Wheat F-Box Protein Gene TaFBA1 Is Involved in Plant Tolerance to Heat Stress

Qinxue Li, Wenqiang Wang, Wenlong Wang, Guangqiang Zhang, Yang Liu, Yong Wang and Wei Wang*

State Key Laboratory of Crop Biology, Shandong Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Tai’an, China

Adverse environmental conditions, including high temperature, often affect the growth and production of crops worldwide. F-box protein, a core component of the Skp1-Cullin-F-box (SCF) E3 ligase complex, plays an important role in abiotic stress responses. A previously cloned gene from wheat, TaFBA1, encodes a homologous F-box protein. A Yeast two-Hybrid (Y2H) assay showed that TaFBA1 interacted with other SCF proteins. We found that the expression of TaFBA1 could be induced by heat stress (45°C). Overexpression of TaFBA1 enhanced heat stress tolerance in transgenic tobacco, because growth inhibition was reduced and photosynthesis increased as compared with those in the wild type (WT) plants. Furthermore, the accumulation of H₂O₂, O₂⁻, and carbonyl protein decreased and cell damage was alleviated in transgenic plants under heat stress, which resulted in less oxidative damage. However, the transgenic plants contained more enzymatic antioxidants after heat stress, which might be related to the regulation of some antioxidant gene expressions. The qRT-PCR analysis showed that the overexpression of TaFBA1 upregulated the expression of genes involved in reactive oxygen species (ROS) scavenging, proline biosynthesis, and abiotic stress responses. We identified the interaction of TaFBA1 with Triticum aestivum stress responsive protein 1 (TaASRP1) by Y2H assay and bimolecular fluorescence complementation (BiFC) assay. The results suggested that TaFBA1 may improve enzymatic antioxidant levels and regulate gene expression by interacting with other proteins, such as TaASRP1, which leads to the enhanced heat stress tolerance seen in the transgenic plants.

Keywords: F-box protein, heat stress, wheat, gene expression, transgenic tobacco

INTRODUCTION

Many different environmental cues affect plant growth and development, of which extreme temperature and water deficit are two major factors (Fang et al., 2015). It has been reported that the increased temperature caused by global warming will have adverse effects on crop yields throughout the 21st century (Sato et al., 2016). To cope with these stress conditions, plants need to adopt a series of efficient strategies, which ultimately lead to morphological, physiological, and biochemical changes (Fang et al., 2015). The regulation of gene expression is an important strategy used by plants for adapting to heat stress. Some genes, including those encoding transcription
factors and functional proteins, are involved in heat stress tolerance of plants. For instance, *Arabidopsis thaliana* has a positive regulator of dehydration-responsive element binding protein 2A (DREB2A), the transcriptional regulator DNA polymerase II subunit B3-1 (DPB3-1), and nuclear factor Y subunit C10 (NF-YC10), which play vital roles in abiotic stress tolerance. In addition, the overexpression of DPB3-1 enhances heat stress tolerance without growth retardation. Furthermore, DPB3-1-overexpressing rice showed enhanced heat stress tolerance (Sato et al., 2014, 2016). SNAC3 is a stress-responsive NAC gene and its overexpression in rice resulted in enhanced tolerance to high temperature, drought, and oxidative stress, whereas suppression of SNAC3 by RNAi led to contrasting phenotype when exposed to these stresses (Fang et al., 2015).

In all eukaryotes, the ubiquitin/26S proteasome system is very important in selectively degrading intracellular proteins (Moon et al., 2004; Smalle and Vierstra, 2004; Ho et al., 2008). In this system, the polymeric ubiquitin chains recognized and modified intracellular proteins via an ATP-dependent reaction cascade. Then these intracellular proteins are degraded by the 26S proteasome. Three enzymes participated in the ubiquitination of a target protein. These are the E1 ubiquitin-activating enzymes, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin ligases (Hershko and Ciechanover, 1998; Yan et al., 2013; Zhou et al., 2015). SKP1/CUL1/F-box protein (SCF) E3 ligase is the best characterized class of E3 ubiquitin ligases, among which F-box protein is a key subunit protein that is involved in responses to abiotic stresses (Moon et al., 2004). Hua and Vierstra (2011) reported that the C-terminal WD40 motif or the leucine-rich repeat region of F-box protein binds targeted substrates to provide reaction specificity.

