Dhn6) with the addition of TaHSP90.1E1 (HSE90-MiniDhn6) (Fig. 6).

The promoter truncation and HSE mutation were also performed for the TaGAAP promoter using a xylanase (xynA) gene as a reporter for quantitative analysis of TaHsfA6f transactivation activity (Fig. 7). TaGAAPe1 is located downstream of a putative TATA box in the TaGAAP promoter. Removal of a 282-bp upstream fragment (containing TaGAAPe2) in the 1045-bp TaGAAP promoter (GAAP–763xynA construct) did not significantly affect its promoter activity induced by the Ubi1 promoter-driven TaHsfA6f effector construct (Fig. 7). Mutation of the TaGAAPe1 element (GAAGCCTCCTGAATCTTC) by site-direct

Fig. 4. Transactivation analysis of GFP reporter genes driven by the promoters of TaHsfA6f target genes in wheat seedlings. (A) Reporter and effector constructs. (B) Transactivation analysis. GFP foci (green) indicate GFP reporter gene expression. Blue foci resulted from the expression of a co-introduced Ubi1:GUS+ reporter and indicate that tissue sections were transformed with these constructs. The red background is the fluorescence from shoot chlorophyll. The GFP expression of all these reporter constructs was induced at 36 °C, including those driven by TaHSP promoters (Supplementary Fig. S2). The TaHsfA6f effector construct activated the expression of reporter genes driven by TaGAAP, TaRof1, and TaHSP promoters, but not TaRCA-L promoter at 22 °C. Illustration of GUS foci is given only when GFP reporter expression is essentially undetectable in the tissue section.
mutagenesis to GAA
GC
CTC
CTG
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C resulted in a GAAP–763 ΔHSExynA reporter construct that was no longer transactivated by TaHsfA6f (Fig. 7). These data indicate that the TaGAAPE1 element is a functional HSE required for TaGAAP transactivation by TaHsfA6f.

Overexpression of TaHsfA6f confers thermotolerance

Although the main aim of this study was to elucidate the role of TaHsfA6f in regulation of genes involved in wheat heat adaptation, relative thermotolerance of transgenic lines overexpressing TaHsfA6f under the control of the drought-inducible HV A1s promoter was also investigated using PEG-induced dehydration stress to induce the transgene expression. Both transgenic lines and wild-type Bobwhite at the 5-day-old seedling stage were pre-treated with PEG for 2 d and then exposed to heat stress at 45 °C for 2 h and followed by recovery at 16 °C/20 °C (night/day) for three weeks with inclusion of further 2 d of PEG treatment at the start of a recovery period. As shown in Fig. 8, transgenic lines recovered from heat injury better than Bobwhite and had longer shoots and more newly grown roots. In contrast, without PEG treatment all seedlings from both TaHsfA6f transgenic lines and Bobwhite were killed by a 2-h 45 °C treatment (Supplementary Fig. S6). No difference in thermotolerance was observed between TaHsfA6f transgenic lines and Bobwhite when they were treated at 42 °C for 2 h without PEG treatment. Furthermore, TaHsfA6f transgenic seedlings had similar growth to Bobwhite without any treatment or with PEG treatment only (Supplementary Fig. S6). These data indicate that the improved thermotolerance in TaHsfA6f transgenic lines in comparison with wild-type plants under heat- and PEG-treated conditions is attributed to induction of TaHsfA6f transgene expression by PEG.

It is interesting to see the improved thermotolerance in TaHsfA6f transgenic lines with relatively low-level mRNA accumulation of TaHsfA6f target genes (Fig. 2) in comparison with their highly induced levels at the early stage of heat induction by treatment at a heat acclimation temperature of 36 °C (Fig. 3). This may be due to the discrepancy in speed
TaHsfA6f regulates heat stress protection genes and amount between mRNA and protein accumulation in the cells during heat stress. Therefore, an experiment was performed to look at the protein level of heat induction of TaHSP17 promoter-driven GFP reporter gene during the course of heat treatment. As shown in Supplementary Figure S7, no GFP foci were detected in samples treated at 45 °C or 42 °C for 2 h at any monitored time points. When samples were placed at a heat acclimation temperature (36 °C), GFP foci became detectable only after 4 h of the heat treatment although the mRNA levels of many HSP genes were increased by 10 000-fold after 1.5 h at 36 °C (Fig. 3). We also introduced TaHSP17 promoter-driven GFP reporter gene to the seedlings...
of the A6f-17 line that were pre-treated with 15% PEG for 2 d to induce the accumulation of TaHsfA6f transgene protein; GFP foci were also detectable after 4 h of post bombardment without heat treatment. The level of GFP protein, as estimated by GFP foci intensity, was much higher in the samples that were co-bombarded with HV A1s promoter-driven TaHsfA6f effector gene with 15% PEG treatment during the 24-h post-bombardment period, compared with A6f-17 samples. This is due to that the copy number of TaHsfA6f effector gene introduced by particle bombardment in a transient expression system is much higher than that in stably transformed transgenic lines. The data from this experiment suggested that the thermotolerance level of wheat plants at above 42 °C without heat acclimation is probably based mainly on the basic levels of thermo-protectants present in the cells, as the high temperature above 42 °C apparently impaired protein synthesis in wheat cells. Pre-treatment of the TaHsfA6f overexpressing lines with PEG for 2 d is expected to provide a sufficient time to accumulate considerably higher TaHsfA6f target gene proteins in the cells of TaHsfA6f transgenic plants than Bobwhite during heat treatment at 45 °C, which resulted in better thermotolerance at 45 °C in the TaHsfA6f transgenic lines than wild-type plants.

