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An ABF5b-HsfA2h/HsfC2a-NCED2b/POD4/HSP26 module integrates multiple signaling pathway to modulate heat stress tolerance in wheat

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Summary

Heat stress caused by increasing global temperature has become a major factor limiting yield in wheat. Heat shock transcription factors (Hsfs), as the primary regulators in plant responses to heat stress, play essential roles in modulating both basal and acquired thermotolerance in plants. However, the underlying molecular mechanisms remain to be elucidated. By analysing the wheat transcriptome after subjecting wheat to heat treatments for different time intervals, we identified gene *TaHsfA2h* that showed a significant positive regulatory response to heat stress. Heat stress tolerance was enhanced by overexpression of *TaHsfA2h* and constrained by its RNA interference. RNA-seq analysis demonstrated that the overexpression of *TaHsfA2h* significantly enhanced the expression levels of genes involved in ABA and ROS signalling pathways. Additionally, we identified *TaABF5b*, a critical regulatory factor in the ABA signalling pathway, as being capable of modulating the expression of *TaHsfA2h*. Notably, TaHsfA2h interacted with TaHsfC2a both *in vivo* and *in vitro*. Similarly, overexpression of *TaHsfC2a* significantly enhanced heat stress tolerance, whereas knockout dramatically reduced tolerance. The presence of TaHsfC2a significantly enhanced the regulatory activity of TaHsfA2h. TaHsfA2h and TaHsfC2a can co-regulate the expression levels of heat stress tolerance-related genes, including *TaNCED2B*, *TaPOD4* and *TaHSP26*, thereby enhancing wheat's tolerance to heat stress. Overall, our findings revealed a positive regulatory function of the ABF5b-HsfA2h/HsfC2a-NCED2b/POD4/HSP26 module on wheat heat stress tolerance. This discovery further expanded the functionality of a plant heat stress response model, providing a theoretical foundation for the development of heat-tolerant wheat varieties.

Introduction

Crop species are frequently challenged by a range of abiotic stresses that impede their growth and development. In recent years, high temperature has emerged as a significant obstacle to crop yield and agricultural sustainability in China (Cui *et al.*, 2019; Valliyodan *et al.*, 2017; Yu *et al.*, 2019). The initial perception of external heat signals in plants is mediated by phytochromes (Chen *et al.*, 2022; Jung *et al.*, 2016). The signals of heat stress are transmitted through multiple pathways, including Ca^{2+}, ROS, NO, H_2S, light signals, phospholipid signalling, epigenetic modification, the proteasome degradation system, subcellular location-specific changes and hormone signalling (Higashi and Saito, 2019; Kan *et al.*, 2023; Lam *et al.*, 2019; Zhu *et al.*, 2023). In response to high temperature stress, plants enhance photosynthetic protection mechanisms and reduce transpiration rates. They also optimize nutrient uptake and transport to safeguard heading stage and grain-filling processes (Liu *et al.*, 2018; Ren *et al.*, 2021; Wang *et al.*, 2010; Zhang *et al.*, 2022). Plant heat stress response pathways can mitigate cellular damage induced by elevated temperatures and facilitate acclimation to high temperature conditions (Ding *et al.*, 2020). Heat shock transcription factors (Hsfs) and heat shock proteins (HSPs), crucial core components

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of heat stress response (HSR) play a pivotal role in the transcriptional regulatory mechanism triggered by high temperature stress.

Heat shock proteins are key molecular chaperones that can be upregulated in response to heat stress. The role of heat shock proteins in responding to heat stress is to maintain protein stability, facilitating proper folding and degrading misfolded proteins (Baniwal *et al*., 2004; Kotak *et al*., 2007). Expression levels of HSPs are regulated by Hsfs (Ding *et al*., 2020; Liu *et al*., 2013a), a cohort of DNA-binding proteins that play a pivotal role in facilitating rapid heat-induced modifications in gene expression (Andrasi *et al*., 2021). The Hsfs in plants were categorized into A, B and C classes based on the length of amino acid sequence insertions and their positioning within two heptad repeats (HR) structures (Guo *et al*., 2016). Hsfs have a modular organizational structure comprising multiple conserved domains. The N-terminal DNA-binding domain (DBD) is primarily responsible for recognizing heat shock elements (HSEs, 5′-nGAAnnTTCn-3′) within the target promoter (Scharf *et al*., 2012). The oligomerization domain (OD) responsible for protein interaction is located adjacent to the DBD domain. In addition, the DBD and OD domains contain a nuclear localization signal (NLS) and a nuclear export signal (NES), as well as transcription activator domains (AHA) and transcription repressor domains (RD) (Scharf *et al*., 2012). The DBD domain is the most conservative, ensuring its fundamental role as Hsf; in contrast, the domain of OD and AHA exhibits variability, reflecting functional variation (Wang *et al*., 2018a). Generally, Class A Hsfs are characterized by the presence of AHA domains and either nuclear localization signals (NLS) or nuclear export signals (NES) (Wu *et al*., 2018). Class B Hsfs contain an NLS domain and an RD domain (Ikeda *et al*., 2011), whereas Class C Hsfs feature NLS domains while lacking both transcriptional activation and inhibition domains (Berz *et al*., 2019). The disparity in structure and function results in variations in the functionality of Hsfs.

The function of Hsfs has been studied in multiple plant species, such as *Arabidopsis*, tomato, wheat, soya bean, rice and maize (Andrasi *et al*., 2021). HsfBs are generally considered to be inhibitors of HsfAs (Scharf *et al*., 2012) and the functions of HsfCs are analogous to those of HsfAs. HsfA genes primarily exert a positive regulatory function in abiotic stress response; however, their transcriptional activities are modulated by other interacting proteins. For example, interaction between AtHSBP and AtHsfA1b results in the suppression of the HSE-binding capacity of AtHsfA1b, thereby negatively regulating heat stress tolerance in *Arabidopsis* (Hsu *et al*., 2010). Interaction of ZmHsfA2b-II with ZmHsfA2b-I in maize impedes the binding ability of ZmHsfA2b-I to heat stress elements (HSEs) (Song *et al*., 2024). Additionally, the activities of HSFA transcription factors on downstream genes are also influenced by ubiquitin and phosphokinase proteins. For example, the transcriptional activity of TaHsfA1 can be impeded by ubiquitination during prolonged exposure to high temperatures, resulting in a partial decrease in the functionality of the TaHsfA1 protein, consequently compromising the plant's heat stress response (HSR) (Wang *et al*., 2023). HsfA family genes are not only associated with heat resistance but also play a pivotal role in alleviating various other abiotic stresses. In cotton, the drought stress tolerance and yield can be regulated by GhHSFA6B-D through its regulation of *GhIPS1-A* expression (Yu *et al*., 2024a). However, the functional role of HsfA in abiotic stresses and its association with other types of HSFs in heat stress response remains unclear.

Common wheat holds significant importance in China's agricultural industry as a staple food crop; however, wheat production is currently facing a threat due to the adverse effects of global warming, particularly in terms of dry and hot air in spring. This significantly impacts grain filling and hence yield and end-use quality of wheat (Zhao *et al*., 2017). In this study, we demonstrate that the *TaHsfA2h* allele can significantly improve the heat tolerance of wheat by regulating the expressions of heat tolerance-related genes. Additionally, it demonstrates interaction between the TaHsfA2h and TaHsfC2a proteins, with further evidence suggesting that TaHsfC2a exerts regulatory control over the transcriptional activity of TaHsfA2h. Moreover, we demonstrated genetically that *TaHsfC2a* can also increase the heat tolerance of plants. The findings of our study highlight the crucial role played by the TaHsfA2h-TaHsfC2a module in regulating heat tolerance in wheat, thereby expanding our understanding of the interplay between Class A and Class C heat shock proteins.

Results

Characterization and expression of *TaHsfA2h*

The molecular mechanism underlying heat tolerance in plants was investigated by exposing the wheat to heat treatment and extracting RNA after 0, 6 and 12 h for transcriptome analysis (PRJNA1206325). A subsequent GO enrichment analysis on the differentially expressed genes (DEGs) identified a substantial number of pathways responding to heat stress at the 0 h and 12 h time points (Figure 1a). Correlation analysis indicated that the class A Hsfs showed the highest correlation with heat stress pathway genes (Figure S1a–e). *TaHsfA2h* (TraesCS2B02G105100), which was significantly induced by heat stress, was isolated by further screening of differential genes associated with class A Hsfs (Figure 1b). Examination of the function and expression pattern of *TaHsfA2h* transcripts by quantitative real-time PCR (qRT-PCR) demonstrated that heat stress induced *TaHsfA2h* expression, with transcripts reaching a peak level at 1 h post heat treatment (Figure 1c). Exogenous application of ABA induced expression levels of *TaHsfA2h*, with a peak at 1 h (Figure 1d), and *TaHsfA2h* transcripts were suppressed by 2 mM tungstate, which is an ABA biosynthesis inhibitor (Figure 1e). Moreover, pretreatment with tungstate significantly suppressed heat-induced *TaHsfA2h* transcription (Figure 1f).

Transgenic *Arabidopsis* plants with GUS under the control of the *TaHsfA2h* promoter ($P_{TaHsfA2h}$:GUS) were generated to further investigate the functionality of TaHsfA2h. Under heat and ABA treatments, the GUS expression levels in $P_{TaHsfA2h}$:GUS transgenic *Arabidopsis* seedlings were significantly higher than those under normal conditions (Figure 1g). Transient expression analysis in wheat mesophyll protoplasts performed to analyse the cellular location of TaHsfA2h showed that GFP-TaHsfA2h and mCherry-TaHRC fusion proteins were exclusively located in nuclei (Figure 1h) whereas the GFP control was uniformly distributed throughout the cells (Figure 1h). Moreover, transcriptional activity assays showed that yeast strains harbouring a BD-*TaHsfA2h* construct could grow on the selective media SD/-Trp/-His and SD/-Trp/-Ade/-His, whereas 10 mM 3-amino-1,2,4-triazole (3-AT) inhibited the strong transcriptional activation activity (Figure S2).

