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Wheat Chloroplast Targeted sHSP26 Promoter Confers Heat and Abiotic Stress Inducible Expression in Transgenic *Arabidopsis* Plants

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Abstract

The small heat shock proteins (sHSPs) have been found to play a critical role in physiological stress conditions in protecting proteins from irreversible aggregation. To characterize the chloroplast targeted sHSP26 promoter in detail, deletion analysis of the promoter is carried out and analysed via transgenics in *Arabidopsis*. In the present study, complete assessment of the importance of CCAAT-box elements along with Heat shock elements (HSEs) in the promoter of sHSP26 was performed. Moreover, the importance of 5' untranslated region (UTR) has also been established in the promoter via *Arabidopsis* transgenics. An intense GUS expression was observed after heat stress in the transgenics harbouring a full-length promoter, confirming the heat-stress inducibility of the promoter. Transgenic plants without UTR showed reduced GUS expression when compared to transgenic plants with UTR as was confirmed at the RNA and protein levels by qRT-PCR and GUS histochemical assays, thus suggesting the possible involvement of some regulatory elements present in the UTR in heat-stress inducibility of the promoter. Promoter activity was also checked under different abiotic stresses and revealed differential expression in different deletion constructs. Promoter analysis based on histochemical assay, real-time qPCR and fluorimetric analysis revealed that HSEs alone could not transcribe GUS gene significantly in sHSP26 promoter and CCAAT box elements contribute synergistically to the transcription. Our results also provide insight into the importance of 5UTR of sHsp26 promoter thus emphasizing the probable role of imperfect CCAAT-box element or some novel cis-element with respect to heat stress.

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Introduction

High temperature stress is one of the most common abiotic stress among many of the world crops, reducing both yield and quality of crops, and there is a need to increase productivity for warmer areas of the world. Worldwide several breeding and molecular approaches are being utilized to impart heat tolerance in crop cultivars. It is known that plants synthesize a set of evolutionarily conserved proteins called Heat Shock Proteins (HSPs) upon heat stress, and many groups have produced thermo tolerant plants by overexpressing these HSPs. The HSP family has been classified into five groups depending on their molecular weight: HSP100, HSP90, HSP70, HSP60 and small HSPs [1]. The expression level of *Arabidopsis thaliana* Heat Shock Factor (AtHsf) was successfully altered and thus HSPs were overexpressed in *Arabidopsis* plants [2]. These small HSPs are known as stress proteins and these stress proteins were found to protect photosynthesis in cells during various abiotic stresses like heat, salt, drought, osmotic, oxidative, and other photoinhibitory stresses [3–11]. Small HSPs has also been found to be regulated at specific plant developmental stages like embryogenesis, fruit maturation and pollen development, other than abiotic stresses [12–13]. Jiang et al. [14] characterized *RcHSP17.8*, a cytosolic class I sHSP, from *Rosa chinensis* by producing transgenic *Arabidopsis thaliana* plants that constitutively expressed *RcHSP17.8* and these plants exhibit increased tolerance to multiple abiotic stresses such as heat, salt, osmotic and drought stress. They also showed the same effect in *Escherichia coli* and yeast by overexpressing recombinant *RcHSP17.8* in both these species as well.

A powerful and more sensitive approach for measuring the activity of any heat shock promoter is by fusing the promoter of a plant heat shock gene to GUS (β-glucuronidase) reporter gene thereby allowing to measure the developmental and tissue specific expression with and without heat stress [15–16]. Transgenic *Arabidopsis* plants were produced by Takahashi et al. [17] which contained the promoter of *HSP18.2* gene fused to the GUS gene and histochemical analysis was carried out. They showed that heat stress induced the GUS gene activity in almost all the organs of the plant. Similarly, heat shock induced GUS activity was also observed in transgenic *Arabidopsis* plants when the promoter of HSP 81–1 gene was fused to the GUS gene [18]. Crone et al. [19] did a detailed analysis of the expression of the GUS gene when fused with small heat shock protein gene promoter, *Glycine max HSP17.5E (GmHSP17.5E)* in all the organs and tissues of the flower.
as a function of development with and without heat stress. They found that the promoter of *GmHSP17.3E* is not uniformly expressed after heat shock in different floral tissues. For example, expression could be seen in all the developmental stages of sepals but not in petals, and the expression is even complex in pistil and anthers. They observed GUS expression in style and upper portion of ovary, but not in lower part of ovary or ovules. Similarly in stamens, GUS induction could be seen only in filament or in the vascular tissue from the filament into the anther but not in other tissues of anther or microspores. However, in vegetative tissue, heat shock induces its response in all the tissues and organs of young plant. A detailed study to examine GUS activity in different organs at different temperatures was done by Moriwaki et al. (16). They observed the expression pattern of the Arabidopsis HSP 18.2-GUS gene chimera at the recovery period following heat shock treatment in transgenic *Nicotiana plumbaginifolia*. They optimised the HS temperature in anthers, petals and capsules to be 42°C in immature seeds, it is 36°C in placenta of capsules, it is 39°C. Thus, they showed organ and different developmental stages specific heat stress inducibility. The usefulness of a heat shock promoter for studying gene functions and also for studying cis-acting transcriptional elements is discussed in detail in transgenic zebrafish [20], where HSP70 promoter has been used for manipulating transgenes in zebra fish embryos.

Rice MT (Metallothionein) promoter has been analyzed in transgenic *Arabidopsis* using GUS as a reporter [21]. Six promoters of seed storage glutelin gene showed the expected spatial expression pattern within the endosperm [22]. Full length or truncated pine ACC oxidase gene promoters showed distinct patterns of expression when responded to IAA (Indole-3-Acetic Acid) and wounding stress [23]. The promoter of *Arabidopsis thaliana* gene AtGILTpro-(Gamma Interferon-responsive Lysosomal Thiol reductase) was fused to the uidA reporter gene and was selected as a useful seed coat outer integument (including mucilaginous layer)-specific promoter for canola [24]. The histochemical advantage of GUS fusion to *Arabidopsis* COR13 (CORONATINE INDUCED) promoter also revealed two integrated cis-regulatory regions required for transcriptional activity in companion cells [25]. Similarly, full length promoter fragments from lemon and lime were investigated by fusing them to GUS reporter gene followed by their transient transformation in tomato floral organs [26]. Promoter analysis of Chalcone synthase from *Populus trichocarpa* showed that it is capable of directing GUS gene expression in both wounded and unwounded leaves [27]. Three different promoters could also induce GUS expression in abiotic stress like ABA and salt treatments in both vegetative and floral organs in transgenic rice [28].