**Discussion**

This study investigated the regulatory network of TaHsfA6f in wheat. A number of HsfA subclasses are known to have a role in regulation of genes involved in heat stress protection (Mishra et al., 2002; Liu and Charng, 2012; Charng et al., 2007; Schramm et al., 2008; Pérez-Salamó et al., 2014; Kotak et al., 2007b; Xue et al., 2014). Constitutive overexpression of HsfA members from several other plant species lead to up-regulation of heat inducible genes in transgenic plants under non-stress conditions (Prändl et al., 1998; Li et al., 2005; Nishizawa et al., 2006; Ogawa et al., 2007; Yokotani et al., 2008).
Hwang et al., 2008; Liu and Chang, 2013). However, involvement of subclass HsfA6 members in regulation of plant adaptation to heat stress is currently unknown. In Arabidopsis, HsfA6a expression is not significantly influenced by heat, but is up-regulated by abscisic acid, salt, or drought stress (Hwang et al., 2014). This study showed that TaHsfA6f was a transcriptional activator, acted as a positive regulator of several classes of heat stress protection genes, and contributed to thermotolerance in wheat. TaHsfA6f was expressed mainly in green organs (leaf, stem, and hull) under non-stress conditions and was highly heat-inducible during the early hours of heat stress, but not by PEG-induced dehydration stress, indicating its potential role in protecting these organs during heat stress. The heat induction pattern of TaHsfA6f was similar to those of the HSP genes, the expression levels of which are attenuated after prolonged heat exposure (5-h heat treatment) (Xue et al., 2014). Expression analysis of transgenic lines overexpressing TaHsfA6f under the control of the drought-inducible HVA1s promoter revealed that TaHsfA6f positively regulated expression of a number of HSP and other classes of known-function heat stress protection genes that include TaGAAP, TaRof1, TaRCA-L, TaGST, HSA32, and TaGalSyn. Expression of all of these TaHsfA6f-up-regulated genes was strongly induced by heat treatment with a similar heat-responsive pattern to that of TaHsfA6f with the exception of TaRCA-L.

Among these TaHsfA6f-regulated genes, the role of HSPs in heat protection is well known (Wang et al., 2004; Basha et al., 2010). Other proteins that have been shown to have a positive impact on thermotolerance are Rof1, galactinol synthase, GST, HSA32, and RCA. Rof1 (FKBP62) is a member of the multidomain FK506 binding protein family and is known to physically interact with HSP90.1 in Arabidopsis (Meiri and Breiman, 2009). Overexpression of Rof1 in Arabidopsis leads to improved long-term acquired thermotolerance, whereas knockout mutation of Rof1 in Arabidopsis reduces thermotolerance (Meiri and Breiman, 2009). Galactinol synthase catalyzes the first step in the synthetic pathways of raffinose family oligosaccharides, which are compatible solutes with a role in protecting plant cells against several abiotic stresses including heat stress (Panikulangara et al., 2004). GST has a role in alleviating oxidative stress (Gill and Tuteja, 2010). Reactive oxygen species are elevated during heat stress (Mittler et al., 2012), which leads to oxidative stress. HSA32 is another important protein up-regulated by heat in plants (Chang et al., 2006). A knockout mutation study has shown that HSA32 is essential for long-term acquired thermotolerance in Arabidopsis (Chang et al., 2006, 2007). HSA32 can slow the turnover of HSP101 and is involved in prolonging the memory of heat acclimation (Wu et al., 2013). Rubisco activase is a member of an AAA family of proteins (a class of chaperone-like ATPases) and has been considered as a specific catalytic chaperone for maintaining Rubisco in active form (Portis, 2003). High temperature promotes inactivation of Rubisco activase, which subsequently reduces the amount of the active form of the Rubisco enzyme and hence the photosynthesis rate. It has been shown that a decrease in Rubisco activase leads to increased thermosensitivity of photosynthesis (Sharkey et al., 2001). Rubisco activase mRNA level has been reported to be down-regulated in cotton during heat stress (DeRidder and Salvucci, 2007), but in rice the Rubisco activase large isofrom protein level is up-regulated by heat with a slight decrease in the Rubisco activase small isofrom protein level (Wang et al., 2010). Rubisco activase large and small isoforms in rice are derived from the same gene by alternative splicing and there is no significant change in the total Rubisco activase mRNA level in rice during heat stress (Wang et al., 2010). Transgenic rice plants overexpressing a Rubisco activase large isofrom showed higher thermotolerance than wild-type plants (Wang et al., 2010). The NCBI sequence database search and analysis revealed two Rubisco activase genes in wheat: a large isoform (TaRCA-L, AF251264) and a small isoform (TaRCA-S, DQ984669) encoded by two separate nuclear genes. This study showed that expression of TaRCA-L was markedly up-regulated by heat in wheat leaves. Heat up-regulation of TaRCA-L expression could partly compensate its heat-promoted inactivation. In addition, during heat stress RCA has been proposed to have another chaperone role in potential protection of protein synthesis machinery in the chloroplast against heat inactivation (Rokka et al., 2001).