Overexpression of *TaHsfA2h* in *Arabidopsis* enhanced heat stress tolerance in transgenic plants

Transgenic *Arabidopsis* plants (*TaHsfA2h*-OE1, -OE2 and -OE3) were generated to determine the biological significance of

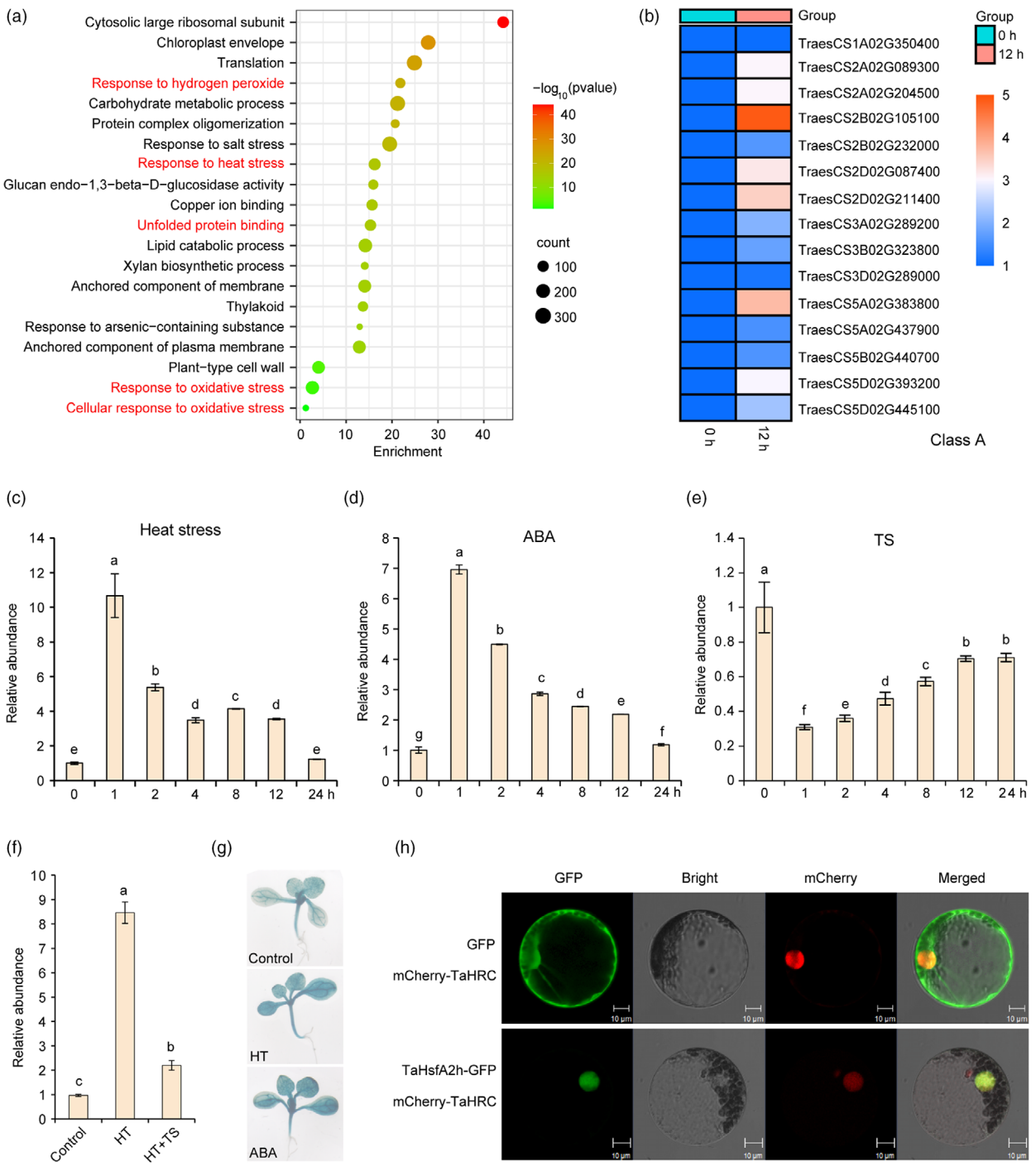

Figure 1 Characterization and expression patterns of *TaHsfA2h*. (a) GO analysis of DEGs in wheat at 0 h vs 12 h post heat treatment. (b) Expression of class A heat shock factor genes in wheat at 0 h vs 12 h post heat treatment. (c–e) The expression profiles of *TaHsfA2h* in the leaves of 7-day-old wheat subjected to heat (HT) (c), ABA (d), tungstate (TS) (e) treatments at the indicated times. *TaHsfA2h* transcript at 0 h was set at 1. (f) The expression profiles of *TaHsfA2h* under the treatment of HT and HT + TS. *TaHsfA2h* transcript for control conditions was set at 1. The expression levels were normalized to that of *TaActin*. SD for three biological replicates is represented by error bars. Significant differences at $P < 0.05$ are indicated by different letters above the columns. (g) Staining of transgenic *Arabidopsis* plants overexpressing $P_{TaHsfA2h}$:GUS assessed under control, heat (HT) and ABA treatments. (h) Subcellular localization of GFP and GFP-TaHsfA2h in wheat protoplasts. The fusion protein mCherry-TaHRC was localized in the nuclei of cells. Three biological replications were performed for each experiment. SD for three biological replicates is represented by error bars. Significant differences at $P < 0.05$ are indicated by different letters above the columns.

TaHsfA2h. *TaHsfA2h* transcripts were significantly higher in the *TaHsfA2h*-OE lines compared with WT lines (Figure S3a,b). Heterologous expression of *TaHsfA2h* conferred higher tolerance to heat stress in the acquired thermotolerance (AT) and thermotolerance (BT) assays (Figure S3a–c). In contrast, the WT and *TaHsfA2h*-OE lines did not exhibit different viabilities in the

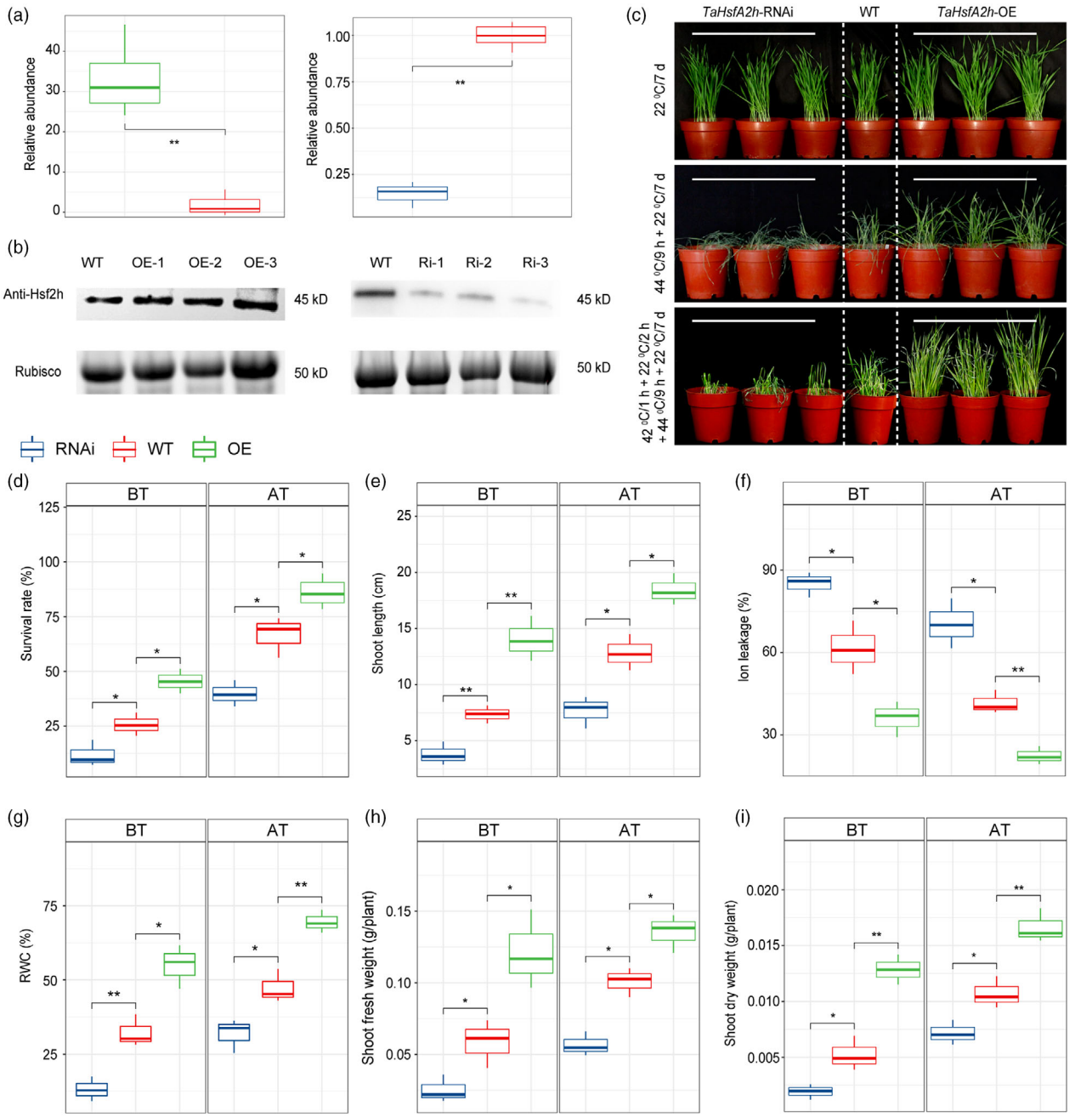

Figure 2 Heat stress tolerance of *TaHsfA2h*-RNAi, wild-type (WT) and *TaHsfA2h*-OE wheat seedlings. (a) Transcripts of *TaHsfA2h* evaluated by qRT-PCR. *TaHsfA2h* transcript in WT plants was set at 1; expression levels were normalized to that of *TaActin*. (b) Protein levels detected in OE and RNAi lines by western blotting using the TaHsfA2h-specific antibody. The protein levels were normalized to that of Rubisco. (c) The basal and acquired thermotolerance of RNAi, WT and OE wheat plant phenotypes assessed under control and heat treatment conditions. (d–i) Survival rate (d), shoot length (e), ion leakage (f), relative water content (RWC) (g), shoot fresh weight (h) and shoot dry weight (i) in RNAi, WT and OE plants subjected to heat treatments. Error bars indicate standard error; * denotes a significant difference when $P < 0.05$, while ** indicates a significant difference at the level of $P < 0.01$.

control condition (Figure S3a–c). Additionally, we introduced an *Arabidopsis* mutant of *AtHsfA2*, a homologous gene to *TaHsfA2h*, to further analyse the functionality of *TaHsfA2h*. The *AtHsfA2* knockout mutant exhibited pronounced susceptibility to heat stress (Figure S3d–g). However, the heat-sensitive phenotype was alleviated following the introduction of *TaHsfA2h* (Figure S3d–g). These results indicated that *TaHsfA2h* positively regulated the tolerance of plants to heat stress.

