Biochemical and Biophysical Research Communications 455 (2014) 139–146

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cell cycle transcription factor E2F2 mediates non-stress temperature response of *AtHSP70-4* in *Arabidopsis*

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ARTICLE INFO

Article history:
Received 15 October 2014
Available online 22 October 2014

Keywords:
Arabidopsis thaliana
Temperature sensitive
AtHSP70-4 promoter
cis-Element
E2F2

ABSTRACT

AtHSP70 expression exhibits both stress and non-stress temperature response, however, the molecular mechanisms underlying these temperature signaling pathways remain elusive. Here we performed truncation and deletion assay to investigate the *cis*-elements within the promoter region of *AtHSP70-4* (AT3G12580). And found the region between −1000 and −1100 bp from the translation initiation site (TIS) was indispensable for the non-stress temperature response of *AtHSP70*. Further deletion assay of several candidate motifs within this region suggested that one 'GCGCCAAA' sequence played the critical role. This motif was found as the reverse DNA-binding motif of cell cycle transcription factor E2F family. EMSA assay verified one number of Arabidopsis E2F family—E2F2 could bind to *AtHSP70-4* promoter *via* this motif. These results indicated the temperature regulated expression of *AtHSP70-4* may be mediated by cell cycle transcription factors and participate in plant acclimations to non-stress temperature changes.

1. Introduction

Among all the environmental factors that affect plant growth, temperature plays a core role [1,2]. For most crops, normal temperature responses are of primer importance to maintain the biomass yield [3,4]. In rice, appropriate temperatures are needed for almost all the development processes like tiling, flowering, pollen fertility, heading and final filling of seed grain [5–9]. Generally, each plant species has a temperature spectrum in which the normal growth will be maintained. Beyond this spectrum, plant need to accumulate itself to too low or too high temperature even after acclimatization [10]. Upon being exposed to unexpected temperature stress, diverse chaperones genes, previously designated as heat shock protein genes, will be induced to expressed for the stress tolerance [11–15]. In *Arabidopsis thaliana*, one typical example is the *HEAT SHOCK PROTEIN 70* (*AtHSP70*) gene family, which helping plant adapt to adverse circumstance [16–19]. This gene family consist of 18 members, and most members can be induced by both heat and cold shocks [20]. Sung et al. found that the transcription of *AtHSP70* members could increase dramatically within the 0.5–1.5 h after heat shock, then resume to the original levels with heat shock last for longer time [16]. As for cold shock, up to 12 h or even more continuous stimulus is needed to initiate increased transcription [16,21].

Among the *AtHSP70* family members, the expression of *AtHSP70-4* (AT3G12580) was found to exhibit both non-stress and stress temperature responses. Unlike the stress temperature responses which generally exhibit short-term impulse of transcription increase [22,23], the expression of *AtHSP70-4* fluctuated positively relate to the non-stress temperature change for long time [11,16,24,25]; von [26], accordingly, *AtHSP70-4* was used as an output of non-stress temperature response [27].

To date, two distinct pathways for perception of the non-stress temperature change have been identified in *Arabidopsis thaliana*. Using *AtHSP70-4* promoter-luciferase reporting system, Kumar et al. found that H2A.Z-containing nucleosome was responsible for almost half of non-stress temperature responses in *Arabidopsis*. The replacement of histone variant H2A.Z with H2A in the transcription start nucleosome could trigger temperature-responsive gene expression [27–29]. Besides, the original light signaling transcription factor *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*) is also found essential for high temperature responses, including petiole and hypocotyl elongation, and flowering induction [30–35]. However, little connection between these two temperature-perception pathways was found yet.

Abbreviations: TIS, Translation initiation site; EMSA, Electrophoretic Mobility Shift Assay; *HSP70, HEAT SHOCK PROTEIN 70.*

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http://dx.doi.org/10.1016/j.bbrc.2014.10.083
0006-291X/

Here deletion assay of promoter was employed to investigate the *cis*-elements within *AtHSP70-4*. And found the −1000 to −1100 bp region from the TIS of *AtHSP70-4* was indispensable for non-stress temperature response. Further investigation identified that one 'GCGCCAAA' box was the core element of this region. Interestingly, this box exhibited as a reverse DNA-binding motif for E2F transcription factor family [36–38], which is responsible for DNA replication in cell cycle [39], suggesting temperature sensitive expression of *AtHSP70-4* may be mediated by cell cycle transcription factors and participate in plant acclimations to non-stress temperature changes.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) was used in all of the promoter analysis experiments. The plants were cultivated under a 16-h-light/8-h-dark photoperiod with light levels of ~300 illumination intensity, at 23 °C. All seeds were stratified for 4 day in the dark at 4 °C prior to growth.

2.2. Plant transformation

A promoter sequence of *AtHSP70-4* was obtained and truncated by PCR and cloned into pCAMBIA3300. Transgenic plants were generated via floral-dip transformation [40]. The transgenic seedlings were screened on MS medium containing 25 mg/L hygromycin. At least 3 independent homozygous lines of every transgenic plants were used in this study.