HSP26 has been well characterized from *Saccharomyces cerevisiae* [29]. Chaperone assays were performed at different temperatures that show that there is temperature dependent dissociation of HSP26 complex into smaller active species and then reassociation of this complex for functional activation of this chaperone [30]. The thermodynamic and kinetic characteristics of structural changes when HSP26 is heat activated showed that its temperature sensing is a function of its middle domain that changes its confirmation in response to temperature [31]. To determine the role of chloroplast localised small HSP26 in heat sensitive and heat tolerant variant of bentgrass (*Agrostis stolonifera cv. palustris*), different isoforms of HSP26 gene were isolated and their structure and expression were characterized [32].

In a previous study from our lab, we have cloned chloroplast targeted sHSP26 from bread wheat (*Triticum aestivum*) and characterized it via transgenic *Arabidopsis* plants [33]. Transgenic *Arabidopsis* plants overexpressing sHSP26 were shown to be substantially tolerant than wild type plants under continuous moderate high temperature regimen. The HSP26 promoter was also functionally characterized in rice transgenics. In the present study, the promoter of TaHSP26 is characterized by deletion analysis of the promoter via *Arabidopsis* transgensics confirming the inducibility of this promoter under heat and other abiotic stresses.

**Results**

**Sequence Analysis of the TaHSP26 Promoter**

A 1514 bp TaHSP26 promoter with 112 bp 5’ UTR is designated as 1625 bp full length promoter that was reported in the previous study by Chauhan et al. [33] by PLACE database [34] (http://www.dna.affrc.go.jp/PLACE) (Fig. S1). Promoter analysis revealed the presence of several transcription factor-binding sites associated with various environmental signals. For example- there are several MYC-rd22 sites in the promoter that respond to drought stress and ABA (abscisic acid) signalling. The upstream region of *TaHSP26* contains all the three types of HSEs: Perfect-type (nGAAnnTTGnnGAAAn), Gap-type (nGAAnnTTGnnGAAAn and Step-type (nTTCnn3 bp)nGAAnn(5 bp)nTTCnn) [33–37]. Two Stress Responsive cis-acting Elements (STREs) (AGGGG) were found at −599 bp and −781 bp, believed to be involved in mediating the general stress response. These sequences are found in the promoter of many stress-responsive genes and are found to be induced under heat stress, osmotic stress, low pH and nutrient starvation [38]. Three CCAAT- box elements were also found in this promoter at −721 bp, −1209 bp and −1435 bp sites, respectively. In an earlier report, these CCAAT box regions were reported to be essential for gene expression, while deletion of the CCAAT box region (deletion −811/−63) reduced the strength of the nos promoter by many folds [39].

**TaHSP26 Promoter Deletion Constructs**

To gain further insights into the functional role of TaHSP26 promoter region, a series of deletions were made by designing primers that truncate promoter fragments. Since CCAAT box elements were found to enhance expression of chimeric heat shock genes in transgenic tobacco [40], a series of deletions were generated removing CCAAT box elements gradually. Moreover, to assess the importance of 5’ Untranslated Region (UTR), UTR region was completely deleted in some of the constructs. Thus, a total of eight constructs were generated; [Full Promoter with UTR (Pro26+UTR), full promoter without UTR (Pro26-UTR), Del 1, Del 2, Del 3, Del 4, Del 5 and Del 6].

- **Full Promoter with UTR** - 1625 bp - includes all the known stress and development related elements, i.e. 3 CCAAT box, 3 HSEs, 2 STREs, 5 Myc- rd22 and UTR.
- **Full Promoter without UTR** - 1514 bp - includes all the known stress and developmental related elements, 3 CCAAT box, 3 HSEs, 2 STREs, 5 Myc- rd22 but UTR deleted.
- **Del 1** - 1302 bp - includes 2 CCAAT box, 3 HSEs, 2 STREs, 5 Myc- rd22, 1 CCAAT box deleted and includes UTR.
- **Del 2** - 885 bp - includes 1 CCAAT box, 3 HSEs, 2 STREs, 2 Myc- rd22, 2 CCAAT box deleted and includes UTR.
- **Del 3** - 530 bp - includes no CCAAT box, 3 HSEs, no STREs, no Myc- rd22, all 3 CCAAT box deleted and includes UTR.
- **Del 4** - 1190 bp - includes 2 CCAAT box, 3 HSEs, 2 STREs, 5 Myc- rd22, 1 CCAAT box and UTR deleted.
- **Del 5** - 773 bp - includes 1 CCAAT box, 3 HSEs, 2 STREs, 2 Myc- rd22, 2 CCAAT box and UTR deleted.

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**Del 6** - 418 bp - includes no CCAAT box, 3 HSEs, no STREs, no Myc- rd22, all 3 CCAAT box and UTR deleted.

Schematic representations of all the *TaHSP26* deletion constructs used for transformation of *Arabidopsis* are shown in Fig. 1. All these constructs were PCR amplified using region-specific primers from *TaHSP26* promoter (Fig. S2) and cloned into plant transformation GATEWAY™ vector pMDC164 mediated by pENTR™/D-TOPO. The constructs were then transformed in *Arabidopsis* via floral dip method [41].

*TaHSP26* Promoter Activity in Plants after Heat Stress

The temporal and spatial distribution of GUS in *TaHSP26* promoter carrying *Arabidopsis* plants were investigated in T4 generation grown in culture-room conditions. Transgenic plants harbouring full-length promoter of *TaHSP26* gene were analysed histochemically under control and heat-stressed conditions (37°C, 2 hrs). Full seedlings were observed for GUS expression; a blue colored end product was observed exclusively in the heat-stressed transgenics and no GUS activity was detected in seedlings of control plants. Shoot and root tissues of full-length promoter transgenics showed intense GUS staining, sections of both the tissues were observed under bright field using fluorescence microscope (Leica, Germany) (Fig. S3). Three independent transgenic lines with consistently high levels of GUS expression were selected for further analysis.