Heat stress tolerance in wheat is enhanced by overexpression of *TaHsfA2h* and weakened by RNA interference

TaHsfA2h transgenic wheat lines (*TaHsfA2h*-OE-1, -OE-2 and -OE-3) and RNA interference lines (*TaHsfA2h*-RNAi-1, -RNAi-2 and -RNAi-3) were generated to investigate the function of *TaHsfA2h* (Figure 2a). Western blotting assays using a TaHsfA2h-specific

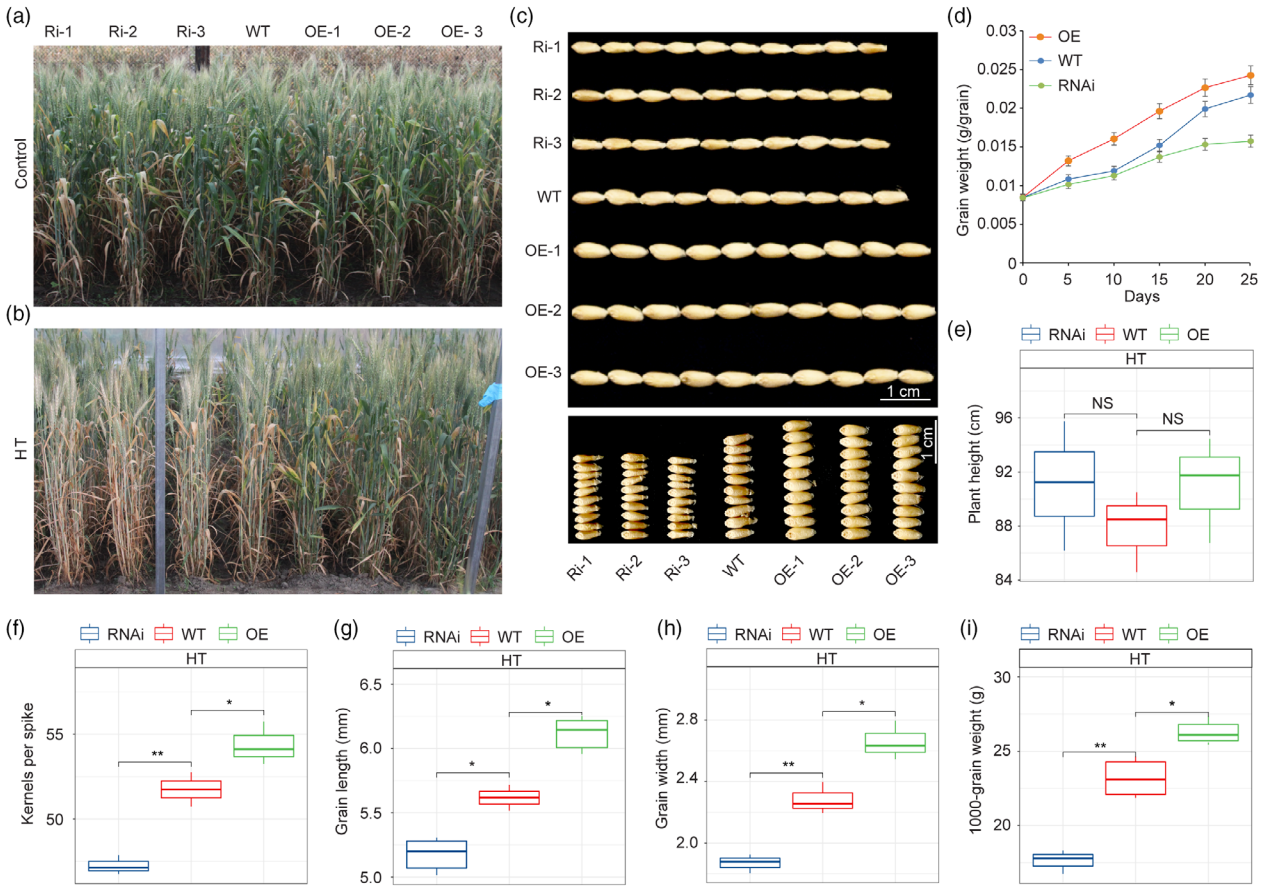

Figure 3 Thermotolerance of *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE wheat plants evaluated at the grain filling stage under control and heat treatments in the field. (a, b) Phenotypes of RNAi, WT and OE wheat plants under control condition (a) and heat stress treatment (b) in the field. (c) Heat stress treatment mature seeds from RNAi, WT and OE lines. (d–i) Agronomic trait analysis of rate of grain filling (d), plant height (e), kernels per spike (f), grain length (g), grain width (h) and 1000-grain weight (i) of RNAi, WT and OE wheat plants under heat stress conditions in the field. Error bars indicate standard error, * denotes a significant difference when $P < 0.05$, while ** indicates a significant difference at the level of $P < 0.01$.

antibody revealed significant increases and decreases in the levels of TaHsfA2h protein with overexpression and RNA interference, respectively, compared to the level in the WT (Figure 2b).

To explore the association between thermotolerance and *TaHsfA2h*, 7-day-old seedlings of *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE lines were subjected to heat stress. *TaHsfA2h*-RNAi lines exhibited more wilting than the WT in both basic thermotolerance (BT) and acquired thermotolerance (AT) assays, whereas most *TaHsfA2h*-OE plants showed only mild injury (Figure 2c). Lower ion leakage and higher relative water content were detected in TaHsfA2h-OE plants compared to the WT (Figure 2d–i). After recovery for 1 week, the survival rate of *TaHsfA2h*-OE plants was higher than that of the WT (Figure 2d–i). Moreover, the shoot length and fresh and dry weights of *TaHsfA2h*-OE plants were higher than those of the WT (Figure 2d–i). *TaHsfA2h*-RNAi plants showed opposite results (Figure 2d–i). There was no significant difference among the *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE lines under control conditions (Figure 2d–i).

The thermotolerances of *TaHsfA2h*-OE, WT and *TaHsfA2h*-RNAi wheat plants at grain filling in the field paralleled the results obtained with seedlings. After 16 days of heat stress treatment, *TaHsfA2h*-OE lines showed enhanced thermotolerance compared with WT plants, whereas *TaHsfA2h*-RNAi plants displayed severe wilting (Figure 3a–c). Although the plant height of *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE wheat plants was similar (Figure 3d–i), the grain traits of *TaHsfA2h*-OE plants were significantly superior to the WT and the *TaHsfA2h*-RNAi lines were inferior (Figure 3d–i). No significant difference was noted among *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE lines under control conditions (Figure S4). Analysis of the grain filling rates showed that the time at which *TaHsfA2h*-RNAi plants reached maximum filling was significantly earlier than that of the WT and *TaHsfA2h*-OE lines (Figure 3d). These results demonstrated that overexpression of *TaHsfA2h* in wheat enhances tolerance to heat stress and that RNA interference compromises tolerance.

TaHsfA2h regulates the transcript levels of heat-related genes

RNA sequencing (RNA-Seq) assays were conducted to analyse genes differentially expressed (DEGs) between *TaHsfA2h*-OE and WT wheat plants under heat stress conditions (PRJNA1250611). Gene Ontology (GO) analysis identified multiple pathways associated with heat stress (Figure 4a). Furthermore, correlation analysis between the heat-induced and *TaHsfA2h* overexpression-mediated wheat transcriptomes revealed a significant overlap of multiple heat stress-responsive genes and pathways (Figure 4a and Figure S5). This finding highlighted the

critical role of TaHsfA2h in regulating wheat's heat stress tolerance. The pathways associated with response to oxidative stress, biosynthesis of abscisic acid (ABA), and response to heat stress were analysed. The expression levels of the DEGs within these pathways were analysed (Figure 4b). TaPeroxidase4 (*TaPOD4*), *TaNCED2b* and *TaHSP26*, which exhibited significant