Some papers have reported E3 ligases function in heat response. For example, the expression of the plant E3 ligase BnTR1 could significantly increase the thermostolerance of *Escherichia coli* (*E. coli*) and BnTR1 expression induced the accumulation of heat shock proteins. Niu et al. (2016) reported that their findings indicated the expression of BnTR1 confers thermoprotective effects on *E. coli* cells. SIZ1 is a well-characterized Small Ubiquitin-like Modifier (SUMO) E3 ligase, Zhang et al. (2017) reported that SUMO conjugations were clearly induced by high temperature. Overexpression of SIZ1 SUMO E3 ligase (SISIZ1) could enhance the tolerance to heat stress in tomato and the level of heat-shock protein (HSP) 70 at high temperatures was increased in transgenic plants. The rice (*Oryza sativa*) HEAT TOLERANCE AT SEEDLING STAGE gene, OsHTAS, is a RING Finger Ubiquitin E3 Ligase and plays a positive role in heat tolerance in rice. Liu et al. (2016) suggested OsHTAS functions in leaf blade to enhance heat tolerance through modulation of hydrogen peroxide-induced stomatal closure. Also, *Oryza sativa* Heat and Cold Induced 1 (OsHCl1) is a RING E3 ligase gene which is highly up-regulated by heat and cold stress. The ectopic expression of YFP fused OsHCl1 in *Arabidopsis* showed a heat-tolerant phenotype and increased survival rate under heat stress (Lim et al., 2013). Moreover, it has been reported that the F-box protein transport inhibitor response 1 (TIR1) auxin co-receptor accumulated rapidly after being induced by increased temperature, and this response was dependent on the molecular chaperone HSP90 (Wang R.H. et al., 2015). *AtCHIP* is a U-Box-Containing E3 Ubiquitin Ligase gene in *Arabidopsis*, Yan et al. (2003) stated the transcript of *AtCHIP* was up-regulated by high temperatures. The overexpression of *AtCHIP* in *Arabidopsis* rendered plants more sensitive to high-temperature treatments. All those suggested the important role of different type E3 ligases in the regulation of plant heat tolerance.

Wheat is a universal and important food crop, and improving its resistance to stresses has become an important research theme around the world. Previously, we obtained an F-box gene TaFBA1 from wheat (*Triticum aestivum* L.). The expression of this gene in wheat was induced by different stresses, including NaCl, drought and ABA treatment in wheat (Zhou et al., 2014). We transformed this gene into tobacco to improve our understanding of the role played by TaFBA1 in plant stress tolerance, and to gain insight into the mechanism underlying its control. We found that the transgenic tobacco lines overexpressioning TaFBA1 displayed an ameliorative performance when exposed to drought (Zhou et al., 2014) and salt stresses (Zhao et al., 2017), and the oxidative stress tolerance of the transgenic plants was increased (Zhou et al., 2015). In the present study, we analyzed how TaFBA1 affected heat tolerance. The possible molecular mechanisms underlying this phenomenon were studied by detecting the interaction of TaFBA1 with other proteins.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Tobacco plant variety NC 89 (*Nicotiana tabacum* cv) was used as the wild type (WT). According to the methods described previously by Zhou et al. (2014), the transgenic tobacco plants were produced and identified. Three homoyzous transgenic tobacco lines: (OE), OE-8, OE-15, and OE-18, were selected. The WT and OE lines were grown in vermiculite under greenhouse conditions with a photoperiod of 16-h light/8-h dark and at 25°C. The germinating wheat seeds were placed on a homemade gauze that could be installed on enamel trays filled with Hoagland solution. Then the seeds were grown under a 16-h-light/8-h-dark cycle at 25°C and 70% relative humidity (light intensity 300 μmol m⁻² s⁻¹).

### Stress Treatments

After the wheat seedlings had been grown hydroponically in Hoagland solution for a week, they were subjected to 45°C heat treatment. The second leaf was harvested at 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h after 45°C treatment and then the mRNA expression levels of TaFBA1 and abiotic stress response protein 1 (TaASRP1) were detected by real-time PCR.

Eight-week-old tobacco plants were used to analyze the expression profiles of stress-related genes. The *NiFBW2* and *NtASRP1* responses to heat stress were analyzed by exposing the tobacco plants to 45°C for 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h in the light.

Eight-week-old tobacco plants were also exposed to 45°C for 6 h in the light and then their plant physiological parameters were measured. After heat treatment at 45°C for 6 h, the adult
tobacco plants were moved to normal condition (16-h-light/8-h-dark cycle at 25°C) to grow for 3 days, and then recovery plants were obtained.

Furthermore, 14-day-old tobacco plants were exposed to 45°C for 6 h to analyze reactive oxygen species (ROS) accumulation.