**Effect of Different Deletions of TaHSP26 Promoter on Heat-shock Responsiveness in Transgenic Arabidopsis**

In continuation to the Pro26+UTR and Pro26-UTR constructs, 6 deletion constructs were also undertaken to measure the *TaHSP26* activity under various abiotic stresses. Deletion constructs were analysed histochemically in three-independent transgenic lines for each construct. The results showed that plants...
harbouring construct Del-1, 2 and 3 showed a remarkable increase in the GUS expression in comparison to Del-4, 5 and 6, but, the GUS levels of all these constructs were relatively low when compared to the full-length promoter (Fig. 2d–f). Among Del-1, 2 and 3, Del-1 showed higher amount of GUS activity in terms of intense blue colour followed by Del-2 and then, Del-3. Histological analysis showed that further deletions of the promoter (in absence of UTR) results in gradual reduction of GUS gene expression relative to full-length promoter without UTR, Pro26-UTR (Fig. 2g & h). Among Del-4 and Del-5, Del-4 showed comparatively higher GUS levels compared to Del-5. Further reduction of the promoter size to 418 bp, resulted in complete absence of GUS activity in Del-6 (Fig. 2). Thus, plants carrying progressive deletions of the promoter resulted in decreasing pattern of GUS activity and ultimately no detectable GUS. The control plants did not show any GUS activity and the three lines of respective deletion constructs analysed by GUS histochemical assay showed similar expression pattern.

GUS Transcript Activity by TaHSP26 Promoter and its Deletions in Response to Heat Stress

To analyse the GUS at the transcriptional level, quantitative RT-PCR was performed in two-week-old transgenic Arabidopsis plants. Various organs analysed were leaf, root, stem, flower, young siliques, and mature siliques. GUS transcript was also more visible in heat-stressed root and flower tissue. This full promoter of TaHSP26 with UTR contained many stress and developmental related elements, i.e. 3 CCAAT box, 3 HSEs, 2 STREs, 5 Myc- rd22. The high GUS induction response could be due to a synergistic effect of heat-stress responsive elements in the promoter. In construct Del-1 of TaHSP26 promoter, it was observed that the GUS transcript has reduced by the deletion of one CCAAT box present at position −1435. There is a 100-fold reduction of GUS transcript in Del-1, when compared to the full-length promoter (Fig. 3b). The leaf tissue upon heat-stress showed GUS transcription induction about 350-fold higher as compared to the control leaf tissue. GUS transcript was also many folds higher in heat-stressed stem and mature siliques. Gene expression levels decrease further when the promoter is deleted to point −773 excluding 2 CCAAT boxes, in addition to UTR. Del-5 thus consisted of 1 CCAAT box, 3 HSEs, 2 STREs, and 5 Myc- rd22. GUS transcript levels if any, almost indistinguishable to that of control (Fig. 3h). Thus, we infer that without UTR and with no CAAT box element present in the shortest promoter fragment, HSEs alone could not transcribe GUS gene significantly.

Quantitative Estimation of GUS Driven by TaHSP26 Promoter and its Deletions in Response to Heat Stress

Quantitative measurement of GUS activity was also determined in two-week-old Arabidopsis transgenics plants. The same tissues used for real-time PCR for GUS transcript were used for protein extraction, i.e. leaf, root, stem, flower, young siliques and mature siliques in transgenic control as well as heat stressed (37°C, 2 hr) plants (Fig. 4). Three independent transgenic lines for each construct were analysed for fluorimetric estimation of GUS protein. Three technical replicates were also taken for each tissue analysed. The activity of GUS was expressed in nmol of 4-MU/mg protein/h. The results quantitate the GUS protein and revealed that the highest amount of GUS activity of 300 units was observed in full-length promoter with UTR, Pro26+UTR (Fig. 4a). All the deletion fragments that contained UTR (Del-1, Del-2, and Del-3), progressive deletion of the three CCAAT box elements resulted in decrease of GUS protein as quantified by the fluorimetric values of 250, 100 and 80 nmol (Fig. 4b–d). Deletion of UTR from the full-length promoter resulted in a dramatic decrease in GUS protein levels; a decrease of 300 units as compared to the full-length promoter (Fig. 4e). Further, gradual deletion of the three CCAAT box elements from the promoter in absence of UTR (Del-4 and Del-5), resulted in even more reduction in the GUS protein level (Fig. 4f–g). In Del-6, though histochemical staining did not show any GUS expression, yet the fluorimetric analysis revealed some marginal GUS activity (Fig. 4h).

5’ UTR Mediated Expression of TaHSP26

To assess the possible involvement of 5’ UTR in the regulation of expression of TaHSP26, two expression cassettes of full-length promoter were generated; one with UTR and other without UTR. Ten-independent transgenic lines from each construct were
analysed for the presence of GUS gene expression. Three lines with consistent expression for the GUS gene were selected for further analysis.

The levels of GUS activity were assayed histochemically in the Pro26+UTR and Pro26-UTR constructs in three-week-old Arabidopsis seedlings when heat-stressed at 37°C for different time durations. The results showed that transgenic plants harbouring TaHSP26 promoter with or without UTR showed immediate appearance of the blue colour of GUS activity as early as 10 min; while no GUS staining was observed under control conditions (Fig. 5). However, a visibly reduced GUS staining was observed for transgenic plants harbouring full length promoter without 5' UTR as compared to transgenic plants harbouring full-length promoter with 5' UTR. Increase in the duration of heat stress at an interval of every 10 min till 2 hrs. was given and seedlings were observed histochemically. Consistent with the earlier observations, transgenic plants harbouring full-length promoter with UTR showed progressive increase in the GUS activity and the plants showed maximum GUS activity after 2 hrs of HS (Fig. 5A). Similarly, plants harbouring full length promoter without UTR also showed an increase in the GUS expression and the induction was maximum again at 2 hrs (Fig. 5B). However, there was a visible difference in the intensity of blue color in both the constructs at all the different time points of heat stress.