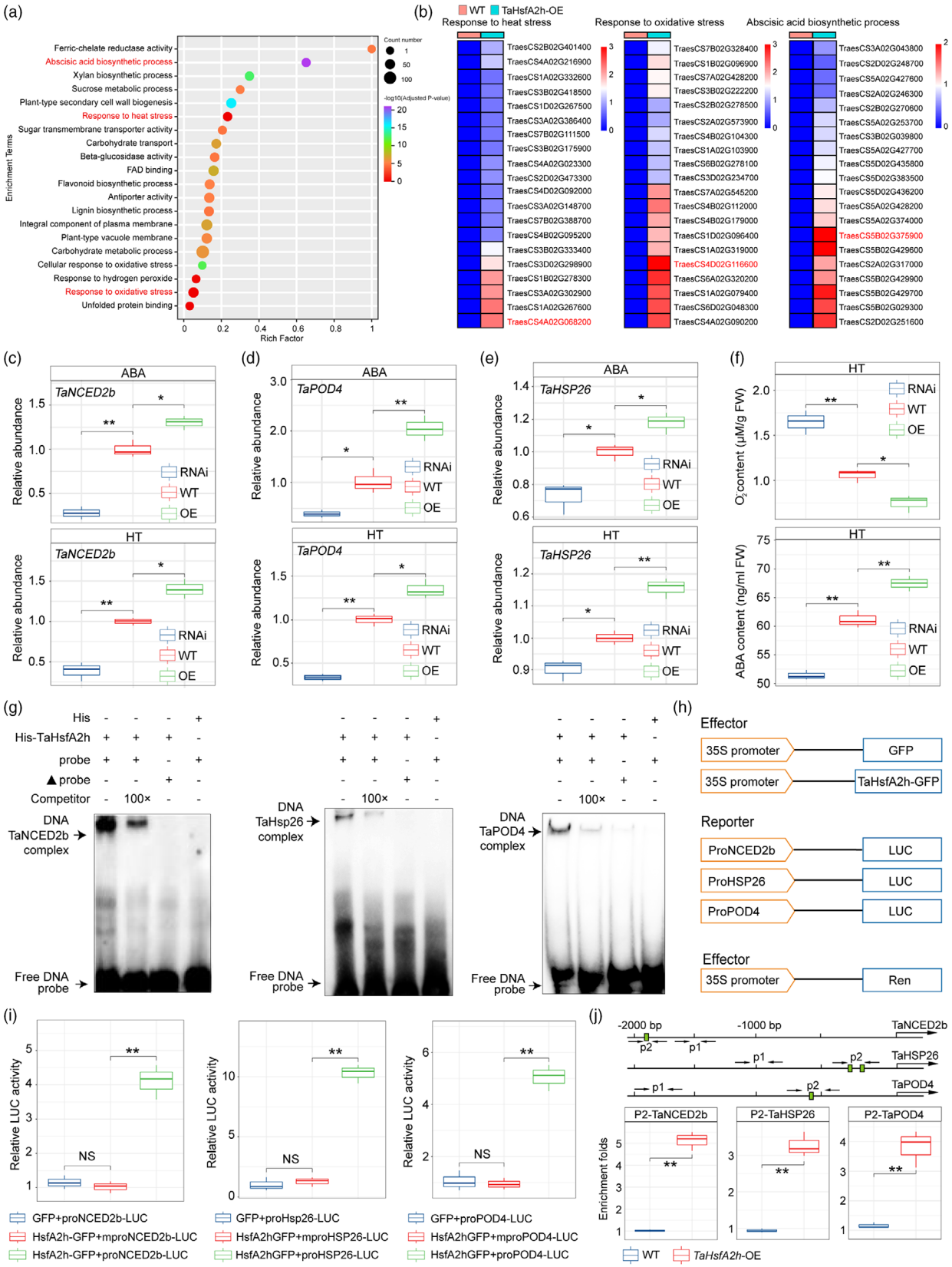

Figure 4 Differentially expressed genes in WT and *TaHsfA2h*-OE plants under heat stress. (a) GO analysis of DEGs related to heat stress in *TaHsfA2h*-OE lines vs WT. (b) Expression pattern of DEGs related to response to heat stress, response to oxidative stress and abscisic acid biosynthetic process. (c–e) Expression profiles of *TaNCED2b* (c), *TaPOD4* (d) and *TaHSP26* (e) under ABA and heat treatments. The transcript of each DEG under the control treatment was set at 1. (f) Analyses of $^{-}O_2$ and ABA content in RNAi, WT and OE plants under heat stress. (g) EMSA assay showing that TaHsfA2h binds to the HSE in *TaNCED2b*, *TaHSP26* and *TaPOD4* promoters. (h, i) Transcriptional activation assays of TaHsfA2h on the *TaNCED2b*, *TaHSP26* and *TaPOD4* promoters using a dual-luciferase system in wheat protoplasts. (j) Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis of TaHsfA2h targeting the *TaNCED2b*, *TaHSP26* and *TaPOD4* promoters. P1 and P2 were used for ChIP analysis. The promoter regions harbouring the HSE are indicated by green bars. Bar, 500 bp. Fragment levels were evaluated based on the amplicon enrichment in ChIPed samples relative to that in the input samples. Levels were normalized to that in WT plants in all trials. Error bars indicate standard error, * denotes a significant difference when $P < 0.05$, while ** indicates a significant difference at the level of $P < 0.01$.

differences in these DEGs, were selected for further investigation (Figure 4b). The relationships among these three candidate genes and *TaHsfA2h* gene under ABA and heat stress were investigated by qRT-PCR analysis. *TaHsfA2h*-OE in wheat significantly enhanced the expression levels of all three candidate genes under heat stress conditions (Figure 4c–f). Conversely, *TaHsfA2h*-RNAi led to a decrease in the expression levels of the three candidate genes under heat stress conditions (Figure 4c–f). The similar results were observed for ABA (Figure 4c–f). There was no significant difference in the control conditions (Figure S6a). These results indicated that all three candidate genes can be targeted by TaHsfA2h.

Electrophoretic mobility shift assays (EMSA) to verify the regulatory relationship between *TaHsfA2h* and the above three candidate genes showed that TaHsfA2h-His fusion protein could bind to heat shock elements (HSE) in the promoters of each gene (Figure 4g). The binding capacity of a TaHsfA2h-His fusion protein to HSE elements in each gene was significantly reduced by the addition of unlabelled competitors, and complete loss of binding ability occurred when biotin-labelled mutated probes were used (Figure 4g). Additionally, LUC activity assays in wheat protoplasts demonstrated significant enhancements in LUC activity driven, respectively, by *ProTaPOD4*, *ProTaNCED2b* and *ProTaHSP26* when TaHsfA2h-GFP was present in the reaction (Figure 4h,i). However, the presence of TaHsfA2h-GFP did not increase LUC activity driven by the mutant promoters (*mProTaPOD4*, *mProTaNCED2b* and *mProTaHSP26*) in the three candidate genes (Figure 4h,i). Chromatin immunoprecipitation (ChIP) assays to further investigate the interactive relationship between TaHsfA2h and the promoters of these three candidate genes revealed 3.1–5.2-fold enrichment of HSE-containing fragments (P2-*ProTaNCED2b*, P2-*ProTaHSP26* and P2-*ProTaPOD4*) in *TaHsfA2h*-OE plants compared to the WT (Figure 4j). However, no significant difference was observed for control DNA fragments, including P-*TaActin*, P1-*TaNCED2b*, P1-*TaHSP26* and P1-*TaPOD4* lacking the HSE sequence (Figure S6b,c). These findings suggested that TaHsfA2h regulated the expression of *TaPOD4, TaNCED2b* and *TaHSP26* by binding the HSEs in their promoters.

TaHsfA2h modulates heat stress tolerance in wheat by regulating genes involved in ROS scavenging

RNA-seq results showed that overexpression of *TaHsfA2h* enriched the pathway of response to oxidative stress in wheat (Figure 4b). Further analysis showed that expression of multiple wheat POD family members, particularly *TaPOD4*, was upregulated in *TaHsfA2h*-OE plants under heat stress (Figure S7). POD proteins scavenge ROS and maintain cellular osmotic balance under stress conditions (Pan *et al*., 2020). To further analyse the relationship between POD and heat stress response, we generated *Arabidopsis* lines overexpressing *TaPOD4*. Under heat stress conditions, *Arabidopsis* seedlings overexpressing *TaPOD4* exhibited enhanced heat stress tolerance compared to WT plants, whereas no significant difference was detected under control conditions (Figure S8a–c). Analysis of physiological and biochemical indexes after 3 h of heat stress treatment showed that ion leakage and superoxide anion contents in the *Arabidopsis* lines overexpressing *TaPOD4* were lower than in WT plants (Figure S8d,e). Additionally, after a 5-day recovery period, the survival rates of *TaPOD4* overexpression lines were higher than those of WT plants (Figure S8c). These results indicated that *TaPOD4* positively regulated ROS scavenging and wheat heat stress tolerance.

Superoxide anion contents were reduced in *TaHsfA2h*-OE wheat plants compared to WT plants under heat stress conditions (Figure 4f). *TaHsfA2h*-RNAi wheat plants exhibited opposite trends, with an increased superoxide anion content compared to WT plants (Figure 4f). To investigate the potential role of *TaHsfA2h* in maintaining ROS homoeostasis, the *TaHsfA2h*-OE, WT and *TaHsfA2h*-RNAi lines were subjected to oxidative stress with or without the oxidative stress inducer methyl viologen (MV) (Zeng *et al*., 1996). After 3 days of MV (30 or 50 μM) treatment, the *TaHsfA2h*-OE lines had lower superoxide anion content and longer shoots in comparison to WT, whereas *the TaHsfA2h*-RNAi line displayed opposite results (Figure S9a–c). No significant difference was noted among the *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE lines under control condition (Figure S9a–c). These results suggested that *TaHsfA2h* had a regulatory role in enhancing the tolerance of wheat to heat stress by modulating oxidative stress pathways.

TaHsfA2h regulates heat stress tolerance via the ABA signalling pathway

Transcriptome analysis indicated that *TaHsfA2h* could regulate heat tolerance through the ABA signalling pathway. Further analysis showed that *TaHsfA2h* regulated the ABA synthesis pathway by modulating the expression of *TaNCED2b* (Figure 4c). Analysis of ABA contents showed that wheat plants overexpressing *TaHsfA2h* exhibited increased ABA content compared to the WT plants, whereas a decreased ABA content was observed in *TaHsfA2h*-RNAi plants (Figure 4f). We also assessed the sensitivities of *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE plants to ABA. There was no significant difference among *TaHsfA2h*-RNAi, WT and *TaHsfA2h*-OE plants under normal conditions (Figure S10a–c), but in the presence of ABA, the shoot and root lengths of *TaHsfA2h*-OE plants were shorter than those of WT plants (Figure S10a–c). *TaHsfA2h*-RNAi wheat plants exhibited an ABA-insensitive phenotype, characterized by increased shoot and

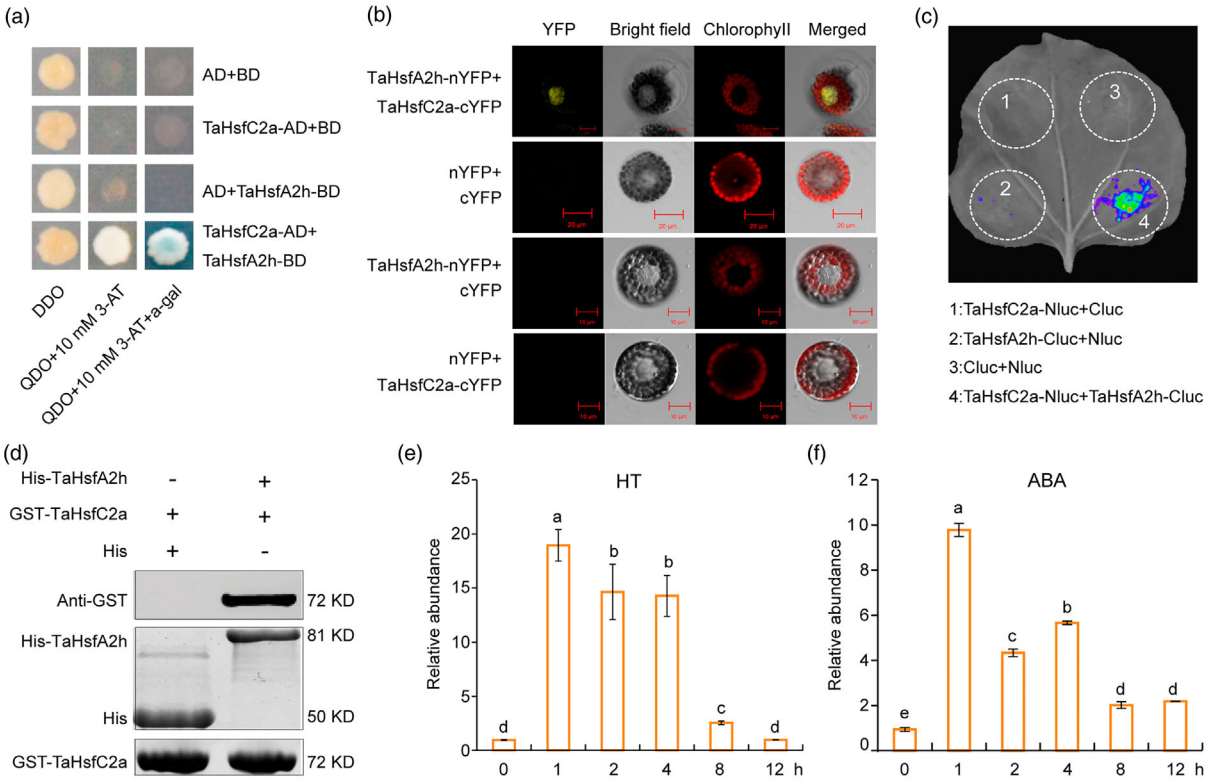

Figure 5 TaHsfA2h interacts with TaHsfC2a. (a–d) Verification of interaction between TaHsfA2h and TaHsfC2a by yeast two-hybrid (a), Bimolecular fluorescence complementary (BiFC) (b), Luciferase complementary imaging (LCI) (c) and pull-down (d) assays. (e, f) Expression profiles of *TaHsfC2a* in leaves of 7-day-old wheat seedlings under heat treatment (HT) (e) and ABA (f). SD for three biological replicates is represented by error bars. Significant differences at $P < 0.05$ are indicated by different letters above the columns.

root length compared to WT plants (Figure S10a–c). *TaHsfA2h*-OE plants under ABA treatment conditions exhibited a significantly reduced stomatal apertures compared to the WT plants. In contrast, *TaHsfA2h*-RNAi plants displayed an increased stomatal aperture compared to the WT (Figure S11). These results showed that the overexpression of *TaHsfA2h* enhanced ABA sensitivity in wheat.