### Root Length Measurements
Wild type and T3 transgenic tobacco seeds were processed using 75% ethanol for 2 min and then disinfected with 3% sodium hypochlorite (NaClO) for 10 min. All the sterilized seeds were rinsed in sterilized deionized water at least five times and moved to MS plates to grow for a week. For heat stress, the plates containing the plants were exposed to 45°C for 24 h. After that, they were subjected to normal growth conditions for 5 days, while the control seedlings were moved to normal condition (16-h-light/8-h-dark cycle at 25°C) all the time. Finally, their root lengths were measured. We randomly selected 20 seedlings from each line for fresh weight and root length analysis and the experiment was performed using three biological replicates, which produced similar results.

### Chlorophyll Content, Malondialdehyde Content, Electrolyte Leakage, and Photosynthesis-Related Parameter Assays
The chlorophyll content was measured using a UV spectrophotometric method as described by Yang et al. (2009). The malondialdehyde (MDA) content was measured according to Zhou et al. (2014) and electrolyte leakage was detected according to Lutts et al. (1996).

The photosynthetic gas exchange parameters of the tobacco leaves were measured using the procedure followed by Wang B.S. et al. (2015). The maximum photochemical efficiency of PSII (Fv/Fm) was measured according to Tian et al. (2013).

### Proline and Soluble Sugar Content Determination
The proline content was determined according to Ryu et al. (2014) and the soluble sugar content was determined according to Zong et al. (2009).

### Measurement of Antioxidative Enzyme Activities and O2− and H2O2 Levels
The peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) activities were measured as described in a previous study by Wang et al. (2012).

The H2O2 accumulation was measured using 3,3’-diaminobenzidine (DAB) (Giacomelli et al., 2007). Qualitative testing of O2− was undertaken using nitroblue tetrazolium (NBT) following the method from Rao and Davis (1999). The H2O2 concentration and O2− production rate were measured according to Hui et al. (2012).

### Trypan Blue Staining Analysis Assay
The analysis of the trypan blue staining was carried out according to Koch and Slusarenko (1990).

### Immunological Analyses of HSP70 Abundance and Protein Carbonylation Assay
Eight-week-old transgenic tobacco and WT plants were subjected to 45°C heat treatment for 6 h. Then they were used in the immunological analyses of HSP70 abundance and protein carbonylation. Total protein was extracted from the tobacco leaves and its concentration was determined by a dye-binding assay (Bradford, 1976). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on 10% and 14% gradient gels were adopted to separate the proteins, and then the separated proteins were transferred to a PVDF membrane (Millipore, Saint-Quentin, France). After that, protein carbonylation was detected using the anti-dinitrophenyl (DNP) antibody (Sigma, St. Louis, MO, United States) and HSP70 proteins were detected with the HSP70 antibody (Sigma). The quantitative proteins analysis was performed on a Tanon GIS system (Tanon, Shanghai, China). The measurement of protein carbonylation levels was carried out according to Tian et al. (2014).

### Protein Interaction Assay
The yeast two-hybrid (Y2H) assays were performed as described previously (Lee et al., 2007). The complementary DNA (cDNA) fragments of Cullin were subcloned into the pGBK7 or pGADT7 vectors. The cDNA fragments of Skp1 and TaFBA1 were subcloned into the pGBK7 vector, respectively. And then they were co-transformed into the yeast strain Y2H Gold using the lithium acetate-mediated transformation method (Ito et al., 1983). Transformant candidates were selected on SD-Leu-Trp, SD-Leu-Trp-His, and SD-adenine-His-Leu-Trp media in order to evaluate the interaction between bait and prey proteins.

The BD Matchmaker library construction and screening kit (Clontech, Dalian, China) was used for the Y2H assays. The coding region of TaFBA1 was inserted into the GAL4 DNA-binding domain vector pGBK7 to generate a bait vector. The prey cDNA library was from wheat. The positive pGBK7 clones were selected on synthetic dextrose/-Leu/-Trp/-His/-Adenine/-Leu/-Trp/-His media in order to further identify the sequence. All protocols were carried out according to the manufacturer’s instructions.

For the bimolecular fluorescence complementation (BiFC) assay, TaFBA1 and TaASRP1 were respectively fused to the C terminus and the N terminus of YFP. Both constructs were coexpressed transiently in Arabidopsis leaf protoplasts via PEG transformation. The fluorescence signal was detected by confocal microscopy (Leica). The whole protocols were carried out referring to the methods in Tang et al. (2016).

### Quantitative RT-PCR Assay
qRT-PCR was carried out in a 25 ml reaction volume with Super Real Premix Plus (TIANGEN, Beijing, China). Quantitative analysis was performed using the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, United States). This method normalizes the expression of a specific gene versus a control reference with the formula 2^−ΔΔCT. In this study, the mRNA levels
for one stably expressed genes NtActin, were evaluated as control genes for qRT-PCR analyses. The information from all of the genes in the qRT-PCR experiments are listed in Supplementary Table S1.