Quantitative RT-PCR of GUS transcript was also analysed of both the constructs (Pro26+UTR, Pro26–UTR) for three-week-old Arabidopsis seedlings that were frozen immediately at −80°C after heat stress was given to them at various intervals. The analysis revealed that under the same heat-stress durations, construct Pro26+UTR showed a 20-fold-increase in expression when compared with the construct Pro26-UTR after 2 hrs of HS (Fig. 6a–b). Quantitative estimation of the GUS protein revealed that deletion of the UTR resulted in reduced induction of the GUS reporter gene by approx. 50-fold (Fig. 6c–d). After 2 hrs of

Figure 2. Histochemical localization of GUS gene activity in transgenic Arabidopsis plants containing full TaHSP26 promoter with and without 5' UTR and six different deletion constructs in two-week-old seedlings. (a) Control (transgenic without heat stress); (b–i) transgenics with heat stress at 37°C for 2 hrs; (b) TaHSP26 promoter with UTR; (c) TaHSP26 promoter without UTR; (d–f) Del 1, 2, 3 with UTR; (g–i) Del 4, 5, 6 without UTR.

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Figure 3. Analysis of Pro26 promoter activity in different tissues of two-week-old transgenic *Arabidopsis* plants. GUS transcript was analyzed by quantitative RT-PCR in full promoter as well as all the deletions lines. Three individual transgenic plants (T1, T2, T3) from each line were analyzed. Standard error bars are shown.

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Figure 4. Analysis of Pro26 promoter activity in different tissues of two-week-old transgenic Arabidopsis plants. Quantitative measurement of GUS activity was determined using protein extracts from different tissues. Three individual transgenic plants (T1, T2, T3) from each line were analyzed. Standard error bars are shown.
doi:10.1371/journal.pone.0054418.g004
HS, Pro26+UTR construct showed GUS activity of 400 nmole 4 MU/hr/mg protein, while that of Pro26-UTR construct showed GUS activity of 22.6 nmole 4 MU/hr/mg protein. Thus, a dramatic difference in gene-expression was observed when UTR was deleted from TaHSP26 full-length promoter.

Activity of TaHSP26 Promoter and its Deletions under other Abiotic Stresses

To carry out a comparative analysis of TaHSP26 promoter and its deletion constructs under different abiotic stresses, GUS reporter gene activity was investigated histochemically in two-week-old Arabidopsis transgenic plants. Plants were treated with three different abiotic stresses for 24 hrs: for drought stress, plants were exposed to 2% mannitol; for salt stress, plants were treated with 150 mM salt (NaCl) solution; for cold stress, plants were kept at cold room (4°C) for 24 hr. Next day, plants were incubated overnight in GUS assay buffer at 37°C and histochemically analysed. Control plants were not treated to any of the abiotic stresses, and did not show GUS staining in any of the promoter constructs. Histochemical analysis showed that the constructs with UTR responded tremendously to drought, salt and cold stress as well. The blue colour of GUS activity was highest in the full-length promoter (Pro26+UTR), and to lesser levels in other deletion constructs also (Fig. 7).

GUS activity was also checked in full-length promoter construct without UTR and the promoter deletions that are without UTR (Del-4, Del-5 and Del-6) (Fig. 8). It was observed that deletion of UTR from the promoter caused a decrease in the GUS staining in

Figure 5. GUS histochemical assay showing induction of GUS gene governed by TaHSP26 promoter in three-week-old Arabidopsis transgenics when heat stressed at different time-points. A. Pro26 promoter with UTR showed GUS activity at different time points of heat stress. B. Pro26 promoter without UTR showed GUS activity at different time points of heat stress. Control taken is transgenic Arabidopsis plant without heat stress. C = Control (non heat-stressed transgenic), HS = Heat Stress (37°C, 2 hrs.).
doi:10.1371/journal.pone.0054418.g005
all the three abiotic stresses (drought, salt, and cold). This again reveals the importance of UTR or other cis-element present in UTR that are responsive to abiotic stresses. Construct Del-4 and Del-5 showed GUS induction in drought stress and salt stress but no GUS staining was observed in cold stress. However, in Del-6 construct, no GUS levels were detected in any of the three stresses studied. In all TaHSP26 promoter constructs, histochemical staining of two-week-old Arabidopsis seedling under simulated drought and salt stress displayed a similar GUS staining pattern as was in the case of heat-shock treated plants. However, only in case of cold stress, there was differential expression observed in case of Del-4 and Del-5 and no GUS induction was observed.

Discussion

We have previously analysed the expression of a wheat chloroplast targeted sHSP, TaHSP26 in different tissues of wheat representing major growth stages and abiotic stresses [42]. Along with vegetative tissues, TaHSP26 transcript was found to be highly inducible by heat-stress in flower and developing seed tissues. Role of TaHSP26, in conferring heat stress tolerance and during seed development has been shown earlier by Chauhan et al. 2012 [33]. In the present study, promoter of TaHSP26 is further characterized by deletion analysis via Arabidopsis transgenics and confirming the inducibility of the deletion constructs in heat stress. Not many reports are available that allowed gene expression in transgenic plants induced only by external factors. One such report by Freeman et al. [43] used GUS reporter gene to demonstrate the heat induction of barley Hvhsp17 gene promoter in transgenic wheat. Gus gene was induced only in heat stressed tissue and was expressed in all tissues and organs tested.