2.3. Plasmid constructs

2.3.1. AtHSP70-4 promoter truncation constructs

The *AtHSP70-4* promoter fragment from −1.5 kb to the TIS was obtained by PCR of genomic DNA (Fig. 1A and B) and cloned into pMD™18-T vector (Takara, Japan). Then the promoter fragment was inserted into the derived binary vector pCAMBIA3300 in front of the *GFP* reporter gene. The truncated *AtHSP70-4* promoter sequences including −1 kb, −500 bp and −200 bp from the TIS respectively (Fig. 1B), were made by PCR using the primers listed in Table 1, cloned into pMD™18-T, and then cloned into pCAMBIA3300 to form truncated *AtHSP70-4* promoter-driven GFP, designated *pHSP70*^{1K}*:GFP*, *pHSP70*^{500}*:GFP* and *pHSP70*^{200}*:GFP*.

2.3.2. AtHSP70-4 promoter deletion constructs

Using *pHSP70*$^{1.5k}$*:GFP* construct as template, amplifying deleted *AtHSP70-4* promoter fragments by PCR with forward and reverse primers (shown in Table 1), to generate deletion constructs designated *pHSP70*$^{\triangle 1}$*:GFP*, *pHSP70*$^{\triangle 2}$*:GFP*, *pHSP70*$^{\triangle 3}$*:GFP*, *pHSP70*$^{\triangle 4}$*:GFP*, *pHSP70*$^{\triangle 5}$*:GFP* (Fig. 1C).

2.3.3. E2F2-GST protein expressing constructs

The CDS of *E2F2* was amplified by PCR using a set of oligonucleotide primers, 5′-CGGGATCCATGGCCGCGACATCAAACTCAGGCGAAGA-3′ and 5′-GCGTCGACTCAGCTGTTGAAGTTGCTCCATAAATCT-3′, and double-digested with *Bam*HI and *Sal*I, then ligated to pGEX-4T-1 and transformed into *Escherichia coli* BL21.

2.4. Observation of GFP fluorescence

Fully expanded leaves from the transgenic plants were dissected and laid on a microscopy slide. Adding one drop of sterile water, a slide was covered carefully without squeezing and imaged under Zeiss LSM 710 confocal microscope (Zeiss, Germany). As for

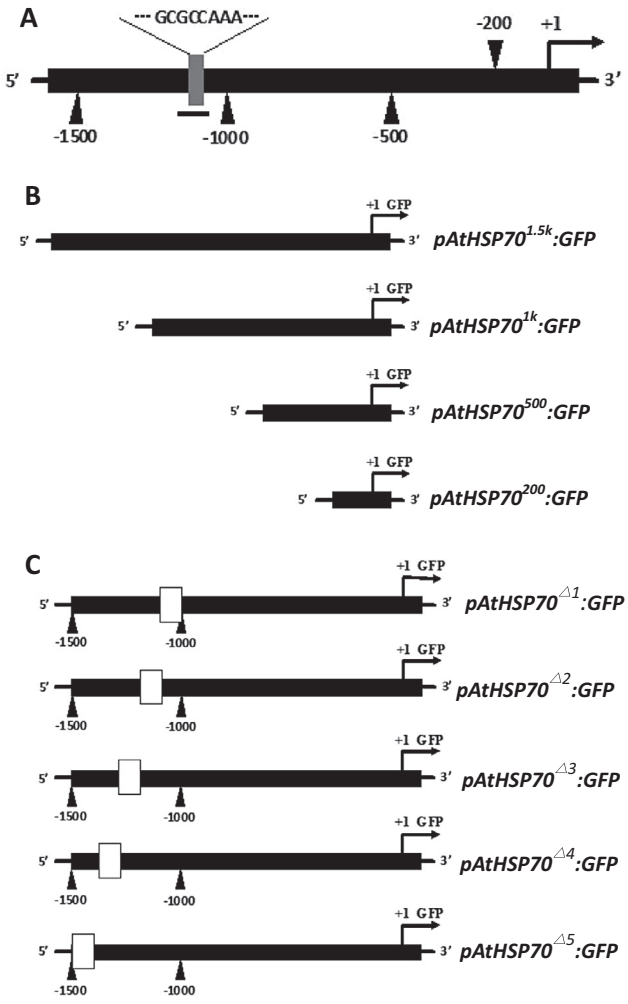

Fig. 1. Peomoters construction employed in transgenic plant. (A) Diagram of the *AtHSP70-4* promoter region showing the relative positions of the core cir-element. The core cir-element is marked by gray rectangles, and relative positions and sizes of the probe are indicated by black line. (B) The truncated *AtHSP70-4* promoter fragments for driving *GFP* expression. (C) The deletion *AtHSP70-4* promoter fragments for driving *GFP* expression, deleted zone is marked by blank rectangles.

Table 1
PCR primers in this study.