Exogenous application of ABA induced the expression level of *TaHsfA2h* with a peak at 1 h (Figure 1d) indicating that its expression is modulated by the ABA signaling pathway. To further investigate the association between *TaHsfA2h* and the ABA signal pathway, a yeast one-hybrid (Y1H) assay utilizing the *TaHsfA2h* promoter to screen for regulatory proteins indicated that TaABF5b had the potential to function as a regulatory protein of *TaHsfA2h*. Investigation of the promoter of *TaHsfA2h* revealed the presence of six ABA-responsive elements (ABRE) (Figure S12a). Yeast one-hybrid assays showed that co-transformation of *proTaHsfA2h* and TaABF5b in yeast competent cells (Y1H gold) caused a significant increase in β-galactosidase activity (Figure S12b). QRT-PCR indicated that heat stress induced the expression of *TaABF5b*, which reached a peak level at 1 h after heat treatment (Figure S12c). Relative LUC activity analysis demonstrated a significant enhancement in the LUC activity driven by the *TaHsfA2h* promoter in wheat protoplasts upon ABA treatment (Figure S12d). LUC activity driven by the *TaHsfA2h* promoter was further enhanced in wheat protoplasts in the presence of TaABF5b (Figure S12d). These results indicated that TaABF5b regulated the expression level of *TaHsfA2h*. Additionally, an analysis of the correlation between TaABF5b and wheat heat stress tolerance in transgenic *Arabidopsis* revealed that overexpression of *TaABF5b* significantly enhanced heat stress tolerance (Figure S12e–i). These results suggested that *TaHsfA2h* regulates heat stress tolerance in wheat through the ABA signalling pathway.

TaHsfA2h interacts with TaHsfC2a in wheat

Yeast two-hybrid (Y2H) assays were employed in a search for the interacting protein of TaHsfA2h. TaHsfC2a (TraesCS7A02G355300) was identified as a potential candidate. Growth of yeast cells co-expressing TaHsfA2h-BD and TaHsfC2a-AD on the deficient medium was observed to be robust, indicating strong interaction between TaHsfA2h and TaHsfC2a in yeast cells (Figure 5a). Bimolecular fluorescence complementation (BiFC) assays indicated that TaHsfA2h interacted with TaHsfC2a in the nucleus (Figure 5b). Split luciferase (LUC) complementation imaging (split-LCI) assays indicated that LUC fluorescence signals were detectable in *N. benthamiana* leaves only within the co-injection area of TaHsfC2a-nLUC and TaHsfA2h-cLUC (Figure 5c). In addition, GST-pulldown assays demonstrated that TaHsfA2h-His protein had a significant ability to pull down the TaHsfC2a-GST protein *in vitro*, compared to the control group (His-tag protein) (Figure 5d). These results showed that TaHsfA2h interacted with TaHsfC2a *in vitro* and *in vivo*. The transcript patterns obtained from quantitative real-time PCR (qRT-PCR) data demonstrated that heat stress induced the expression of *TaHsfC2a* transcripts, reaching a peak level at 1 h after heat

treatment (Figure 5e). Exogenous application of ABA induced expression of TaHsfC2a with a peak level at 1 h (Figure 5f).

Heat stress tolerance in wheat is enhanced by overexpression of *TaHsfC2a* and weakened by its knockout

To explore the biological role of *TaHsfC2a* in plant heat tolerance response, we produced two triple knockout lines (*TaHsfC2a*-KO-3 and -KO-10) in wheat by CRISPR/Cas9-mediated genome editing, and generated two different types of *TaHsfC2a* wheat knockout mutations (Figure 6a). We also developed two *TaHsfC2a* overexpression lines (*TaHsfC2a*-OE-3 and -OE-10). The *TaHsfC2a*-KO, WT and *TaHsfC2a*-OE seedlings were subjected to heat stress, no significant difference was apparent under normal conditions (Figure 6b); however, under heat treatment, the survival rate of *TaHsfC2a*-OE plants was higher than that of WT, whereas the survival rate of *TaHsfC2a*-KO was lower (Figure 6b–d). Furthermore, fresh weight, electrolyte leakage and chlorophyll content of *TaHsfC2a*-OE plants were higher than for the WT lines, whereas *TaHsfC2a*-KO plants had opposite effects (Figure 6e–j). We then investigated the thermotolerance of *TaHsfC2a*-KO, WT and *TaHsfC2a*-OE plants at the grain filling stage in the field. Under normal conditions, no differences were observed among the *TaHsfC2a*-KO, WT and *TaHsfC2a*-OE lines (Figure S13). After heat stress treatment, the thermotolerance of *TaHsfC2a*-OE plants was superior to that of WT plants, whereas *TaHsfC2a*-KO plants were inferior (Figure 6k, l). In addition, the grain length, grain width and 1000-grain weight of *TaHsfC2a*-OE plants were significantly larger compared to WT plants, and the same traits in *TaHsfC2a*-KO wheat plants were smaller (Figure 6m–r). No significant difference was observed in plant height among *TaHsfC2a*-KO, WT and *TaHsfC2a*-OE plants under heat stress conditions (Figure 6m–r). The time at which *TaHsfC2a*-KO plants reached maximum filling rate was significantly earlier than that of WT and *TaHsfC2a*-OE plants (Figure 6m). These results indicated that *TaHsfC2a* had a role in enhancing the tolerance of wheat to heat stress. Consistent with the *TaHsfA2h* transgenic plants, the shoot and root lengths of *TaHsfC2a*-OE plants were shorter than WT plants, whereas *TaHsfC2a*-KO plants exhibited an ABA-insensitive phenotype (Figure S10d–f). These results suggested that the overexpression of both *TaHsfA2h* and *TaHsfC2a* significantly increased sensitivity to ABA.

TaHsfC2a enhances regulation of TaHsfA2h

To investigate the impact of TaHsfC2a on the transcriptional activity of TaHsfA2h, we conducted a transient activity assay in *N. benthamiana* leaves by introducing downstream target gene promoters of TaHsfA2h. LUC fluorescence activity analysis in tobacco leaves revealed that the presence of *TaHsfC2a*-GFP significantly enhanced the LUC activity driven by promoters of downstream target genes of TaHsfA2h (Figure 7a). This was further augmented by introducing *TaHsfC2a*-GFP into the reaction (Figure 7a) suggesting that TaHsfC2a enhances TaHsfA2h activity. The impact of TaHsfC2a on the regulatory activity of TaHsfA2h was further investigated in wheat protoplast cells, where we observed that co-transformation of *TaHsfA2h*-GFP with a LUC reporter gene driven by the promoters of downstream target genes in wheat protoplasts enhanced LUC activity, and this was further increased by introducing *TaHsfC2a*-GFP into the system (Figure 7b–d). These findings further demonstrated that TaHsfC2a can enhance the regulatory activity of TaHsfA2h. TaHsfA2h and TaHsfC2a interact to improve the heat tolerance of plants (Figure 8).

Discussion

Common wheat, a polyploid species that arose from interspecific hybridization and whole-genome duplication, is a globally significant food crop (Ramirez-Gonzalez *et al.*, 2018). Ongoing global warming presents a substantial threat to maintaining current wheat yields. Heat shock factors (Hsf) were identified as transcriptional regulators controlling the transcription of heat shock proteins (HSPs), which play a crucial role in maintaining protein homoeostasis (Akerfelt *et al.*, 2010). The Hsf-HSP mode represents a quintessential heat-tolerant conduit (Kan *et al.*, 2023). Our findings demonstrate that the positive regulation of heat stress tolerance by *TaHsfA2h* in wheat is achieved by precise control over the expression of *TaHSP26* (Figure 4e), which plays a pivotal role in enhancing the plant capacity to withstand high temperatures (Chauhan *et al.*, 2012). Our results highlight the significance of the TaHsfA2h-TaHSP26 module in regulating heat stress tolerance; however, the ABA signal pathway regulates the Hsf-HSP model (Cohen *et al.*, 2021). *ABF2*, *ABF3* and *AREB3* are crucial transcription factors in the ABA signal transduction pathway and specifically bind to ABRE within the *HSFA6A* promoter to induce its expression (Hwang *et al.*, 2014). *HSFA6b* regulates downstream heat stress-related genes by the ABA signalling pathway to enhance heat tolerance (Huang *et al.*, 2016). We demonstrated that *TaABF5b* plays a crucial role in modulating the expression level of *TaHsfA2h* (Figure S12), thereby highlighting the significance of the ABA signalling pathway in regulating heat stress tolerance. Interestingly, we observed a significant increase in the ABA content in *TaHsfA2h*-overexpression wheat lines compared to the WT plants under heat stress conditions (Figure 4f). Subsequent analysis revealed that the regulation of *TaNCED2b*, a key enzyme in the ABA synthesis pathway, is mediated by *TaHsfA2h*, further revealing the significance of the ABA signalling pathway in regulating heat stress response and implying that the regulation of wheat heat stress tolerance by *TaHsfA2h* is partially dependent on the ABA signalling pathways.