Based on our results as shown by histochemical assay, quantitative RT-PCR and fluorimetric analysis, it could be inferred that without UTR and with no CAAT box element present in the shortest promoter fragment, HSEs alone could not transcribe GUS gene. As was also shown by Haralampidis et al. [44] the cis-elements present in promoter region of AtHSP90-1 contribute in a combinatorial manner to regulate the expression in development, suppression, or stress conditions. They concluded that the two stress responses (heat stress and arsenite treatment) may involve common but not necessarily the same regulatory elements. Our results clearly demonstrate that TaHSP26 promoter is highly heat inducible and Heat Shock Elements (HSEs) alone are not sufficient for heat-shock inducibility, CCAAT box elements contribute synergistically to the transcription of heat shock genes. Same was reported earlier by Rieping and Schoffl (1992) [40] in soybean Gmhsp 17.3
Heat inducible CAT (chloramphenicol acetyltransferase) activity was detected when three CCAAT boxes and a single HSE were reconstituted in the HS promoter; however deletion of the CCAAT box sequences reduced CAT activity five-folds. Deletion of the CCAAT box region reduced the nos promoter strength many folds as was shown earlier by An (1987) in transgenic tobacco [39]. The CCAAT box elements are one of the most common regulatory elements present in 30% of eukaryotic promoters and are conserved in promoters of the heat-shock genes [45]. This fact was thoroughly studied in promoters of HSP70, which are the most well studied among heat shock genes [46]. In yeasts, plants and mammals, NF-Y binds to CCAAT box in most of the promoters and activates it [47]. The importance of CCAAT box has been shown by Landsberger et al 1995 where they conclude that CCAAT box maintains the promoter in open chromatin configuration so that HSF could rapidly activate after thermal stress [48]. In vivo footprinting experiments in mouse cells by Abravaya et al. [49] showed that CCAAT box elements are constitutively protected prior to the heat shock whereas HSEs bound to the HSFs after heat shock has been given. In CCAAT-less constructs, the promoter remains in a closed nucleosomal configuration, thus does not allow HSFs to bind to HSEs and activate transcription after heat stress induction [48]. Mutation analysis has also showed that the basal transcriptional activity of human Hsp70 promoter in vitro was primarily dependent on the CCAAT-box element located at −65 [50]. Thus, as evident by our results, we can also hypothesize that in wheat plants, CCAAT box elements may contribute in maintaining the open chromatin configuration so as to allow HSFs to bind to HSEs after heat

Figure 7. Analysis of Pro26 promoter activity in two-week-old Arabidopsis transgenic seedlings under three different abiotic stresses. Transgenic lines with UTR were analyzed for simulated drought (mannitol 2%, 24 hr), salt (150 mM, 24 hr) and cold stress (4°C 24 hr). doi:10.1371/journal.pone.0054418.g007
induction. Also, in the case of TaHSP26 promoter, 5' UTR has contributed significantly to the heat-shock inducibility. This indicates that the heat-stress responsive elements required for the expression of the gene are also located in this region which needs to be characterized further.

Similarly, importance of HSE for the heat shock induction of the apx1 gene was confirmed by mutational analysis [51]. In vitro analysis of the interaction between tomato HSF and the apx1 promoter confirmed that HSE represents a functional HSF-binding site. Thus, confirming that HSE is responsible for the heat shock induction of the gene and also contributing partially to oxidative stress induction. Also, developmental induction of HaHsp17.6G1 promoter was abolished when any mutation was performed in its HSE [52].

Our results have also shown a dramatic difference in gene-expression when UTR was deleted from TaHSP26 full-length promoter. In a similar report, Karthikeyan et al. [53] studied the effect of 5' UTR intron and the role of putative cis-elements present in AtPht1;4 (Arabidopsis phosphate transporter 1:4) promoter on gene expression. Experimental analyses showed that the 5'UTR intron is essential for AtPht1; 4 expression in root tips besides enhancing the level of expression in roots during Pi starvation. When 5' UTR (112 bp) of TaHSP26 was submitted to PlantCare database [54], we found few interesting cis-acting elements. One of the important elements was an imperfect CAAT box element; others are TATA box, I-box, GATA motif and CBFHV. I-box and GATA motifs are the conserved sequences present upstream of light regulated genes. They are required for light regulated tissue-specific expression. Light regulation at the

Figure 8. Analysis of Pro26 promoter activity in two-week-old Arabidopsis transgenic seedlings in three different abiotic stresses. Transgenic lines without UTR were analyzed for simulated drought (mannitol 2%, 24 hr), salt stress (150 mM, 24 hr) and cold stress (4°C, 24 hr). doi:10.1371/journal.pone.0054418.g008
transcriptional level has already been demonstrated in chloroplast targeted proteins [53]. Light has been shown to regulate the expression of small Hsps like Hsp 22 [56]. In fact, it has also been proposed that Hsp induction is primarily not because of elevated temperatures rather oxidizing environment of high light [57], and HSP70B also has been found to play an important role in PSI repair process [58]. Other important element found is the CBFHV which is crucial for drought stress. These CBFs are also known as dehydration-responsive-element (DRE) binding proteins (DREBs). It is a well-known fact that a high degree of overlap occurs between genes that are induced by different stresses. So, in response to one particular stress, transcription factors responsive to both the stresses have been found to be induced. DREB2A is one such transcription factor, which has been found to be one of the main regulators of drought and heat response [59]. This group found a novel splice variant of DREB2A that lacked the interacting domain (with RCD1-Radical Induced Cell Death-1) and was induced during senescence and heat shock treatment. Thus, we assume that the drastic reduction in GUS levels due to the deletion of 5’ UTR could be mainly because of the imperfect CAAT box element or the light responsive elements or the drought responsive elements present in it. It could be that one of the light/drought responsive elements has also some important role as heat stress elements as well, but is not yet characterized with respect to heat stress.