Primer name	Sequence (5′–3′)
pHSP70F-1.5k	CACCTCTGTGTGTGATGGTCC
pHSP70F-1k	CACCTAGATACAATAAGGCAACA
pHSP70F-500	CACCACAAAGTTTTGAGTCAAA
pHSP70F-200	CACCAGTTACTATTTGACAATTTA
pHSP70F$^{\triangle 1}$	TGTAGTCTCTTCTGTGGACTTAGATACAATAAGGCAACA
pHSP70R$^{\triangle 1}$	TGTTGCCTTATTGTATCTAAGTCCACAGAAGAGACTACA
pHSP70F$^{\triangle 2}$	TCTGGCTCTGCCTCTGGCTCTTGTATTTGGTGAAATTGAA
pHSP70R$^{\triangle 2}$	TTCAATTTCACCAAATACAAGAGCCAGAGGCAGAGCCAGA
pHSP70F$^{\triangle 3}$	ATTCCCACTGGGATCATCCCTAGTTGAGTTTAGGGAGCTG
pHSP70R$^{\triangle 3}$	CAGCTCCCTAAACTCAACTAGGGATGATCCCAGTGGGAAT
pHSP70F$^{\triangle 4}$	CGTTTTAGGTTTTTACATAAATTTTTTTGGTCTTTTTTTT
pHSP70R$^{\triangle 4}$	AAAAAAAAGACCAAAAAAATTTATGTAAAAACCTAAAACG
pHSP70F$^{\triangle 5}$	ATTCTCAACTTGTTCTCCAA
pHSP70R-co	GGAAGAGAAGGCAGAGAGGT
p*GFP*-RT-F	ATGGTGAGCAAGGGCGAGGAG
p*GFP*-RT-R	TTACTTGTACAGCTCGTCC
pE2F2-F	CGGGATCCATGGCCGCGACATCAAACTCAGGCGAAGA
pE2F2-R	GCGTCGACTCAGCTGTTGAAGTTGCTCCATAAATCT

p*HSP70R*-co = the 3′ end common primer for PCR amplifying *AtHSP70-4* promoter; p*HSP70F*$^{\triangle 1}$ *to* p*HSP70F*$^{\triangle 5}$ = the 5′ primers respectively contained deleted −1100 to −1000, −1200 to −1100, −1300 to −1200, −1400 to −1300, −1500 to −1400 region.