A number of heat protection genes directly regulated by Hsfs have been experimentally demonstrated in previous studies in several plant species, which are mainly HSP genes (Rojas et al., 2002; Kotak et al., 2007b; Singh et al., 2012; Xue et al., 2014) and galactinol synthase (Panikulangara et al., 2004; Pillet et al., 2012). This study identified a TaHsfA6f regulatory network and a number of direct target genes regulated by TaHsfA6f. Analysis of promoter sequences of ten TaHsfA6f up-regulated genes revealed that all these genes, except TaRCA-L and TaHSP16.9b, contain at least one high-affinity TaHsfA6f-binding element. TaHsfA6f showed a high binding affinity to the typical HSE (GAAnnTTCnnGAA) as well as a sequence of GAAnnCTCnnGAA. Results obtained from transactivation analysis of reporter genes driven by the promoters of TaHSP16.8, TaHSP17, TaHSP17.3, TaHSP90.1-A1, TaGAAP, and TaRof1 also support that these TaHsfA6f up-regulated genes are direct target genes of TaHsfA6f. The reporter gene driven by the TaRCA-L promoter was also heat-inducible, but not by TaHsfA6f in transactivation analysis. The transactivation data together with the absence of a high-affinity TaHsfA6f element in the TaRCA-L promoter suggest that TaRCA-L is likely to be an indirect target gene of TaHsfA6f.

Most significantly, two direct TaHsfA6f target genes (TaRof1 and TaGAAP) identified in this study have not been experimentally demonstrated previously for any Hsf members from other plant species. Comparative analysis of relative transactivation strength of TaHsfA6f with TaHsfA1b and TaHsfA4e showed that TaGAAP and TaRof1 reporter genes were preferentially regulated by TaHsfA6f among these three TaHsfA subclass members. TaRof1 protein shares 73.3% and 91.7% amino acid identities with previously characterised wFKBP73 and wFKBP77 in wheat (Blecher et al., 1996; Kurek et al., 1999). wFKBP77 is also a heat-inducible gene, whereas wFKBP73 is not (Kurek et al., 1999). The involvement of GAAP in plant adaptation to high temperatures has
not been reported to date. Studies from mammalian systems suggest that GAAP has a role in suppressing programmed cell death (Gubser et al., 2007; Saraiva et al., 2013). This study showed that the GAAP gene in wheat was highly heat-inducible (>1000-fold increase with 1.5-h heat treatment at 36 °C) and its heat induction pattern is similar to that of TaHSA32. Further evidence of TaHsfA6f being a direct transcriptional activator of TaGAAP and TaHSP90.1-A1 was from promoter truncation and HSE mutation analyses. Transactivation of TaGAAP and TaHSP90.1-A1 by TaHsfA6f relied on the presence of a high-affinity TaHsfA6f-binding HSE in their promoters. These analyses provide substantial evidence that TaHsfA6f is a transcriptional activator that directly regulates the heat-inducible expression of TaGAAP and TaHSP90.1-A1. Furthermore, this study showed that the addition of the TaHSP90.1E1 element to a drought-inducible promoter resulted in a reporter gene that was inducible by heat or TaHsfA6f, thus demonstrating that a functional HSE is sufficient for turning a non-heat-inducible promoter into a heat-inducible one.