Conclusions

Since wheat is hexaploid and the genome is large and unknown, to functionally analyse promoter of wheat chloroplast targeted sHSP26 gene for abiotic stress tolerance, Arabidopsis has been chosen. The results reported herein offer a picture of the mechanism underlying TaHSP26 mediated regulation of heat-tolerance via characterization of TaHSP26 promoter by deletion analysis. The results provide a basis for understanding of how this TaHSP26 promoter activity is directly related to the numbers of CCAAT box elements in promoter under heat stress. Moreover, since UTR is of primary interest in promoters, this study highlights the role of 5’UTR in enhancing GUS gene expression in heat and other abiotic stresses. In conclusion, TaHSP26 has association with heat-tolerance in wheat and its promoter offers a possibility of inducible gene expression of otherwise abiotic stress sensitive genes in molecular breeding of superior wheat cultivars for changing environments especially high temperature.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana (Col 0) were used for raising transgenics in the present study. Wild-type seeds were spread in pots containing soilrite for the generation of full grown plants and were kept in the culture room conditions. For plating of seeds onto Murashige-Skoog (MS) medium, they were first surface sterilized with 2.0% sodium hypochlorite for 10 minutes and then washed with sterile RO water for 3–4 times and finally suspended in 0.1% (w/v) agar. The seeds were then plated onto half-strength MS medium containing 2% sucrose and 0.8% agar, pH 5.8. The plates were kept at cold room (4°C) for 24 hr for uniform seed germination and were then kept at culture room maintained at 22±1°C with 16:8 hr light and dark regime with a light intensity of 100-125 μmol m−2s−1. For raising successive generations, MS medium was supplemented with 15 mgL−1 hygromycin and 150 mg L−1 augmentin. Twenty-day mature seedlings were transferred to soil for further maturation of plants.

Development of TaHSP26 Promoter Deletion Constructs and Plant Transformation

The promoter region of 1625 bp was cloned in the plant transformation GATEWAY vector pMDC164 mediated by pENTR™/D-Topo cloning system [33]. Region-specific primers were designed from full length TaHSP26 promoter and, a series of deletions were made that truncate promoter fragments thus, removing CCAAT-box elements gradually. Primers were also designed so as to remove UTR element from some of the constructs, and all the 8 constructs were PCR amplified using these region-specific primers. Sequences of the primers used are given in Table S1. All the deletion constructs were cloned into plant transformation GATEWAY™ vector pMDC164 mediated by pENTR™/D-TOPO. The constructs were transformed in Arabidopsis via floral dip method [41]. At T1 generation, the putative transformants were confirmed for the presence of transgene of the respective deletion construct by PCR. Deletion specific primer (Forward) and GUS-reporter gene specific marker (Reverse) was used for amplification from the deletion constructs. Binary vectors harbouring various deletion fragments were used as positive control.

High Temperature and other Abiotic Stress Treatment

Homozygous transgenic plants were germinated on half-strength MS medium in Petriplates containing 15 mg L−1 hygromycin. Two-week-old full seedlings of 8 different deletion constructs were subjected to heat stress at 37°C for 2 hrs and then analysed histochemically. From soil grown mature plants, various tissues (leaf, root, stem, flower, young siliques, mature siliques) were harvested after high temperature treatment at 37°C for 2 hrs. Five individual transgenic plants from each line were analyzed. For time-course experiment, three-week old transgenic seedlings were subjected to heat stress of 37°C for different time-points. For each experiment after heat-stress, the tissue was analyzed by GUS histochemical assay and the tissue was also frozen in liquid nitrogen and stored at −80°C for RNA and protein isolation. For drought stress, salt stress and cold stress, two-week old seedlings were subjected to 2% mannitol for 24 hrs, 150 mM NaCl for 24 hrs and 4°C for 24 hrs respectively and then analyzed.

Histochemical GUS Assay

Histochemical GUS staining was performed as described in the protocol by Jefferson et al. [60]. The tissues were first given heat stress at 37°C for 2 hrs. and then analyzed histochemically. The tissues used were seedling tissue, leaf, root, stem, flower, young siliques, mature siliques. Transgenic plant without heat stress was used as control. All the samples were incubated at 37°C for 24 hrs in the GUS buffer. After the GUS staining, tissues were treated with ethanol and acetic acid (3:1) to remove chlorophyll from the GUS stained tissue.

Quantitative RT-PCR Analysis

Total RNA was isolated from different tissues of two-week old Arabidopsis transgenic seedlings and other tissues from mature plants i.e. Leaf, root, stem, flower, young siliques, mature siliques using RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer’s instructions including on-column DNase-I treatment to remove genomic DNA contamination. GUS transcript was analysed in all the deletion constructs. First strand cDNA was synthesized from 2 μg of total RNA employing the high-capacity cDNA archive kit (Applied Biosystems, USA). Reaction constitutes cDNA samples along with 200 nM of each primer and SYBR Green PCR master mix (Applied Biosystems, USA) and run on the
Abi Prinm 7000 Sequence Detection System and Software (PE Applied Biosystems). Amplification of cDNA was confirmed by melting curve analysis. Actb, was used as an internal control for the quantification of mRNA levels of the constructs. All reactions had at least three biological and three technical replicates.

Fluorimetric GUS Assay

The transgenic lines which were positive for GUS histochemical staining were taken for further fluorimetric analysis. Total protein was extracted from the T$_2$ transgenic plants and final concentration of 6 mgm protein was taken for estimation of GUS protein. Fluorimetric assay was done according Jefferson et al. [60]. The substrate used was 4-methylumbelliferyl β-D-glucuronide. The protein was incubated with the substrate at 37°C for 15 hrs. and the reaction was stopped by 0.2 M Na$_2$CO$_3$ in the dark. The reaction product 4-methyl umbelliferone (4-MU) was estimated by a Dyna Quant TM 200 fluorimeter (Hoefer Pharmacia Biotech Inc., California, USA). The assay was done in triplicates for each of the biological sample taken.

Statistical Analyses

Statistical analysis was done by calculating mean value for all the three replicates. Standard error was also calculated based on these replicates. Readings were calculated in pmol/2 ml. The final readings were calibrated in nmol/mg/hr.