**Statistical Analysis**
All experiments and determinations were conducted at least in triplicate. The data processing system procedures (DPS, Zhejiang University, China) were used to perform statistical analyses. Statistical significance was evaluated using a t-test at the 0.05 or 0.01 probability levels.

**RESULTS**

**Confirmation That Wheat F-Box Protein TaFBA1 Is Part of the SCF Complex**
We tested the interaction among Skp1 (GenBank: AY316293.1), Cullin (GenBank: EMS49792.1) and TaFBA1 to confirm that TaFBA1 (GenBank: JN038382.1) is the key subunit of the SCF complex. Y2H assay revealed that the three proteins could interact with each other (Figure 1). This suggested that the F-box protein coded by TaFBA1 was a subunit of the SCF complex.

**TaFBA1 Expression Pattern Responses to Heat Stress**
We investigated TaFBA1 mRNA expression using qRT-PCR to test whether TaFBA1 expression in wheat changes in response to heat stress treatments. The TaFBA1 transcription levels were analyzed in plants that had been grown under normal growth condition and 45°C heat at different time points (0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h). The results in Figure 2 illustrated that TaFBA1 expression was gradually induced by heat stress, and the highest expression level was observed after 6 h of heat treatment. This suggested that TaFBA1 may be involved in a heat responsive pathway.

**Overexpression of TaFBA1 Improved Heat Tolerance in Transgenic Tobacco Seedlings**
The transgenic tobacco lines: OE-8, OE-15, and OE-18, were used to confirm the roles of TaFBA1 in heat stress tolerance responses. Eight-week-old WT and transgenic tobacco lines were kept at 45°C for 6 h and their responses to heat stress were examined. Both the WT and transgenic plants grew well before heat treatment and had reached similar growth stages (Figures 3A,E). However, after exposure to heat stress, significant leaf withering on the WT plants was observed (Figure 3F). The root lengths of the transgenic seedlings were also longer than those of the WT (Figures 3B,D). Furthermore, the fresh weights of the WT plants were consistently lower than those of the transgenic plants (Figure 3C).

**Overexpression of TaFBA1 Improved the Growth and Photosynthetic Capacity of the Transgenic Plants Grown Under Heat Stress**
Under normal conditions, transgenic and WT plants showed no significant phenotypic differences (Figure 4A). After heat treatment, the leaves of the plants showed different degrees of withering and green color loss. Leaves of transgenic plants were less withered than those of the WT plants (Figure 4A). Accordingly, the transgenic plants had higher average chlorophyll contents (Figures 4B,C).

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**FIGURE 1** Identification of the interaction between TaFBA1 and other subunits in the Skp1-Cullin-F-box (SCF) complex by yeast two-hybrid (Y2H) analysis. The interactions between pGADT7-324 and pGBK7-332, pGADT7-322, and pGBK7-332 proteins were used as positive and negative controls. LT, LTH, and LTHA represent the selective media SD-Leu-Trp, SD-Leu-Trp-His, and SD-Leu-Trp-His-Ade, respectively.
FIGURE 2 | TaFBA1 mRNA expression responses to heat stress in wheat. The qRT-PCR analysis of TaFBA1 responses after 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h of normal (A) and 45°C (B) heat treatment by wheat that had been grown in Hoagland solution for 1 week. The data represent the mean ± SE of three biological replicates.

FIGURE 3 | Overexpression of TaFBA1 increased the heat tolerance of three transgenic tobacco lines at the seedling stage. (A,B) Growth status of single transgenic and wild type (WT) plants under normal growth conditions and after 45°C heat stress for 24 h, respectively. (C) Fresh weight of 20 seedlings. (D) Primary root lengths of the seedlings before and after heat treatment. (E,F) Phenotypes of 2-week old tobacco seedlings under normal conditions and after 45°C heat stress for 24 h. The data represent the mean ± SE of three biological replicates. *P < 0.05; **P < 0.01.
The photosynthetic system is usually susceptible to damage induced by heat. Therefore, the effects of heat stress on photosynthetic gas exchange parameters were measured. The data in Figure 4D showed that there were no significant differences in the net photosynthesis rate (Pn) between transgenic and WT plants under normal conditions. After heat treatment, Pn decreased in all lines, but this decrease was lower in the transgenic lines than in the WT plants. After a 3 days recovery period, the Pn value increased slightly, but was still lower than that for the plants grown under normal conditions. However, the Pn of the transgenic plants was higher than that of the WT plants. The results for transpiration rate (E) (Figure 4F) and stomatal conductance (Gs) (Figure 4G) were consistent with those of Pn. Similar results were also recorded for Fv/Fm (Figure 4H) and φPSII (Figure 4I). However, the change in the intercellular CO₂ concentration (Ci) showed a trend opposite to that of Pn (Figure 4E). The results shown in Figure 4 demonstrated that the photosynthetic capacity of the transgenic plants was less sensitive to heat stress than that in the WT plants.