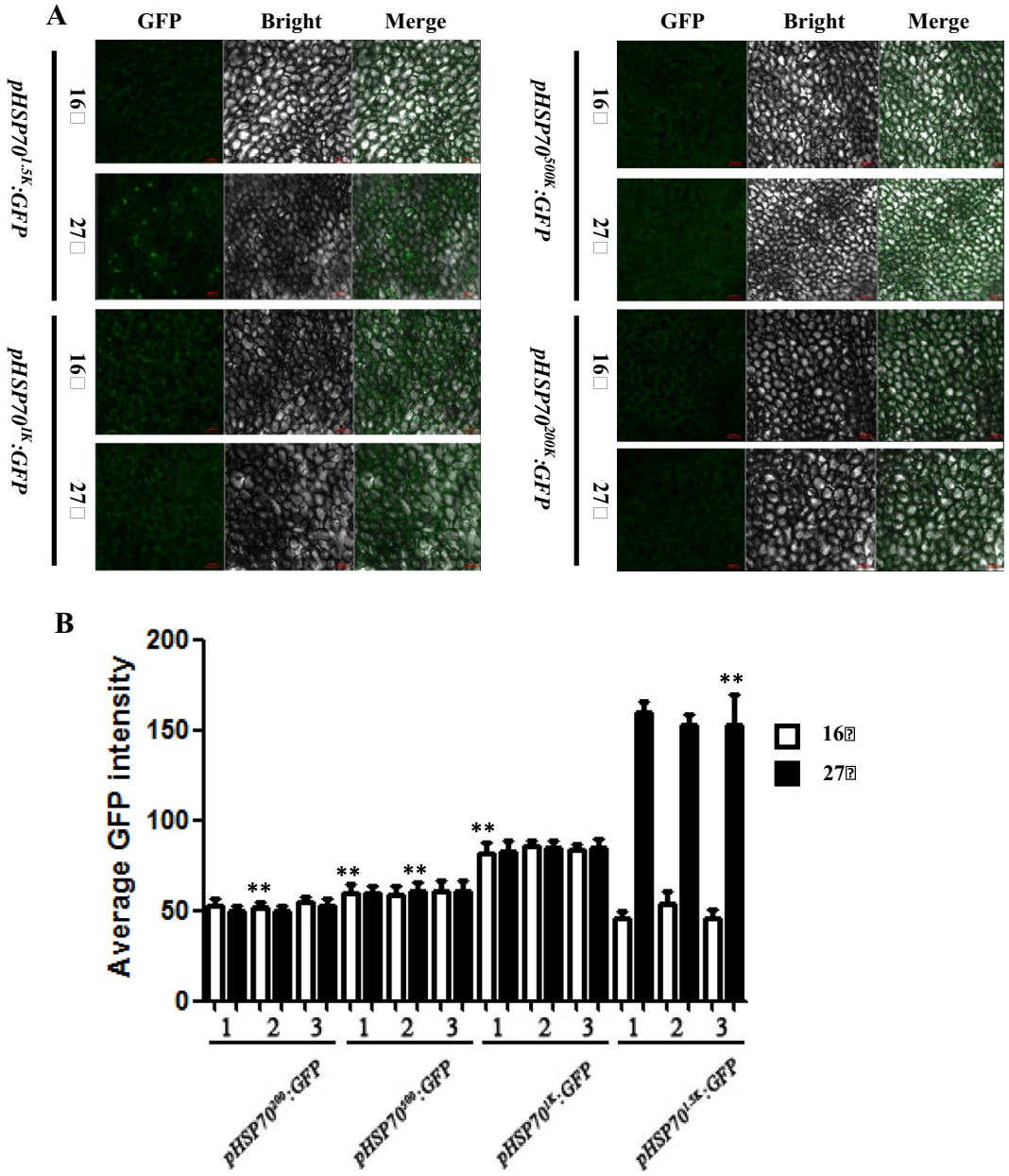

Fig. 2. Diverse truncated and deleted *AtHSP70* promoters driving GFP expression under different temperature in transgenic plants. (A) The images of HSP70-4:GFP fusion protein in Arabidopsis leaf, observed under Confocal microscope. Every image was repeated in three independent experiments using more than three transgenic lines in each experiment. (B) The semi-quantification of the average green intensities by SMART software in the mesophyll ($n > 10$ microscope view areas, $^{**}p < 0.001$, $p < 0.05$, Student's t test) of transgenic plants. Note that 1, 2 and 3 means 3 independent lines of transgenic plants. Bar = 50 μm. (C) The images of HSP70-4:GFP fusion protein in Arabidopsis leaf, observed under Confocal microscope. Every image was repeated in three independent experiments using more than three transgenic lines in each experiment. (D) The semi-quantification of the average fluorescence intensity (y axis) in different transgenic plants by SMART software in the mesophyll ($n > 10$ microscope view areas; $^{**}p < 0.001$, $p < 0.05$, Student's t test) of transgenic plants. Note that 1, 2 and 3 means 3 independent lines of transgenic plants. Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

semi-quantification of the fluorescence intensity, randomly >10 microscope view areas were selected for measuring and normalizing by the SMART software. Statistics of at least 5 leaves per line, 3 lines per transgenic plants.

2.5. Semi-quantification PCR

Fully expanded leaves were collected for extracting total RNA and performing reverse transcription according to Zhang et al. [41]. PCR amplify GFP CDS fragment with GFP CDS (GFP RT-F&R) primers listed in Table 1. Three PCR reactions were repeated independently using *Actin* gene as an internal control.

2.6. Websites for cis-element assay

Website http://bioinformatics.psb.ugent.be was used to analyze putative *cis*-element(s) at the crucial region on the *AtHSP70-4* promoter. Manipulation of assay was performed according to the step suggestions of the website.

2.7. EMSA assay

The EMSA was conducted using LightShift™ EMSA Optimization & Control Kit (Thermo) according to the manufacturer's protocol. The recombinant E2F2-GST protein was purified from *E. coli*