References

1. Lee JH, Hubel A, Schöffl F (1995) Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic Arabidopsis. Plant J 8: 603-612.
2. Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9: 244-252.
3. Heckathorn SA, Downs CA, Sharkey TD, Coleman JS (1998) The small, methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. Plant Physiol 116: 439-444.
4. Lee S, Prochaska DJ, Fang F, Barnum SR (1998) A 16.6-kilodalton protein in the G. cyanothecae sp. PCC 6803 plays a role in the heat shock response. Curr Microbiol 37: 403-407.
5. Lee BH, Won SH, Lee HS, Miyao M, Chung WJ, et al. (2000) Expression of the chloroplast-localized small heat shock protein by oxidative stress in rice. Gene 245: 283-290.
6. Malik MK, Slavin JP, Hwang CH, Zimmerman JL (1999) Modified expression of a carrot small heat shock gene, hsp17.7, results in increased or decreased thermotolerance. Plant J 20: 89-99.
7. Hamilton EW, 3rd, Heckathorn SA (2001) Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas Complex II is protected by proline and betaine. Plant Physiol 126: 1266-1274.
8. Sun W, Bernard C, van de Cotte B, Van Montagu M, Verbruggen N (2001) At-HSP17.6, a temperature-regulated chaperone. EMBO J 18: 6744–6751.
9. Tokor Z, Goloubimoff P, Horvath I, Tovelfova NM, Laitz A, et al. (2001) Synechocystis HSP17 is an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. Proc Natl Acad Sci U S A 98: 3098-3103.
10. Volkov RA, Panchuk II, Mullineaux PM, Schöffl F (2006) Heat stress induced H$_2$O$_2$ is required for effective expression of heat shock genes in Arabidopsis. Plant Cell Biol 61: 733-746.
11. Sato Y, Yokota S (2000) Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, hsp17.7. Plant Cell Rep 27: 407-415.
12. Sato Y, Kuroda I, Takeda Y, Takahashi T, Itoh H, et al. (2000) Complexity of the heat stress response in plants. Curr Opin Plant Biol 10: 310-316.
13. Volkov RA, Panchuk II, Schöffl F (2005) Small heat shock proteins are differentially regulated during pollen development and following heat stress in tobacco. Plant Mol Biol 57: 407-502.
14. Kaska E, Dziedzic P, Zysk E, Kowalczuk A, Wilczek J, et al. (2005) Complexity of the heat stress response in plants. Curr Opin Plant Biol 10: 310-316.
15. Zhang C, Xu J, Zhang H, Zhang X, Shi J, et al. (2005) A cytotoxic class I small heat shock protein, RcHSP17.8, of Rosa chinensis confers resistance to a variety of stresses to Escherichia coli, yeast and Arabidopsis thaliana. Plant Cell Environ 32: 1046-1059.
16. Zhang Y, Garrido D, Eller N, Tupy J, Vicente O, et al. (1995) The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ 18: 139-147.
17. Moriwaki M, Yamaoka T, Wazini T, Kodama T, Igarashi Y (1999) Delayed recovery of β-D-glucuronidase activity driven by an Arabidopsis heat shock promoter in heat-stressed transgenic Nicotiana plumbaginifolia. Plant Cell Rep 19: 96-100.
18. Takahashi T, Naito S, Komeda Y (1992) The Arabidopsis HSP112 promoter/GUS gene fusion in transgenic Arabidopsis plants: a powerful tool for the isolation of regulatory mutants of the heat-shock response. Plant J 2: 751-761.
19. Yabe N, Takahashi T, Komeda Y (1994) Analysis of tissue-specific expression of Arabidopsis thaliana HSP90-family gene HSP81. Plant Cell Physiol 35: 1207-1219.
20. Crone R, Rueda J, Martin KL, Hamilton DA, Mascarenhas JP (2001) The differential expression of a heat shock promoter in floral and reproductive tissues. Plant Cell Environ 24: 869-874.
21. Shoji W, Sato-Maeda M (2008) Application of heat shock promoter in transgenic zebratin. Dev Growth Differ 50: 401-406.
22. Lu S, Gu H, Yuan X, Wang X, Wu AM, et al. (2007) The GS7 reporter-assayed analysis of the promoter activity of烅rice metallothionein genes in rice. Proc Natl Acad Sci USA 104: 6424-6429.
23. Yuan GF, Jia CG, Li Z, Sun B, Zhang LP, et al. (2010) Effect of brassinosteroids on drought stress resistance and abscisic acid concentration in tomato under water stress. Sci Horticulturae 126: 103-108.
24. Wu L, El-Mezaway A, Shah S, et al. (2011) A seed coat outer integument-specific promoter for Brassica napus. Plant Cell Rep 30: 75–80.
25. Tsuwamoto R, Harada T (2011) The Wheat sHSP26 Promoter in Transgenic Arabidopsis. Transgenic Res 16: 177–191.
26. Qiu le Q, Xing YP, Liu WX, Xu XP, Song YR (2008) Expression pattern and activity of six glutelin gene promoters in transgenic rice. J Exp Bot 59: 2417-2424.
27. Yuan GF, Jia CG, Li Z, Sun B, Zhang LP, et al. (2010) Effect of brassinosteroids on drought resistance and abscisic acid concentration in tomato under water stress. Sci Horticulturae 126: 103-108.
28. Sun Y, Tian Q, Yuan L, Jiang Y, Huang Y, et al. (2011) Isolation and promoter analysis of a chalcone synthase gene PtrCHS4 from Populus trichocarpa. Plant Cell Physiol 52: 1661-1671.
29. Ganguly M, Rochoudhury A, Sarkar SN, Sengupta DN, Datta SK, et al. (2011) Inducibility of three salinity/abscisic acid-regulated promoters in transgenic rice with gunA reporter gene. Plant Cell Rep 30: 1617-1620.
30. Chen J, Frige MJ, Franzmann TM, Beiperfing A, Buchner J (2010) Regions outside the alpha-crystallin domain of the small heat shock protein Hsp26 are required for its dimerization. J Mol Biol 398: 122–131.
31. Haidbeck M, Walke S, Stromeier T, Ehrengerer M, White HE, et al. (1999) Hsp26: a temperature-regulated chaperone. EMBO J 18: 6744-6751.

Supporting Information

Figure S1 The sequence of the putative promoter region of HSP26. Some cis-acting elements have been highlighted in the putative promoter sequence through PLACE promoter motif analysis. Three CCAAT BOX1 elements (721, 1209, 1435) are highlighted in green, two of them lie in the antisense-strand while one of them lie in the sense strand.

Figure S2 PCR amplification of different deletions of wheat TaHSP26 promoter for TOPO cloning.

Figure S3 Analysis of control and heat-stressed (a) leaf tissue, and (b) root tissue of Arabidopsis transgenic plants harboring full TaHSP26 promoter under bright field using fluorescence microscope for histochemical localization of GUS activity in control and heat stress tissues, respectively.