**Overexpression of TaFBA1 Alleviated Reactive Oxygen Species (ROS) Accumulation and Heat-Induced Cell Damage**

Reactive oxygen species are usually produced with aerobic respiration, and are usually considered to be toxic molecules that cause oxidative damage to DNA, proteins, and membrane lipids in plant cells (Fang et al., 2015). The intracellular levels of H₂O₂ and O₂⁻ were analyzed by DAB and NBT staining, respectively (Figures 5A,B) to examine the ROS accumulation in both WT and transgenic plants under heat stress. Without heat treatments, both O₂⁻ and H₂O₂ accumulation was low and there were no significant differences between the WT and transgenic plants. After exposure to heat stress, the ROS accumulation level increased in all the plants. However, less H₂O₂ and O₂⁻ existed in the transgenic plants than in the WT plants. After a 3 days recovery period, ROS accumulation had slightly decreased in both the WT and transgenic lines, but was still higher than that under normal conditions (Figures 5C,D).

We used trypan blue staining to investigate the damage to cells. All plants showed similar levels of blue staining under normal growth conditions, but the WT lines showed darker blue staining than the transgenic lines under heat stress (Figure 5E). Ulteriorly, MDA accumulation and electrolyte leakage, which have been reported to be cell damage indicators (Wang G.D. et al., 2015) were assessed to verify that results. After heat treatment for 6 h, the transgenic plants had remarkably lower MDA contents and electrolyte leakage levels than the WT plants did, but there were no significant differences under normal conditions (Figures 5F,G), which suggested that the transgenic plants experienced less lipid peroxidation and were able to maintain a higher membrane stability than WT plants under heat stress.
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TaFBA1 is involved in heat

FIGURE 5 | Reactive Oxygen Species (ROS) accumulation and cell damage in the WT plants and the transgenic lines. The \( \text{H}_2\text{O}_2 \) (A) and \( \text{O}_2^- \) (B) accumulations were detected by histochemical staining using nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively. The top represents plants grown at 25°C and the bottom represents plants treated at 45°C for 24 h. Quantitative analysis of \( \text{O}_2^- \) (C) and \( \text{H}_2\text{O}_2 \) (D). (E) Trypan blue staining. The top represents plants grown at 25°C and the bottom represents plants treated at 45°C for 24 h. (F) malondialdehyde (MDA) content. (G) Electrolyte leakage. The data represent the mean ± SE of three biological replicates. \* \( P < 0.05 \); \*\* \( P < 0.01 \).

Overexpression of TaFBA1 Reduced Protein Carbonylation Levels

The role of TaFBA1 in coping with heat stress at the protein level was investigated by determining the protein carbonylation levels in the WT and transgenic plants using immunoblotting (Figures 6A,B). Both the transgenic and the WT plants performed increased carbonylated protein content after heat stress in Figure 6B. However, significantly less increase was found in the transgenic plants than in the WT plants.
Overexpression of TaFBA1 Increased Antioxidative Enzyme Levels Under Heat Stress

We determined the enzyme activities of SOD, CAT, POD, and APX. There were no obvious differences in enzyme activity levels between the WT and transgenic plants before heat stress, but heat stress changed the activity levels of these enzymes in different ways (Figures 7A–D). For example, the SOD and POD activities were significantly increased by heat stress. After a 3 days recovery period, the activity levels of these two enzymes decreased slightly but they were still higher than those under normal conditions. In contrast, the CAT activity levels were reduced by heat stress. Additionally, APX activity was slightly, but not significantly, increased by heat stress. However, over all, the transgenic plants showed higher enzyme activities than the WT plants did after exposure to heat stress.

Proline and soluble sugars are typical osmolytes that can alleviate osmotic stress induced by heat stress. During heat stress, the concentration of both these osmolytes increased in all lines studied (Figures 7E,F). However, the TaFBA1-overexpressing lines had significantly higher soluble sugar contents than the WT plants (Figure 7E). No significant increases in proline content were recorded for the transgenic lines under heat stress (Figure 7F), however, during the recovery period, both the proline and soluble sugar contents remained high (Figures 7E,F).