Another finding of this study is the identification of a gene that encodes a highly heat-inducible transcription factor (TaC4ZFP) from the ZPR1 zinc finger family and which is regulated by TaHsfA6f. TaC4ZFP transcript level was up-regulated by 60-fold after 1.5-h heat treatment in wheat leaves. TaC4ZFP contains two C4-type zinc fingers, known as the ZPR1 domain. Recently, a ZPR1 protein from wild tomato has been shown to be a transcriptional activator and binds to an ABA-responsive element (Li et al., 2013). Regulation of this family of transcription factors by a Hsf is previously unknown. Future work on characterisation of the TaC4ZFP regulatory network would identify further downstream genes of TaHsfA6f.

It is interesting to note that there was a large difference in the accumulation of target gene transcript levels between early heat induction of target genes in wild-type plants and TaHsfA6f overexpressing transgenic plants, although TaHsfA6f expression levels in transgenic plants (A6f-9 and A6f-17) carrying HVA1s promoter-driven TaHsfA6f transgene under PEG-induced dehydration conditions were almost comparable to its heat-induced level. This discrepancy may be explained by the following two factors. Firstly, many heat-up-regulated genes are regulated by multiple members of the Hsf family. In particular, TaHsfA2 subclass members are phylogenetically close to TaHsfA6 members. A TaHsfA2 member (TaHsfA2b) has also been shown to be a strong transcriptional activator for activation of expression of TaHSP17 and TaHSP90.1-A1 in wheat (Xue et al., 2014). Secondly, it is known that Hsf repressors are active under non-heat stress temperature (Hahn et al., 2011; Scharf et al., 2012), which will reduce the level of functional TaHsfA6f protein in TaHsfA6f transgenic plants. Although the transcript levels of TaHsfA6f target genes in transgenic lines after 3 d of PEG treatment were relatively low, improved thermotolerance of transgenic lines than wild-type plants and can help the recovery of heat-injured plants.

In conclusion, this study, through the identification of TaHsfA6f target genes, shows that TaHsfA6f is one of the regulators of wheat adaptation to heat stress. This is supported by observation of improved thermotolerance of plants overexpressing TaHsfA6f. It is likely that the improved thermotolerance observed in the TaHsfA6f transgenic plants relies on the concerted action of genes regulated by this transcriptional activator. This subclass HsfA6 regulatory network includes three important classes of previously unknown targets: GAAP, RCA-L, and C4ZFP. For improvement of thermotolerance in crop species grown in heat-prone environments, an appropriate HsfA overexpression system needs to be investigated, as constitutive overexpression of a Hsf gene can lead to growth retardation (Ogawa et al., 2007; Zhu et al., 2009). This study used a drought-inducible expression system for overexpression of TaHsfA6f, which may be useful for wheat crops grown in terminal drought stress environments where wheat crops often encounter soil water deficit and occasional heat stress during grain filling. However, the expression level of the TaHsfA6f transgene using the drought-inducible promoter obtained in this study may not be high enough to achieve marked improvement of thermotolerance. Furthermore, the usefulness of this approach for improvement of wheat yield under drought/heat-prone environments awaits further investigation in field trials with appropriate field growth conditions.

Supplementary data
Supplementary data are available at JXB online.

Table S1. Real-time-PCR primers of T. aestivum genes.

Table S2. Genes are up-regulated in TaHsfA6f over-expressing lines.

Figure S1. Analysed promoter sequences of heat up-regulated genes from T. aestivum.

Figure S2. Neighbor-joining phylogenetic tree of wheat HsfA proteins and amino acid sequence alignment of TaHsfA6f with other full-length TaHsfA class members.

Figure S3. Expression of TaHsfA6f and its target genes in wild-type plants in response to PEG treatment.

Figure S4. The heat-induced expression of GFP reporter genes driven by HSP promoters.

Figure S5. Relative strength of TaHsfA6f transactivation activity in comparison with TaHsfA1b and TaHsfA4e.

Figure S6. Illustration of thermotolerance and growth of TaHsfA6f transgenic lines (A6f-9 and A6f-17) and Bobwhite (BW) under various control conditions.

Figure S7. Time course of the appearance of GFP foci produced by TaHSP17 promoter-driven GFP reporter gene under various conditions.
Acknowledgements

We thank Terry Grant for his assistance in maintenance of the controlled environment facility. Authors are also grateful to Kemal Kazan for his valuable comments and suggestions in the preparation of this manuscript. This work was supported by funds from CSIRO.

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