Table S1 List of primers used.

Author Contributions

Conceived and designed the experiments: NK HC PK. Performed the experiments: NK. Analyzed the data: NK HC PK. Contributed reagents/materials/analysis tools: PK. Wrote the paper: NK PK.
31. Franzmann TM, Menhorn P, Walter S, Buchner J (2008) Activation of the chaperone Hsp26 is controlled by the rearrangement of its thermosensor domain. Mol Cell 29: 207–216.
32. Wang D, Luhe DS (2003) Heat sensitivity in a bentgrass variant. Failure to accumulate a chloroplast heat shock protein isoform implicated in heat tolerance. Plant Physiol 133: 319–327.
33. Chashan H, Khurana N, Nijhavan A, Khurana JP, Khurana P (2012) The wheat chloroplastic small heat shock protein (sHSP26) is involved in seed maturation and germination and imparts tolerance to heat stress. Plant Cell Environ 35: 1912–1931.
34. Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27: 297–300.
35. Xiao H, Perisic O, Lis JT (1991) Cooperative binding of Drosophila heat shock transcription factor to arrays of a conserved 5 bp unit. Cell 64: 585–593.
36. Santors N, Johansson N, Thiele DJ (1995) Cooperative binding of Drosophila heat shock transcription factor in Saccharomyces cerevisiae. BioEssays 7: 211–214.
37. Yamamoto A, Mizukami Y, Sakurai H (2005) Identification of a novel class of target genes and a novel type of binding sequence of heat shock transcription factor in Sachafrasscinia roritno. J Biol Chem 280: 11911–11919.
38. Siderius M, Mager WH (1997) General stress response: in search of a common denominator. In: Holmham S, Mager WH, editors. Yeast Stress Responses. Heidelberg: Springer-Verlag. 213–230.
39. An G (1987) A potential Z-DNA-forming sequence is an essential upstream element of a plant promoter. BioEssays 7: 211–214.
40. Rieping M, Schoffl F (1992) Synergistic effect of upstream sequences, CCAAT box elements, and HSE sequences for enhanced expression of chimeric heat shock genes in transgenic tobacco. Mol Gen Genet 231: 226–232.
41. Clough SJ, Bent AF (1998) Floral dip: a simplified method for plant transformation. Plant J 16: 735–741.
42. Chahud H, Khurana N, Tyagi AK, Khurana JP, Khurana P (2011) Identification and characterization of high temperature stress responsive genes in bread wheat (Triticum aestivum L.) and their regulation at various stages of development. Plant Mol Biol 75: 35–51.
43. Freeman J, Sparks CA, West J, Shevery PR, Jones HD (2011) Temporal and spatial control of transegene expression using a heat-inducible promoter in transgenic wheat. Plant Biotechnol J 9: 780–796.
44. Haralampidi K, Milionis D, Rigas S, Hatzopoulos P (2002) Combinatorial interaction of cis elements specifies the expression of the Arabidopsis AtHsp90–1 gene. Plant Physiol 129: 1136–1149.
45. Bucher P (1990) Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J Mol Biol 212: 563–578.
46. Morimoto RJ (1993) Cells in stress: transcriptional activation of heat shock genes. Science 259: 1409–1410.
47. Mantovani R (1999) The molecular biology of the CCAAT-binding factor NF-Y. Gene 239: 15–27.
48. Landsberger N, Wolffe AP (1995) Role of chromatin and Xenopus laevis heat shock transcription factor in regulation of transcription from the X. laevis hsp70 promoter in vivo. Mol Cell Biol 15: 6013–6024.
49. Abravaya K, Phillips B, Morimoto (1991) RI Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. Genes Dev 5: 2117–2127.
50. Morgan WD, Williams GT, Morimoto RJ, Greene J, Kingston RE, et al. (1997) Two transcriptional activators, CCAAT-box-binding transcription factor and heat shock transcription factor, interact with a human hsp70 gene promoter. Mol Cell Biol 7: 1129–1136.
51. Stroozhenko S, Pnies PD, Montagu MV, Inze D, Kishnir S (1998) The Heat-shock element is a functional component of the Arabidopsis APX1 gene promoter. Plant Physiol 118: 1003–1014.
52. Carranco R, Almoguera C, Jordano J (1999) An imperfect heat shock element and different upstream sequences are required for the seed-specific expression of a small heat shock protein gene. Plant Physiol 121: 723–730.
53. Karrhkeyan AS, Ballachanda DN, Raghotheama KG (2009) Promoter deletion analysis elucidates the role of cis elements and 5'TLTR intron in spatiotemporal regulation of AtPh1;4 expression in Arabidopsis. Plant Physiol 136: 10–18.
54. Lescoat M, Delasa P, Thijs G, Marchal K, Moreau Y, et al. (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30: 325–327.
55. Jung SH, Chory J (2010) Signaling between chloroplasts and the nucleus: can a systems biology approach bring clarity to a complex and highly regulated pathway? Plant Physiol 152: 453–459.
56. Ish-Shalom D, Kloppstech K, Ohad I (1990) Light regulation of the 22 kd heat shock protein in Dunaliella salina. Plant Cell Physiol 42: 1389–1406.
57. Rossel JB, Wilson JW, Pugou JF (2002) Global changes in gene expression in response to high light in Arabidopsis. Plant Physiol 130: 1109–1120.
58. Yokihongwattana K, Christ B, Behrman S, Casper-Lindley C, Mels A (2001) Photosystem II damage and repair cycle in the green alga Dunaliella salina: involvement of a chloroplast-localized HSP70. Plant Cell Physiol 42: 1390–1397.
59. Vainonen JP, Jaspar P, Wrzaczek M, Lamminmaki A, Reddy RA, et al. (2012) RCD1–DREB2A interaction in leaf senescence and stress responses in Arabidopsis thaliana. Biochem J 442: 573–581.
60. Korshunova B, Kwon S, Guo L, Reddy RA, et al. (2012) Activation of a heat shock promoter in transgenic Arabidopsis.