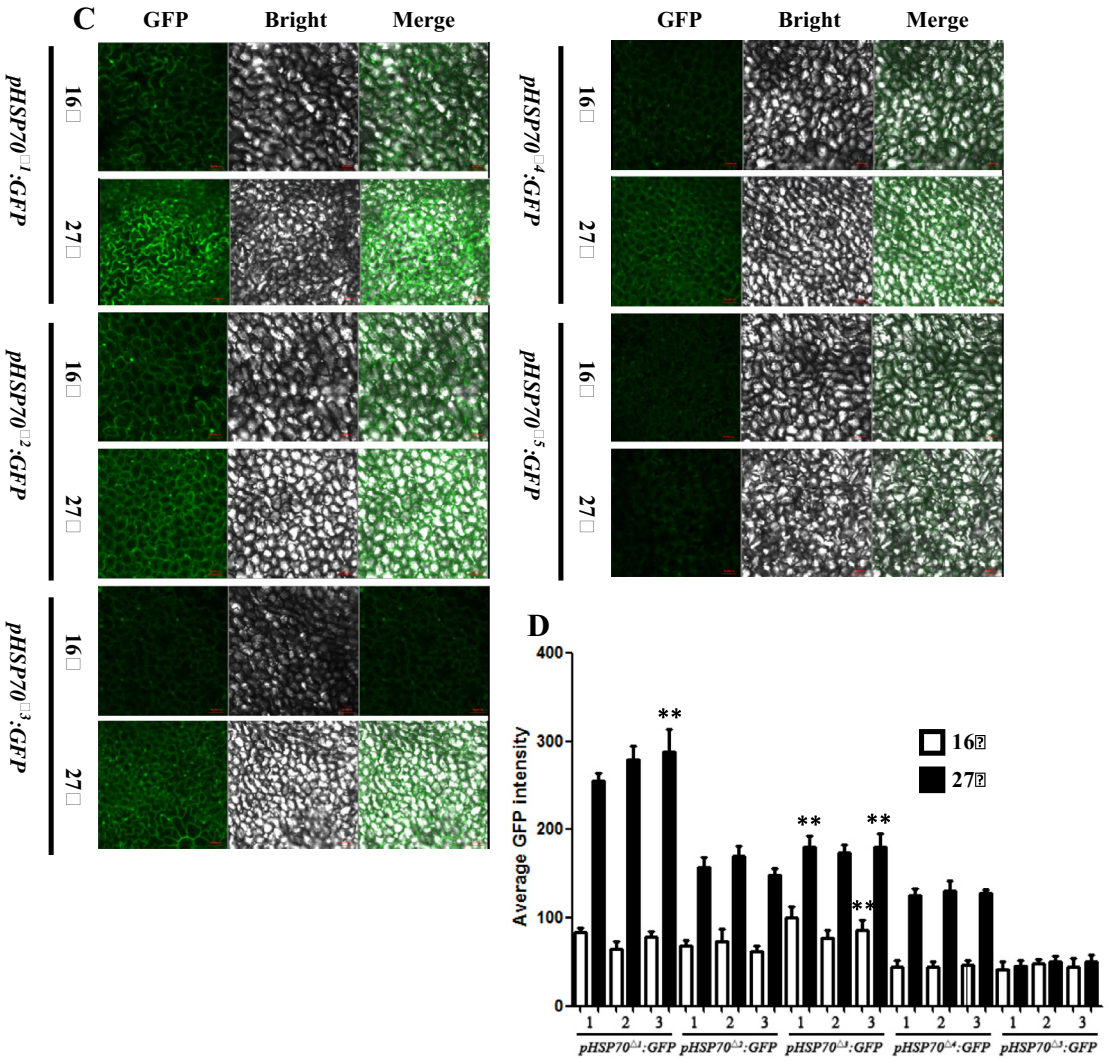

Fig. 2 (*continued*)

BL21strain. The fragment of the *AtHSP70-4* promoter −1027 to −1083 bp from the TIS was artificially synthesized as a probe (Sangon Biotech, Company in Shanghai, China). Biotin-unlabeled fragment of the same sequence was used as competitors.

3. Results

3.1. Truncated assay demonstrated that 1.5 K promoter region was necessary for non-stress temperature-responsive expression of AtHSP70-4 gene

The truncated versions of 1.5 kb, 1 kb, 500 bp and 200 bp *AtHSP70-4* promoter fragments were integrated into eGFP expression constructs and transformed into plants as described by Clough and Bent [40]. As showing in Fig. 2. 1.5 K *AtHSP70-4* promoter region exhibited identical temperature response of eGFP, with approximately 1.5–2 times fluorescence intensity increase at 27 °C compared with 16 °C (Fig. 2A and B), which was further verified by semi-quantification RT-PCR (Fig. 3A). However, the versions truncated promoters, including 1 kb, 500 bp and 200 bp long, just exhibited non-temperature response in eGFP fluorescence expression (Fig. 2 A and B). Thus, the region of −1500 to −1000 bp of promoter was necessary for non-stress temperature-responsive expression of *AtHSP70-4*.

3.2. The −1100 to −1000 bp region of AtHSP70-4 promoter was indispensable for non-stress temperature response

Once it was found that the 1.5 kb promoter region was necessary for the temperature-responsive expression of *AtHSP70*, the deletion constructs of −1100 to −1000 bp, −1200 to −1100 bp, −1300 to −1200 bp, −1400 to −1300 bp and −1500 to −1400 bp were made and had been transformed into plants. The fluorescence detection showed that all transgenic plants exhibited identical GFP expression response to temperature change like the *pHSP70$^{1.5k}$:GFP* transgenic plants, except the plant imparting the deletion −1100 to −1000 bp construct (Figs. 2C, D and 3B). Thus, the region of −1000 to −1100 bp was dispensable for the non-stress temperature response expression of *AtHSP70-4*.

3.3. One 'GCGCCAAA' sequence was the core sequence of the −1100 to −1000 bp region of AtHSP70-4 promoter

Further assays were performed to found the core motifs around the −1100 to −1000 bp region of *AtHSP70-4* promoter. Empirically