Reactive oxygen species (ROS), as crucial signalling molecules, play indispensable roles in regulating plant stress adaptation and immunity. They include hydrogen peroxide (H_2O_2), superoxide anion and hydroxy free radicals, and are excessively induced by abiotic stress (Yu *et al.*, 2024b). Excessive accumulation of ROS is a characteristic cellular response that triggers oxidative reactions in plants, ultimately leading to cellular damage caused by oxidation (Baxter *et al.*, 2014). ROS scavenging systems are activated to maintain homoeostasis of ROS levels within plant cells and to enhance plant tolerance to abiotic stress (Ohama *et al.*, 2017). In this process, the abundance of various antioxidant enzymes (such as POD, SOD and CAT) and antioxidants is increased, leading to the eruption of their activities in plant cells to enhance the antioxidant capacity of plants (Ohama *et al.*, 2017). For example, the soya bean NF-Y transcription factor enhances the activity of *GmSQE1* under stress conditions, thereby increasing the content of sterol and triterpenoid antioxidants and enhancing antioxidant capacity and stress tolerance (Yu *et al.*, 2024b). In rice, STRK1 enhances the phosphorylation activity of CAT-C, thereby increasing its peroxidase activity and improving salt tolerance (Zhou *et al.*, 2018). In maize, ZmPRX1 induces a high level of POD to enhance drought

stress tolerance (Zhai *et al.*, 2024). Similarly, HSFs enhance ROS elimination, thereby mitigating ROS-induced cell damage (Andrasi *et al.*, 2021). Our findings demonstrated that *TaPOD4* positively regulated ROS scavenging and heat stress tolerance in wheat. TaHsfA2h and TaHsfC2a regulate the expression of the downstream gene *TaPOD4*, thereby modulating H_2O_2 contents in plants. This further demonstrates that HsfA enhances the heat tolerance of plants by depending on the ROS scavenging system.

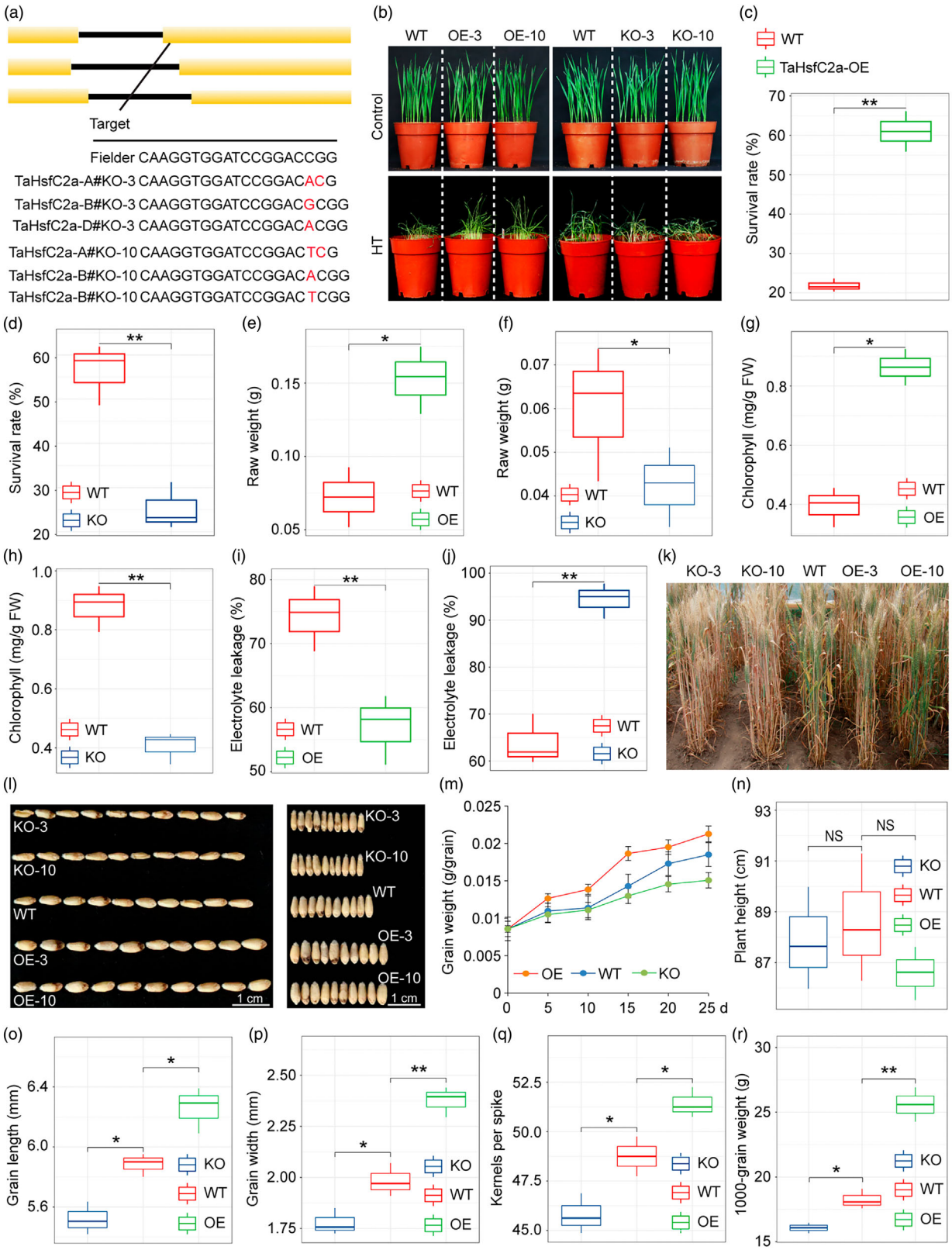

Figure 6 Heat stress tolerance of *TaHsfC2a*-KO, WT and *TaHsfC2a*-OE wheat seedlings. (a) CRISPR/Cas9–mediated mutations in the TaHSFC2a-A, TaHSFC2a-B and TaHSFC2a-D genes. The positions of CRISPR/Cas9 gene editing are indicated by black lines. The red dashes represent base deletions, insertions and substitution. (b) Phenotypes of KO, WT and OE seedlings after heat stress. (c–j) Survival rate (c, d), Raw weight (e, f), chlorophyll content (g, h) and electrolyte leakage (i, j) of *TaHsfC2a* transgenic seedlings following different treatments. (k) Phenotypes of KO, WT and OE wheat plants under heat stress. (l) Seeds from OE lines were fuller than WT and KO lines. (m–r) Agronomic trait analysis: rates of grain filling (m), plant height (n), grain length (o), grain width (p), kernels per spike (q) and 1000-grain weight (r) of KO, WT and OE wheat lines under heat stress in the field. Error bars indicate standard error; * denotes a significant difference when $P < 0.05$, while ** indicates a significant difference at the level of $P < 0.01$.

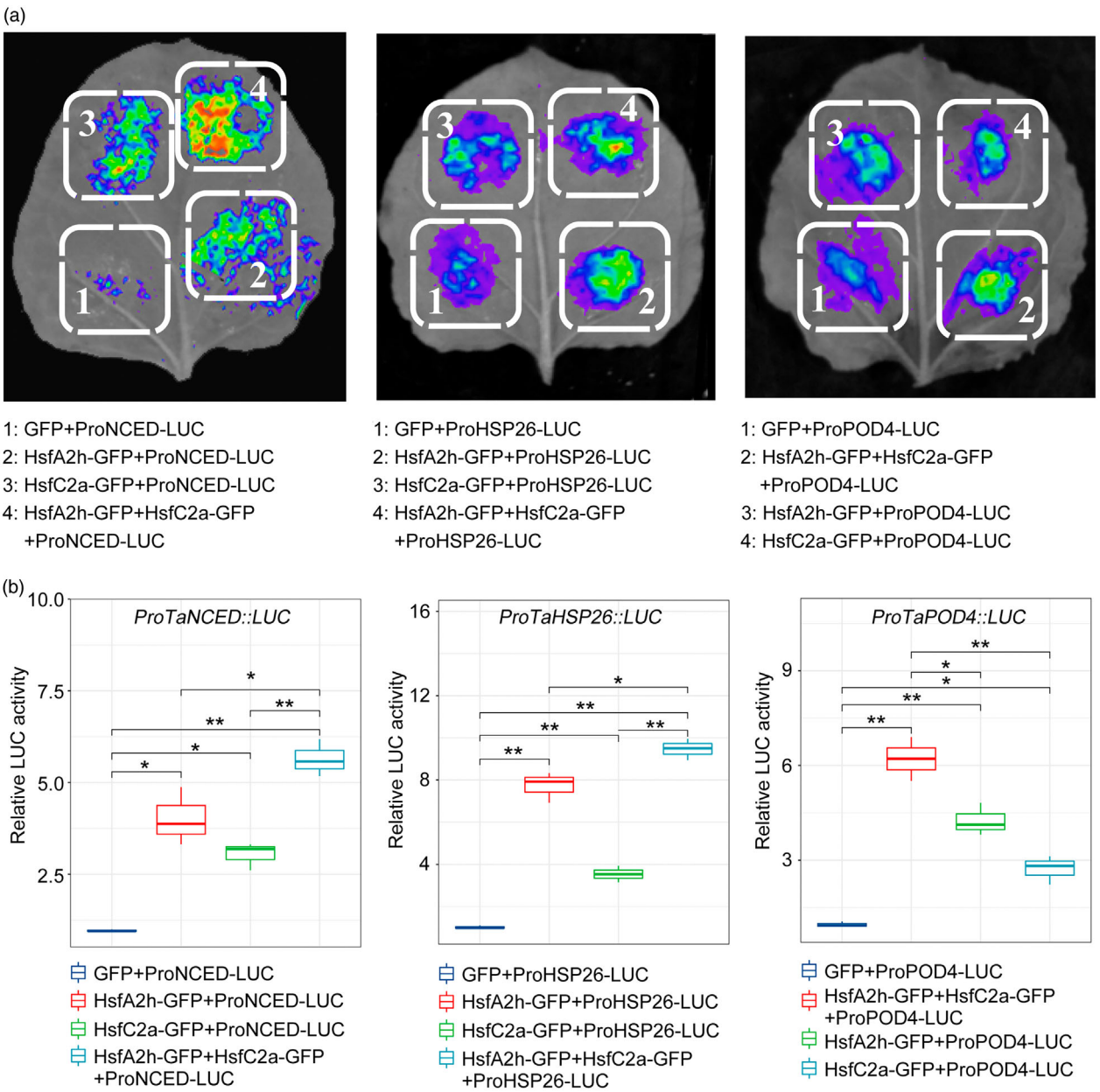

Figure 7 Transcriptional activation activity of TaHsfA2h was modulated by TaHsfC2a. (a) Dual-Luciferase assays showing that TaHsfC2a enhances the regulatory activity of TaHsfA2h in tobacco (*Nicotiana benthamiana*). (b) Dual-Luciferase assays showing that TaHsfC2a enhances the regulatory activities of TaHsfA2h on *TaNCED2b*, *TaHSP26* and *TaPOD4* in wheat protoplasts. Error bars indicate standard error; * denotes a significant difference when $P < 0.05$, while ** indicates a significant difference at the level of $P < 0.01$.