TaFBA1 Overexpression Altered the Expression Levels of Some Stress-Responsive Genes

The positive effect of TaFBA1 on oxidative stress tolerance suggested that TaFBA1 may be involved in the regulation of ROS homeostasis (Zhou et al., 2015). Therefore, qPCR was used to determine the expression levels of several antioxidative related genes encoding SOD, APX, POD, or CAT in the TaFBA1-overexpressing and WT plants (Figures 8A–D). Heat stress upregulated the plant transcript levels for all the analyzed genes, but the three OE lines had higher expression levels than the WT plants, particularly for the expression levels of the SOD, POD, and CAT-related genes (Figures 8A–C). Furthermore, the POD and CAT expressions were still high after the 3 days recovery period, which suggested that these two genes may play important roles in the recovery process after exposure to heat stress. The trend for NtAPX expression was consistent with the APX activity level (Figures 7C, 8D).

We then measured the expression levels of eight heat stress marker genes consisting of six HSP genes (NhHSP101, NhHSP90, NhHSP82, NhHSP70, NhHSP18.2, and NhHSP17.6) and two heat-shock transcription factor (HSF) genes (NhHSF30 and NhHSF24). The expressions of the HSP genes were significantly up-regulated under heat stress as compared with that in the WT plants, but then decreased rapidly after recovery (Figures 8J,K,M–P). The expression trend of NhHSF30 was similar to the six HSP genes (Figure 8I). After heat stress, NhHSF24 expression increased significantly in the transgenic lines, whereas its expression decreased in the WT plants. However, it increased in the WT plants during recovery (Figure 8L).

Some genes associated with proline (NtP5CS) and stress defense proteins (NtLEA5, NtERD10C, and NtERD10D) were significantly up-regulated in transgenic plants under heat stress, and then declined after recovery but their expression levels were still higher than that before treatment (Figures 8E–H).

TaFBA1 Overexpression Increased HSP70 Protein Abundance

From the data in Figure 8, we found that the expression level of NhHSP70 was increased rapidly after heat treatment and the expression level in the transgenic plants was higher than that in WT plants. To explore whether the expression of HSP70 protein had a similar trend as that of the mRNA level of NhHSP70, HSP70 protein level in leaves isolated from transgenic and WT plants were studied. Western blot analysis results revealed that HSP70 protein abundance in transgenic plants showed a significant increase as compared with that in WT plants after 6 h heat stress. (Figures 9A,B). Thus, TaFBA1 overexpression increased the HSP70 protein contents in transgenic lines under heat stress.
TaFBA1 Interacts With an Abiotic Stress Response Protein

Yeast two-hybrid assays were performed to screen for proteins that interact with TaFBA1 in wheat. The full-length cDNA of TaFBA1 was inserted into the pGBKT7 vector as bait. Positive clones were identified based on their survival on restrictive medium [synthetic dextrose (SD)/-His/-adenine (Ade)/-Trp/-Leu]. A total of 104 positive colonies survived on SD/-His/-Ade/-Trp/-Leu restrictive medium and they were then sequenced. Finally, 15 clones were obtained from Triticum aestivum (Supplementary Table S2). In the sequenced colonies, we were particularly interested in a Triticum aestivum stress responsive protein 1 (TaASRP1). The cDNA of TaASRP1 was used as prey to further confirm the interaction between TaFBA1 and TaASRP1. An interaction was found between TaFBA1 and TaASRP1 (Figure 10A). Moreover, this interaction was further confirmed by BiFC assay (Figure 10B).

We then examined the expression level of TaASRP1 in response to heat stress in wheat. The results showed that TaASRP1 expression was induced when the plants were exposed to heat for 2 h, but as the duration of heat stress, its expression was down-regulated while TaASRP1 expression level in control plants was unchangeable (Figure 10C). Furthermore, we found the homologous genes of TaFBA1 and TaASRP1 in tobacco, NtFBW2 and NtASRP1. However, different from TaASRP1 in wheat, the response trend of NtASRP1 to heat was similar to that of NtFBW2 in tobacco (Figure 11).

DISCUSSION

TaFBA1 Is a Subunit of the SCF Complex

F-box proteins are subunits of the SCF complex, which is an E3 ubiquitin ligase. The F-box motif can bind to SKP1 at the N
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The ubiquitin 26S proteasome system (UPS) can degrade non-functional proteins by ubiquitination, in which E3 ligase plays a vital role (Hare et al., 2003; Ding et al., 2015). Various stresses have been reported to induce the expression of E3 ligase genes in plants (Liu et al., 2011; Chen et al., 2013). The F-box may play an important role in the degradation process because it is the key subunit of the E3 ligase SCF complex.