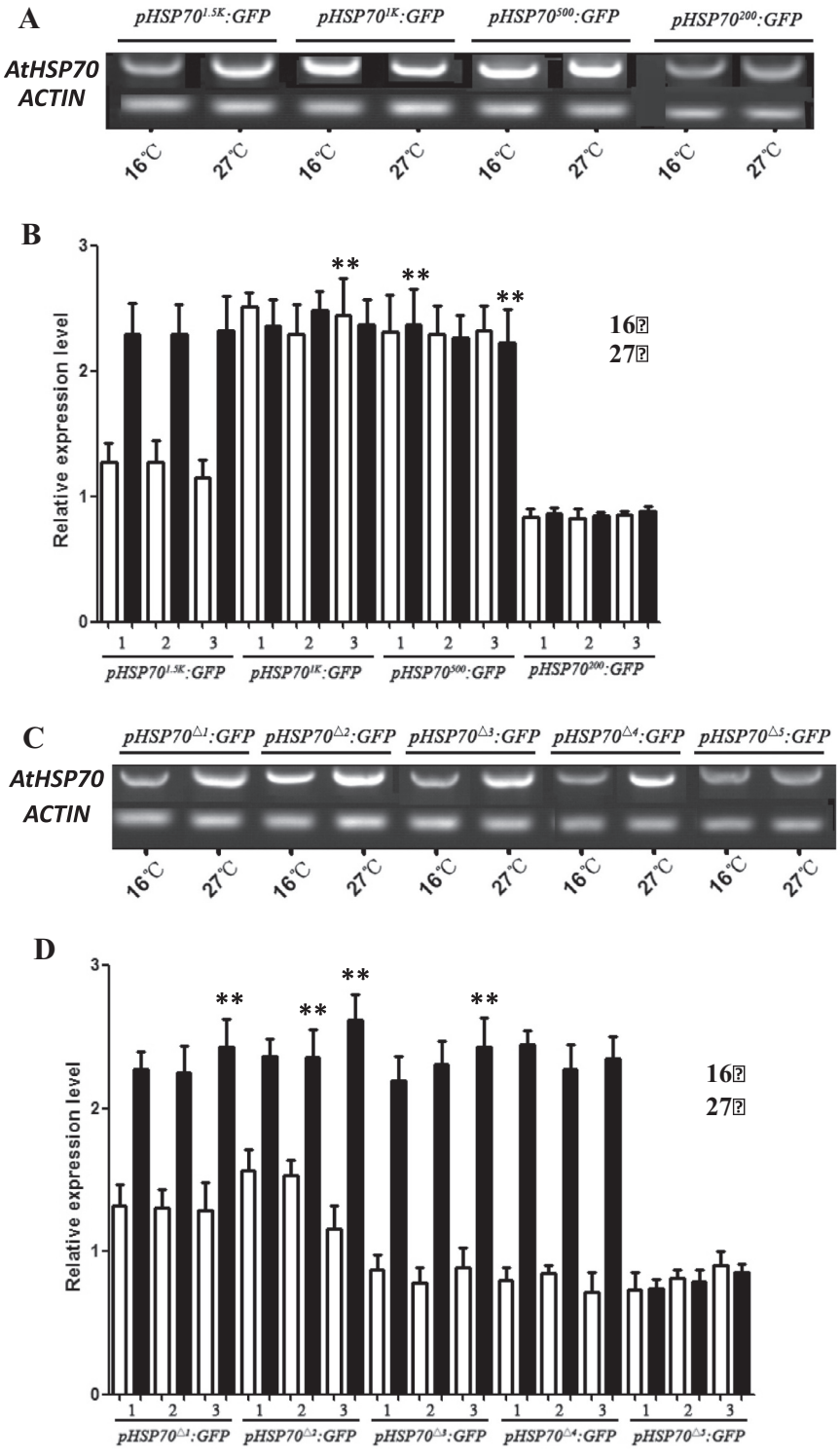

Fig. 3. *GFP* expression in different transgenic plants was detected by RT-PCR. (A) Represented truncated *AtHSP70-4* promoter driving GFP expression in transgenic plants. (B) RT-PCR analysis of *AtHSP70-4*/*ACTIN* expression in truncated *AtHSP70-4* prompter transgenic plants ($n > 10$, ±SD; $^{**}p < 0.001$, $p < 0.05$, Student's t test), 1, 2 and 3 means 3 independent lines of transgenic plants. (C) Represented deletion *AtHSP70-4* promoter driving GFP expression in transgenic plants. (D) RT-PCR analysis of *AtHSP70-4*/*ACTIN* expression in deletion *AtHSP70-4* prompter transgenic plants ($n > 10$, ±SD; $^{**}p < 0.001$, $p < 0.05$, Student's t test), 1, 2 and 3 means 3 independent transgenic lines.

the 70–80 bp flanking sequence around the core motif was also important [42–44], therefore, −900 to −1200 bp region of *AtHSP70-4* was used in searching for the potential *cis*-elements. The potential *cis*-elements within this region were demonstrated in Supplemental Fig. 1. Among them 'GCGCCAAA' were selected to be deleted. As demonstrated in Fig. 4(A–D), 'GCGCCAAA' sequence deletion from 1.5 kb promoter region resulted in abolishment of the *AtHSP70-4* temperature-responsive expression. In contrast, other motif deletion just exhibited a little, even undetectable difference compared with the intact 1.5 k promoter region (data not shown). Thus this 'GCGCCAAA' sequence was the core sequence of the −1100 to −1000 bp region of *AtHSP70-4* promoter.