Additionally, our heat-induced transcriptome analysis identified that several other pathways, including carbohydrate metabolic process and xylan biosynthetic process, were critically involved in the response of wheat to heat stress conditions (Figure 1a). Notably, further investigation revealed that the expression levels of genes enriched in these pathways were significantly

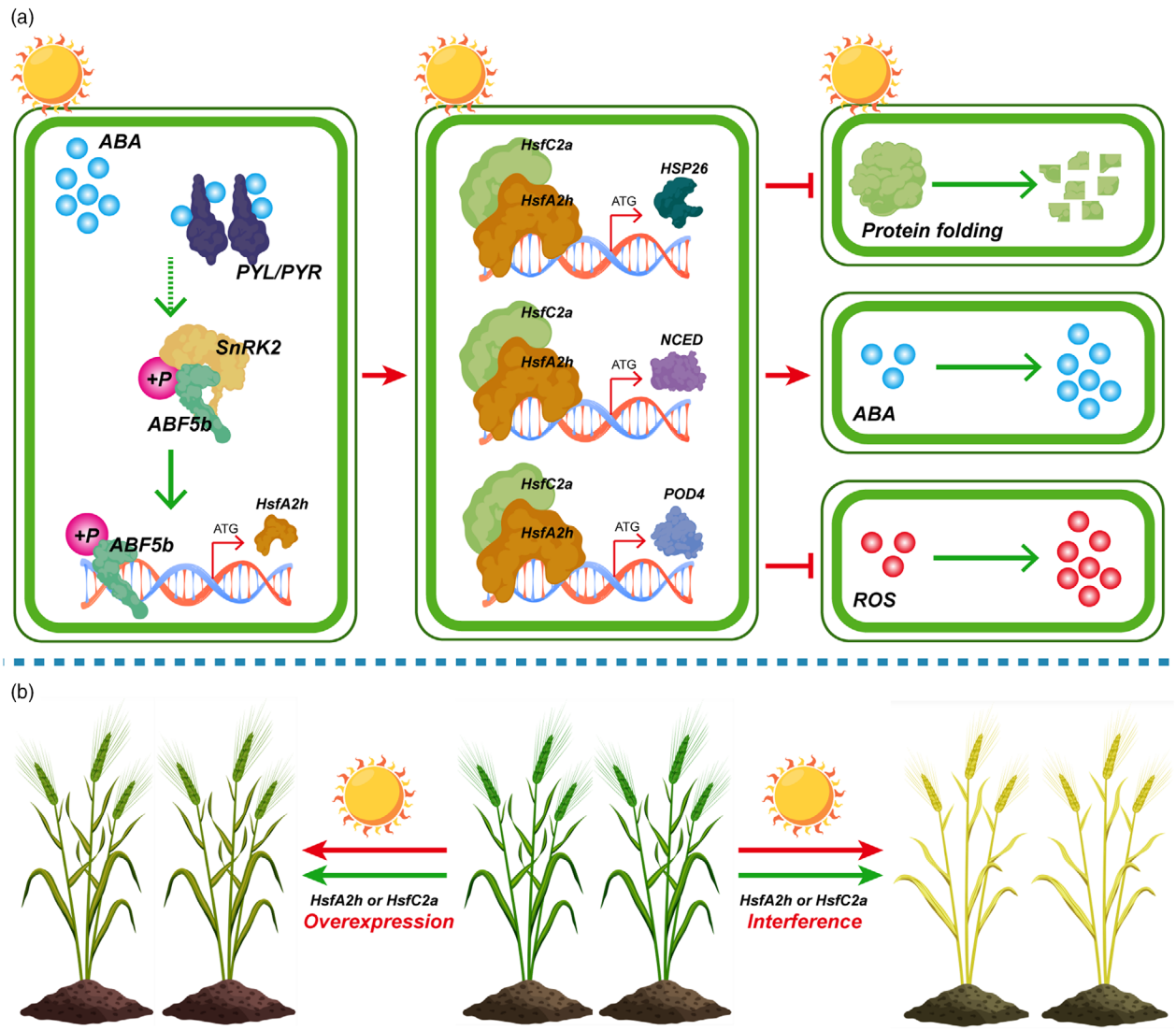

Figure 8 Model for the role of HsfA2h in improving wheat heat stress tolerance. (a) Under heat stress, ABA binds to PYL/PYP receptors, which triggers SnRK2-mediated phosphorylation of ABF5b. ABF5b activates the expression of *HsfA2h*. HsfA2h interacts with HsfC2a to enhance the expression of *Hsp26*, *NCED* and *POD4*, thereby regulating protein folding, ABA synthesis and ROS scavenging in plants. (b) Wheat heat stress tolerance was enhanced by overexpression of *HsfA2h* or *HsfC2a* and constrained by its interference.

upregulated in wheat plants overexpressing *TaHsfA2h* (Figures 1a and 4a and Figure S5). These results indicated that *TaHsfA2h* regulated plant heat stress tolerance through the modulation of multiple physiological processes in plants.

The HsfC, a subclass of Hsfs, possesses a distinctive structure characterized by the absence of transcriptional activation and inhibition domains (Berz *et al.*, 2019; Xue *et al.*, 2014). However, research demonstrates the significant involvement of the HsfC subclass in response to heat stress. In wheat, TaHsfC2a regulated heat stress tolerance by modulating the ABA signal pathway (Hu *et al.*, 2018). Interaction between LlHSFC2 and HSFAs significantly enhanced heat tolerance in lilies (Wu *et al.*, 2024). These findings revealed significant involvement of HsfC in the regulation of heat stress tolerance. HsfC can be induced by various stresses such as drought, high salt, heavy metals and other environmental treatments (Andrasi *et al.*, 2021). This implies a diverse role of HsfC in modulating plant responses to abiotic stress. Although the function of HsfC has similarity with HSFA, the research on its complex regulatory mechanism remains limited (Berz *et al.*, 2019; Xue *et al.*, 2014). In this study, the TaHsfC2a protein was identified through screening for interacting proteins of TaHsfA2h. Like *TaHsfA2h*, overexpression of *TaHsfC2a* enhanced the tolerance in wheat to heat stress, and its effect was compromised by knockout (Figure 6). Further analysis showed that TaHsfC2a enhanced the regulatory activity of TaHsfA2h by interacting with it (Figure 7). These results provided further evidence that the HsfC subclass regulates abiotic stress response in plants by modulating the regulatory activity of its interacting protein. Our study revealed the significant role of TaHsfA2h, a heat shock transcription factor, in wheat. This gene interacts with TaHsfC2a to modulate multiple signalling pathways, especially the ABA and ROS pathways, to enhance tolerance to heat stress. Our results further expand knowledge of the molecular mechanisms underlying the regulation of heat stress response by HsfA and HsfC subclasses of Hsfs,

thereby providing valuable gene resources and a better theoretical foundation for developing heat-tolerant wheat varieties.

Materials and methods

All primers used in various assays are listed in Table S1.

Plant materials and growing conditions

The bread wheat (*Triticum aestivum*) cv Jinhe 991 was used to amplify the coding sequences (CDS) of *TaHsfA2h* and *TaHsfC2a* and as the recipient for generation of the transgenic lines. Wheat grains were sterilized using 2% (w/v) sodium hypochlorite (NaClO) and placed in the plates containing moistened filter paper at 20 °C for 2 days. Uniform seedlings were transferred to half-strength Hoagland solution in a culture room with a 16-h photoperiod at 22/18 °C, 300 μmol m^{-2} s^{-1} light intensity and 50% relative humidity (normal conditions). For heat treatments, 7-day-old seedlings were subjected to 44 °C for 12 h (Hu *et al*., 2018). For ABA and tungstate treatments, roots of 7-day-old seedlings were, respectively, immersed in half-strength Hoagland solution containing 200 μM ABA and 2 mM tungstate for 12 h (Han *et al*., 2004). Leaves of treated wheat plants were collected at 0, 1, 2, 4, 8 and 12 h and flash-frozen in liquid nitrogen for RNA extraction. To investigate the correlation between heat and ABA, seedling roots were immersed in a half-strength Hoagland solution or a 2 mM tungstate solution for 3 h, followed by exposure to 44 °C for 1 h, representing the HT and HT + tungstate treatments, respectively. Seedlings from normal conditions were used as the control. Leaves were sampled and frozen by liquid nitrogen for RNA extraction.

RNA extraction, RT-qPCR

Extraction of the total RNA and synthesis of cDNA were performed as described previously (Cui *et al*., 2019). Quantitative real-time polymerase chain reactions (qRT-PCR) were carried out with TransStart Top Green qPCR SuperMix (TransGen, China) on an ABI 7500 Real-Time PCR system according to the manufacturer's recommendations. Quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method (Cui *et al*., 2019; Le *et al*., 2011). The wheat *β*-actin gene was used as the internal control. qPCR primers are used in Table S2.

Plasmid construction and plant transformation

To generate transgenic wheat lines with *TaHsfA2h* and *TaHsfC2a*, the coding sequences (CDS) of *TaHsfA2h* and *TaHsfC2a* were inserted into transformation vector pWMB110 under the control of the maize ubiquitin promoter to generate pWMB110-*TaHsfA2h* and pWMB110-*TaHsfC2a* constructs. A synthesized fragment containing sense and anti-sense orientations of the 204-bp specific sequence of *TaHsfA2h* flanking the 151-bp intron 1 of maize alcohol dehydrogenase 1 gene (Figure S16) was cloned into the pWMB110 vector to generate the pWMB110-RNAi-*TaHsfA2h* construct. Wheat spikes were harvested 14 days post-anthesis. The grains were surface-sterilized with 70% ethanol for 1 min and subsequently treated with 5% sodium hypochlorite for 15 min, followed by five rinses with sterile distilled water. Fresh immature embryos were carefully isolated and transformed via *Agrobacterium*-mediated gene transfer to generate transgenic plants (Wang *et al*., 2022).

Gene editing to produce a *TaHsfC2a* mutant was performed using CRISPR/Cas9 technology. The sgRNA target sequence for tahsfc2aA/B/D gene editing was designed using an online platform (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2). The sgRNA sequences were inserted into the pBUE411 vector and the resulting constructs were transferred into the EHA105 and subsequently transformed into cultivar Fielder (Wang *et al*., 2022). The transgenic plants were validated by PCR and sequencing.

Preparation and confirmation of TaHsfA2h-specific antibody

A 298–405 aa fragment from the *TaHsfA2h* CDS was inserted into pGEX4T-1 to generate a GST-TaHsfA2h fusion protein to which a GST tag was cleaved. TaHsfA2h protein was injected into rabbits to generate polyclonal antibodies by ABclonal Tech Co (Wuhan). Immunoblot analysis of young wheat leaves was used to determine the specificity of anti-TaHsfA2h. The assay was performed as described previously (Duan *et al*., 2019).