Our previous studies have reported that the stress tolerance of TaFBA1-overexpressing tobacco plants had improved (Zhou et al., 2014; Zhao et al., 2017). Consistent with these findings, in the present study, we also observed that heat stress could induce the expression of the TaFBA1 gene in wheat (Figure 2). We found that leaf wilting was more severe in WT plants than in the transgenic lines after exposure to heat stress or following the recovery process in Figure 3. Furthermore, the root lengths of the transgenic lines were greater than those in WT plants, and the chlorophyll contents and photosynthetic capacity of the former were greater (Figure 4). These results
suggest that TaFBA1 is involved in the improved heat stress tolerance of plants and that its overexpression contributed to this improvement.

An Active ROS-Scavenging System May Be Involved in the Enhanced Heat Stress Tolerance Shown by Transgenic Tobacco

Various stresses, including drought, high salinity, and extreme temperature, may lead to the over-accumulation of ROS, which can cause damage to plants (Apel and Hirt, 2004; Mittler et al., 2012). Furthermore, membranes are usually the target of heat stress. Under heat stress, membranes are particularly susceptible to ROS-initiated lipid peroxidation reactions (Wang G.D. et al., 2015). The results in Figure 5 showed that ROS accumulation in the transgenic plants was less than in the WT plants after heat stress. The trypan blue staining assay revealed that the cell damage level was less serious in TaFBA1-overexpressing plants than in WT plants. Furthermore, ion leakage and the MDA content of the transgenic plants were much lower than those of WT plants (Figures 5C,D), which suggested that the improved heat resistance of the TaFBA1-overexpressing plants may be due to a stronger ability to scavenge ROS under heat stress conditions. When ROS are produced, plants usually need to take measures to scavenge ROS because their over-accumulation reduces plant growth through enzymatic and non-enzymatic systems (de Azevedo Neto et al., 2006). Therefore, the enhanced activities of the antioxidant enzymes, including SOD, POD, CAT, and APX, might be related to the improved heat tolerance shown by transgenic plants (Figure 7). However, APX activity did not increase significantly in this study (Figure 7C).

Heat stress can result in intracellular protein oxidation in plant cells. As the main product of protein oxidation, protein carbonyls often irreversibly damage proteins (Kang et al., 2016). The western blot assay showed that less carbonylated proteins were produced in transgenic plants than in WT plants, which suggested that TaFBA1 contributes to alleviating protein oxidation in the transgenic lines (Figures 6A,B).

In addition, when the transgenic and WT plants were exposed to drought, salt, and methyl viologen (MV), the activities of antioxidant enzymes were higher in transgenic plants than in WT plants after stress treatment (Zhou et al., 2014, 2015; Kong et al., 2016; Zhao et al., 2017). Based on the results presented in this paper, we speculate that antioxidants may play an important role in the responses of transgenic tobacco exposed to these abiotic stresses. In addition, it implied that this might be a common mechanism of the regulation of tolerance response to stress in plants by TaFBA1.

The Interaction of TaFBA1 With Target Proteins and Related Genes Regulation May Be Involved in the Enhanced Heat Tolerance Seen in Transgenic Plants

The molecular mechanism underlying the plant responses to abiotic stresses might be the regulation of the transcriptional activity of some stress-related genes (Zhu, 2001). The overexpression of TaFBA1 led to the induction of many stress-related genes, including NtSOD, NtPOD, NtCAT, NtLEA5, NtP5CS, NtERD10C, and NtERD10D. Figures 8A–H show that the expression levels of these genes were higher in the transgenic plants than in WT plants. However, consistent with the APX enzyme activity level, there was no obvious difference in the NtAPX expression levels between the transgenic lines and WT plants (Figure 8D). This suggested that the up-regulated expression of stress-related genes, with the exception of NtAPX, might contribute to the improved stress tolerance in transgenic plants, but the underlying mechanism needs to be explored further.

The complex network of heat-shock responses (HSRs) includes HSPs, HSFs, secondary messengers, ROS, and phytohormone signaling (Kotak et al., 2007; Mittler et al., 2012; Chao et al., 2017). HSPs and ROS-scavenging enzymes are major functional proteins that are induced by heat stress and they are well known as target genes of HS-responsive transcription factors (TFs) (Ohama et al., 2017). HSPs, including HSP100, HSP90, HSP70, HSP60, and small HSPs, function as molecular chaperones and are induced in HSRs (Kotak et al., 2007; Qu et al., 2013). In the present study, we investigated the expression levels of HSR-related genes, including the HSP genes: NtHSP101, NtHSP90, NtHSP82, NtHSP70, NtHSP18.2, and NtHSP17.6, and the HSF genes: NtHSF30, and NtHSF24 (Figure 8). The results indicated that the induction of NtHSP101, NtHSP90, NtHSP82, NtHSP70, NtHSP18.2, NtHSP17.6, NtHSP30 and NtHSF24 were more significant in transgenic plants than in WT plants (Figures 8I,J,L, M–P). HSP70 is a widely studied HSP (Hahn et al., 2011). In the present study, the abundance of HSP70 differed significantly between the transgenic lines and the WT
TaFBA1 is Involved in Heat Stress Response