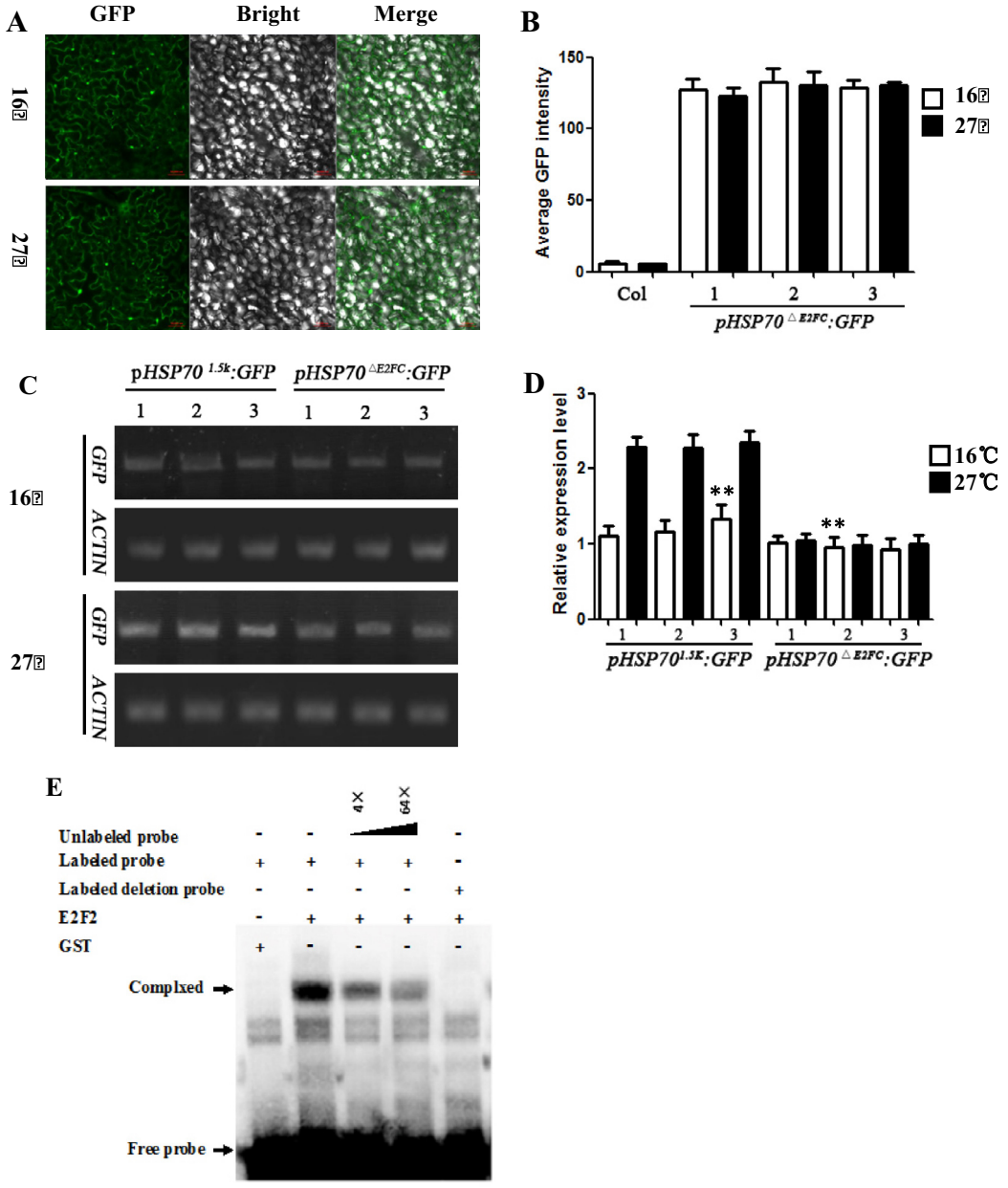

Fig. 4. Functional segments assay of *AtHSP70-4* promoter in transgenic plants. Leaves detected under Confocal microscope. (A) is a p*HSP70*$^{\triangle E2FC}$:*GFP* transgenic plant. (B) Histogram showing average green fluorescence intensities in p*HSP70*$^{\triangle E2FC}$:*GFP* transgenic plant mesophyll ($n > 10$ microscope view areas, ±SD; $p < 0.05$, Student's t test), 1, 2 and 3 means 3 independent transgenic lines. (C) and (D) RT-PCR analysis of *GFP/ACTIN* expression in p*HSP70*$^{1.5k}$:*GFP* and p*HSP70*$^{\triangle E2FC}$:*GFP* transgenic plants under different temperature ($n = 5$, ±SD; $^{**}p < 0.001$, Student's t test), 1, 2 and 3 represent the independent lines of each transgenic plants. (E) EMSA assay to analyze the binding of E2F2 to *AtHSP70-4* promoter. Each biotin-labeled DNA probe was incubated with E2F2 protein. An excess of unlabeled probes was added to compete with labeled promoter sequences. Biotin-labeled probes incubated with GST protein served as the negative control. Labeled deletion probe means the probe without 'GCGCCAAA'. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Arabidopsis E2F2 transcription factor could bind to the core motif

Interestingly, the core 'GCGCCAAA' sequence was proven to be the reverse consensus of the DNA-binding motif of the cell cycle regulatory transcription factor E2F family [37,45]. In Arabidopsis there were 6 members of *E2F* [46]. To test whether these transcription factors bound to the 'GCGCCAAA' sequence of *AtHSP70-4*, EMSA assay was performed by using −1027 to −1083 bp around the 'GCGCCAAA' sequence of *AtHSP70-4* as probe and the −1023 to −11087 bp of *AtHSP70* with the 'GCGCCAAA' deleted as the negative control (Table 2). As a result, the E2F2 (AT1G47870) of Arabidopsis could bind to the 'GCGCCAAA' sequence of *AtHSP70-4*, but no binding to the negative control with the deletion of the 'GCGCCAAA' sequence (Fig. 4E). Thus, it was verified at least *in vitro* the E2F2 transcription factors could interact with the 'GCGCCAAA' core motif of *AtHSP70-4*, which was essential for the temperature-responsive expression of *AtHSP70-4*.