Heat stress treatment

Uniform seedlings were transferred to pots (11 cm in depth, 13.5 cm in diameter) containing a sand/vermiculite (3/1, v/v) mixture. For the control treatment, 7-day-old seedlings were held in a plant growth chamber under normal conditions. For the basal thermotolerance treatment, 7-day-old seedlings were transferred to a plant growth chamber at 44 °C for 9 h when significant differences were observed between transgenic and WT seedlings. For the acquired thermotolerance treatment, 7-day-old seedlings were transferred to a growth chamber at 42 °C for 1 h and 22 °C for 2 h, followed by 44 °C for 9 h and then transferred to normal conditions for 7 days for recovery. RWC and ion leakage were determined as described previously (Guo *et al*., 2006; Lu *et al*., 2009).

To determine whether *TaHsfA2h* and *TaHsfC2a* functioned during grain development, wheat plants grown under normal conditions were transferred to a growth chamber at 35/30 °C 8 h light (300 μmol m^{-2} s^{-1} intensity)/16 h darkness and 50% relative humidity at the beginning of the grain filling stage for 25 days (Stratonovitch and Semenov, 2014; Xu *et al*., 1995). The wheat plants grown under normal conditions were used as the control.

To further determine the function of *TaHsfA2h and TaHsfC2a* in the field, we erected a high temperature canopy covering wheat plants at the beginning of grain filling to ensure the temperature inside the canopy was 4–5 °C higher than outside for 16 days. Wheat plants outside the canopy represented the control.

ABA sensitivity assays

To investigate root growth, germinated seeds of transgenic and WT wheat lines were placed in half-strength Hoagland's solution for 2 days, and then transferred into half-strength Hoagland's solution containing various concentrations of ABA (0, 50, 80) for 5 days. The lengths of shoots and roots were measured by a ruler.

Subcellular localization and bimolecular fluorescence complementation (BiFC) in wheat protoplasts

The CDS of *TaHsfA2h and TaHsfC2a* without termination codons were inserted into the *Bam*HI restriction site of the pJIT16318 vector driven by the CaMV35S promoter. The CDS (without the termination codon) of *TaHRC*, a nuclear-localized protein, was cloned into the pLVX-IRES-mCherry vector to generate a *mCherry-TaHRC* construct (Su *et al*., 2019). The plasmids were extracted and co-introduced into wheat protoplasts as described

previously (Liu *et al*., 2013b). Transfected protoplasts were incubated in darkness at 22 °C for 16–18 h, and GFP fluorescence signals were observed with a confocal laser scanning microscope (LSM 700, Carl Zeiss).

The CDS of *TaHsfA2h* was cloned into 35S::nYFP, and the CDS of *TaHsfC2a* was cloned into 35S::cYFP to generate protein fusion constructs for BiFC assays, following previously described methods. The plasmids were extracted and co-introduced into wheat protoplasts as described previously (Liu *et al*., 2022). Transfected protoplasts were incubated in darkness at 22 °C for 16–18 h; YFP fluorescence signals were observed with a confocal laser scanning microscope (LSM 700).

Luciferase complementary imaging (LCI)

LCI assays of TaHsfA2h and TaHsfC2a were performed as described previously (Yu *et al*., 2021). Candidate TaHsfC2a protein and TaHsfA2h protein were cloned into vectors labelled with nLUC and cLUC, respectively. Luciferase activity was assessed after 48 h.

In vitro pull-down assays

Pull-down assays of TaHsfA2h and TaHsfC2a were performed as described previously (Liu *et al*., 2022).

Yeast one- and two-hybrid assays

The CDS of *TaHsfA2h* and CDS of *TaHsfC2a* were cloned into the pGADT7 and pGBKT7 vector for yeast two-hybrid assays. The bait and prey constructs were co-transformed into the yeast strain AH109, followed by Y2H assays conducted in accordance with the manufacturer's instructions.

Yeast one-hybrid assays were carried out as described previously (Duan *et al*., 2019). The *TaHsfA2h* promoter was isolated from wheat and cloned into the pLacZi vector at the XhoI restriction site to generate a pLacZi-P*TaHsfA2h* construct. The CDS of *TaABF5b* was inserted into the EcoRI restriction site of the pB42AD vector to generate a pB42AD-*TaABF5b* construct. Both constructs were co-transformed into yeast strain Y1HGold. SD-Trp/-Ura was used for the growth of transformants at 30 °C for 3 days and then subjected to X-gal staining to detect the activity of β-galactosidase encoded by LacZ.

Transcriptional activation assays in wheat protoplasts and tobacco

The promoters of target genes were cloned into the LUC reporter vector pGreen II 0800 containing the Renilla luciferase (REN) gene used as an internal control driven by the CaMV 35S promoter. The effector and reporter plasmids were extracted and introduced into wheat protoplasts using PEG4000-mediated transformation. The assays were performed in wheat protoplasts as described previously (Cui *et al*., 2019). The effector and reporter plasmids introduced were into *A. tumefaciens* GV3101 (pSoup). The mixture was injected into the tobacco leaves, and the LUC and LUC/REN levels in the leaves were quantified after 60 h.

Chromatin immunoprecipitation assay (ChIP)

The ChIP assay was performed to determine the enrichment of TaHsfA2h at the promoters of *TaNCED2b*, *TaHSP26* and *TaPOD4* using the anti-TaHsfA2h specific polyclonal antibodies. About 4 g young leaves of WT and OE plants were selected for the ChIP assay. Detailed procedures of this assay were carried out as described previously (Duan *et al*., 2019). ChIP primers are used in Table S3.

Electrophoretic mobility shift assay (EMSA)

The CDS of *TaHsfA2h* was inserted into the expression vector pCold TF to generate His-TaHsfA2h fusion proteins. The partial promoter sequences of genes related to heat stress labelled with biotin were obtained by artificial synthesis and used as probes. Purified His-TaHsfA2h or His protein and probe were mixed with the binding buffer, and the total volume was 20 μL. His protein in combination with the probe was used as the negative control. EMAS was performed as described previously (Dong *et al*., 2010). The HSE and mutated HSE are shown in Figure S15.

Measurements of superoxide anion and ABA contents

Seven-days-old wheat seedlings grown in a half-strength Hoagland solution were exposed to various treatments for 3 h (Control, 44 °C). The levels of superoxide anion ($O_2^{-\cdot}$) were quantified following the protocols outlined in the Superoxide Anion Content Detection Kit (Solarbio Life Science, China). A plant ABA ELISA assay kit (Nanjing Jiancheng) for plants was used to measure the concentrations of ABA (Wang *et al*., 2018b).

Oxidative stress treatment

Three-days-old seedlings were exposed to a half-strength Hoagland solution supplemented with varying concentrations (0, 30 and 50 μM) of methylviologen (MV) for 4 days. Marked differences between transgenic and wild-type (WT) seedlings were subsequently observed. The lengths of shoots and the content of superoxide anion were then quantified.

Generation of transgenic *Arabidopsis* seedlings

The CDS of target genes was cloned into the pCAMBIA1302 vector. T_3 homozygotes were obtained as described previously (Ma *et al*., 2020).

Promoter:GUS analysis

The promoter of *TaHsfA2h* was cloned in a modified pCAMBIA1305 vector. T_3-generation $P_{TaHsfA2h}$:GUS transgenic seedlings were obtained as previously described (Ma *et al*., 2020). Five-day-old seedlings subjected to 37 °C or 0.5 μM ABA solution for 2 h were stained as previously described.

Heat stress treatment of transgenic *Arabidopsis* seedlings

Transgenic *Arabidopsis* seeds were surface-sterilized and stratified as described previously (Ma *et al*., 2020). The seeds were transferred to a greenhouse at 22 °C with a 16-h/8-h light/darkness photoperiod. Five-day-old seedlings were used to perform BT and AT tests as described previously (Charnget *et al*., 2007).

Statistical analysis

All data in this study were analysed using SPSS Statistics 22 (IBM) for a completely randomized design model. Tukey–Kramer tests were used to assess differences among lines at $P = 0.05$.

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Author contributions

Ji-Tong Wei: Methodology, experiment, investigation and writing – original draft. Xiao-Jun Ma: Experiment and investigation. Tai-Fei Yu: Writing – review and editing and data curation. Xiang Gao: Investigation. Lei Zheng: Methodology. Ze-Hao Hou: Resources. Yong-Wei Liu: Resources. Xin-You Cao: Resources. Jun Chen: Supervision. Yong-Bin Zhou: Resources. Ming Chen: Supervision. Qi-Yan Jiang: Resources. You-Zhi Ma: Supervision. Wei-Jun Zheng: Resources and supervision. Zhao-Shi Xu: Conceptualization, funding acquisition, project administration and Supervision.

Conflict of interest

The authors declare no competing interests.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Transcriptome sequencing analysis of heat induced wheat plants.

Figure S2 TaHsfA2h and TaHsfC2a transcriptional activation activity analysis in yeast strains.

Figure S3 Heat stress tolerance analysis in *TaHsfA2h*-OE *Arabidopsis* plants.

Figure S4 Thermotolerance of TaHsfA2h-RNAi and -OE wheat plants were evaluated at the grain filling stage under control treatments in field.

Figure S5 Correlation analysis between the heat-induced and TaHsfA2h-OE wheat transcriptomes.

Figure S6 TaHsfA2h can regulate expression of *TaNCED2b*, *TaPOD4* and *TaHsp26* in normal and ABA add heat stress condition. Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis of TaHsfA2h targeting the *TaNCED2b*, *TaHSP26* and *TaPOD4* promoters.

Figure S7 Expression profiles of multiple POD family members in *TaHsfA2h*-OE wheat through transcriptome sequencing.

Figure S8 Heat stress tolerance analysis in *TaPOD4*-OE *Arabidopsis*.

Figure S9 Oxidative stress tolerance analysis of *TaHsfA2h*-RNAi and -OE wheat plants.

Figure S10 ABA Sensitivity analysis of *TaHsfA2h* and *TaHsfC2a* transgenic wheat plants under control and ABA treatments.

Figure S11 Stomatal aperture analysis of *TaHsfA2h*-RNAi and -OE wheat plants under control and ABA treatments.

Figure S12 TaABF5b directly regulated TaHsfA2h transcripts by binding the *TaHsfA2h* promoter and heat stress tolerance analysis in *TaABF5b*-OE *Arabidopsis* plants.
Figure S13 Thermotolerance of KO, WT and OE *TaHsfC2a* wheat plants were evaluated at the grain filling stage under control treatments in field.
Figure S14 RNAi-TaHsfA2h sequence. The fragment was inserted into *Sma*I and *Sac*I restriction site of pWMB110 vector.
Figure S15 The biotin-labelled probe and mutated probe of *TaNCED2b*, *TaHsp26* and *TaPOD4*.
Table S1 All primers used in various assays.
Table S2 Qpcr primers used in the study.
Table S3 ChIP-qPCR primers used in the study.