**FIGURE 10** Interaction between TaFBA1 and a deducitive stress response protein (named TaASRP1), and the response of TaASRP1 mRNA expression to heat stress in wheat. (A) Y2H analysis of the TaFBA1 and TaASRP1 interaction. The interaction between pGADT7-324 and pGBKT7-332, and pGADT7-322 and pGBK7-332 proteins were used as a positive control and negative control, respectively. LT, LH, and LTH represent the selective media SD-Leu-Trp, SD-Leu-Trp-His, and SD-Leu-Trp-His-Ade, respectively. (B) Confirmation of the interaction of TaFBA1 and TaASRP1 by BiFC in Arabidopsis protoplasts as shown by a yellow fluorescence signal. nYFP, the construct for the YFP N-terminal fusion expression. cYFP, the construct for the YFP C-terminal fusion expression. Bars = 20 mm. Real time-qPCR analysis of wheat TaASRP1 mRNA expression responses after 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h of normal (C) and 45°C (D) heat treatment. The data represent the mean ± SE of three biological replicates.

**FIGURE 11** The response of homologous genes in tobacco to heat. (B) and (D) Real time-qPCR analysis of tobacco NtFBW2 and NtASRP1 mRNA expression responses after 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h of normal (A) and 45°C (C) heat treatment, respectively. The data represent the mean ± SE of three biological replicates.

Plants (Figure 9). In addition, these results suggested that NtHSP70 might play an important role in heat stress tolerance in transgenic tobacco. Moreover, the expression of HSF24 was different between the transgenic and WT plants, indicating the possibility that there is a relationship between TaFBA1 and HSFs. This needs to be studied further.
In the SCF complex, which is a key subunit that targets downstream substrates, the F-box may play a vital role in interactions with proteins. In this study, we carried out a yeast library screening analysis in order to elucidate the possible molecular mechanism underlying the enhancement of tolerance in transgenic plants under heat stress. Among the 15 positive clones acquired from the library screening experiment, we obtained a *Triticum aestivum* stress responsive protein 1 (TaASRP1), and Liu et al. (2015) reported that TaASRP1 response to heat stress. Interested in the function of this protein, we selected it to carry out the next experiments. Both Y2H assay and BiFC assay confirmed the interaction between TaFBA1 and TaASRP1 proteins (Figures 10A,B). The qRT-PCR analysis indicated that TaASRP1 expression was down-regulated after heat treatment (Figure 10C). Therefore, we hypothesized that TaFBA1 regulation of TaASRP1 may be important in heat stress tolerance. The enhanced heat tolerance in TaFBA1-overexpressing plants may be caused by the regulation of TaASRP1 expression. When exposed to continuous heat treatment, the expression level of TaASRP1 showed a decreasing trend (Figure 10). But under heat treatment, the response of *NtASRP1*, homologous gene of TaASRP1 in tobacco, showed opposite trend to TaASRP1 (Figure 10). Based on this result, we speculated that there might be a relationship between TaFBA1 and TaASRP1 in the response to heat stress. Homologous gene mutants of TaFBA1 in *Arabidopsis* could be available for carrying out future studies. In the previous study, we got an *Arabidopsis* homologous gene of TaFBA1 mutant, *atfbw2-4* (Zhao et al., 2017). In addition, we could obtain the information on this relationship by transforming TaASRP1 to homologous mutants *atfbw2-4*. Moreover, TaFBA1 functions as a member of E3 ubiquitin ligase complex, which was related to the degradation of some proteins. Therefore, further studies on whether TaFBA1 degrades TaASRP1 in wheat, mediated by the UPS, are necessary. As for the different response trend to heat between TaASRP1 and *NtASRP1*, we speculated that the difference in genetic background between monocot wheat and dicot tobacco might be responsible for the response difference to heat at transcript level.

**CONCLUSION**

The findings of the present study showed that the overexpression of TaFBA1 improved the heat stress tolerance of transgenic tobacco. The improvement in the ROS-scavenging system through the regulation of stress-responsive gene expression might have contributed to the enhanced stress tolerance shown by the transgenic plants. The expression of HSR related genes was also involved in the regulation of heat stress tolerance in the transgenic lines. The wheat F-box protein, TaFBA1, regulated stress tolerance by interacting with stress-related proteins.

**AUTHOR CONTRIBUTIONS**

QL designed the experiments and performed the experiments. WenqW, WenlW, GZ, YL, and YW provided assistance with the experiments. WeiW provided vital advice on the article. QL wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00521/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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