4. Discussion

4.1. The reverse E2F-binding motif is essential for the non-stress temperature-responsive expression of AtHSP70-4

The dual stress and non-stress temperature-sensitive responses of *AtHSP70-4* transcriptional expression make *AtHSP70-4* a good marker for temperature response output. Kumar and Wigge [27] used *AtHSP70-4* promoter-driven luciferase reporting system to

Table 2
Sequence of probe for EMSA assay.

Probe name	Sequence (5′–3′)
proHSP70F	GAAAAGTTTAGTAGAAATCTGGCTGCGCCAAAGATGATAGAGACTCTAATGGGATC
proHSP70R	GATCCCATTAGAGTCTCTATCATCTTTGGCGCAGCCAGATTTCTACTAAACTTTTC
proHSP70ΔF	AATTGAAAAGTTTAGTAGAAATCTGGCTGATGATAGAGACTCTAATGGGATCCTTA
proHSP70ΔR	TAAGGATCCCATTAGAGTCTCTATCATCAGCCAGATTTCTACTAAACTTTTCAATT

proHSP70F and proHSP70R means the sense and antisense strand of probe containing 'GCGCCAAA'; proHSP70ΔF and proHSP70ΔR means the sense and antisense strand of probe deleted 'GCGCCAAA' region.

screen for temperature perception mutants and found that *ARP6*-dependent H2A.Z-contaning nucleosome was an essential component for most non-stress temperature response in Arabidopsis seedling. On the other side, *AtHSP70-4* exhibited a tissue-specific expression profile at constant regular cultivation temperature. Jung et al. [15] reported that they just detected trace level of *AtHSP70* expression in the aerial parts of the mature plant.

Here we verified that even in the mature leaf tissue, the temperature response of *AtHSP70-4* still remained. Compared with the plant cultivated at 16 °C, *AtHSP70-4* expression would increase about 1.5–2 folds when the plant was cultivated at 27 °C. Furthermore, the 1.5 kb promoter region of *AtHSP70-4* was found indispensable for the temperature response of *AtHSP70-4* expression. Further finer assay on deleted *AtHSP70-4* promoters exhibited that a 'GCGCCAAA' sequence within −1100 to −1000 bp of *AtHSP70-4* promoter was responsible for the temperature response of *AtHSP70-4*. The deletion of 'GCGCCAAA' sequence resulted in abolishment of expression increase of *AtHSP70-4* at higher temperature. In addition, EMISA assay using purified E2F2 protein exhibited that E2F2 protein did bind to the 'GCGCCAAA' sequence of *AtHSP70-4* promoter. Thus, E2F2 was essential for the non-stress temperature response of *AtHSP70-4* expression through recognizing and binding to the 'GCGCCAAA' sequence.

4.2. The characteristics of AtHSP70-4 expression activated by cell cycle transcription factor suggested a link existed between cell cycle and higher temperature acclimation of plant

There are 6 members of E2F family in Arabidopsis thaliana (E2Fa-f) [46,47]. E2F2, however, is the one of the two members whose ectopic expression can cause significant cell proliferation change in *Arabidopsis thaliana* [48]. In the mature leaf, endoreplication rather than cell division activity frequently occurs and is tightly linked to the E2F2 function. Usually a cell which is undergoing proliferation is often hypersensitive to environmental change. During these stages, the biosynthesis levels of macromolecules, such as DNA/RNA, protein etc. are largely reduced, and cellular organelles are often packed and to be ready for even division between two daughter cells. The elevated *AtHSP70-4* expression by E2F2 at higher temperature may help cell get along with cellular activity transition during which cells are vulnerable, especially at high temperature circumstance.

There have been rich reports about the downstream genes and events triggered by E2F gene family. In general, most of these genes are related to cell cycle control and DNA metabolism [49–51]. The previous work of Kumar and Wigge [27] had established the connection between non-stress temperature perception and chromatin remodeling through histone H2A/H2A.Z displacement. Here, our finding further provided a link between temperature perception and E2F activity, and brought in one question whether E2F was involved in the chromatin remodeling of the H2A.Z-containing nucleosome. Alternative, one or some downstream events activated by E2F might facilitate H2A/H2A.Z displacement during temperature perception.

Acknowledgments

We thank Dr. Liu Zhenguo for using confocal instrument, Li Chaobin for technical assistance in EMSA. This work was funded by the National Natural Science Foundation of China (30870225) and Pujiang Project (08PJ1405500) granted to Zhang Wei.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.083.

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