TaGST1 Gene and Its Regulatory Factor WRKY74 Mediate Copper Tolerance by Effecting Glutathione Accumulation in Wheat

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Research

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Abstract

Background: Copper (Cu) is an important plant micronutrient; however, excessive Cu can disturb the protein structure, affect plant growth and development, and pose as a potential human health risk. Glutathione S-transferase (GST) is the key enzyme in glutathione (GSH) synthesis; it plays crucial role in Cu detoxification. Nonetheless, its regulatory mechanisms remain largely unclear.

Results: A Cu-induced *TaGST1* gene had been identified in this study. And the regulatory factor TaWRKY74 had been screen out by Yeast one-hybrid (Y1H) method. Their binding were checked by using another Y1H and luciferase (LUC) assays, indicated that TaWRKY74 bound to the *TaGST1* promoter via W-box sequence. Moreover, *TaWRKY74* or *TaGST1* expression, GST activity, and GSH content were significantly inhibited in transiently TaWRKY74-silenced wheat plants under Cu stress. However, the contents of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and Cu were significantly increased. Further investigation found that transiently ectopic overexpression of *TaWRKY74* increased GSH content, whereas decreased MDA content during Cu stress. Notably, exogenous application of GSH could reversed the adverse effects of transiently TaWRKY74-silenced wheat plants during Cu stress.

Conclusions: These results indicated that TaWRKY74 regulated *TaGST1* expression and affected GSH synthesis under Cu stress, and could be useful to ameliorate Cu toxicity for crop food safety.

Background

With the rapid development of modern industry and agriculture, heavy metal contamination has become a severe worldwide problem as a result of anthropogenic activities [1]. Particularly, although copper (Cu) is among the most toxic heavy metals, it is also vital for plant growth and development due to its important role in various proteins and enzymes required by plants to perform essential metabolic functions such as photosynthesis, respiration, redox reactions, and cell wall remodeling [2–4]. The anthropogenic factors including mining, tanning, refining, manures, fertilizersties have caused excessive soil Cu, which can lead to toxicity and stunt plant growth. And thus, it represents a significant threat to agricultural product, quality, and safety, as well as to human and animal health [5–8]. Therefore, a deeper understanding of the tolerance of Cu on crops is crucial to ensure agricultural product safety.

Although some phenotypic and physiological effects of Cu stress have been characterized [9, 10], its toxic effects on regulatory mechanisms are poorly understood at the molecular level, especially in agricultural crops. Thus, it is urgent to understand the regulatory mechanisms of Cu toxicity in order to develop crops with higher Cu tolerance. Plants have evolved effective Cu tolerance mechanisms, including compartmentalization, exclusion, sequestration, chelation, biotransformation, and repair to mitigate Cu phytotoxicity [11, 12]; Additionally, a series of studies involving the physiological and biochemical responses of Cu tolerance mechanisms have been conducted to improve the efficiency of phytoemediation approaches [9, 10, 13–15]. Previous studies on the molecular responses to Cu in plants have focused on the discovery and functional analysis of some stress-related amino acids, resistance

proteins or genes, and signalling molecules [16–19]. Moreover, rapidly changing tolerance proteins are reported fundamental for the interpretation of the molecular mechanisms related to environmental stress resistance [1, 19]. Therefore, enzymatic and nonenzymatic molecular mechanisms play important roles in the plant responses to Cu stress.

Enzymatic (catalase, CAT; peroxidase, POD; superoxide dismutase, SOD; etc) and nonenzymatic (glutathione, GSH; ascorbate, ASA; etc) immune response mechanisms have been identified as heavy metal chelators for Cu detoxification [19–21]. Glutathione S-transferases (GSTs) play a vital role in nonenzymatic detoxification and catalyzes the conjugation GSH to xenobiotic substrates [22–24]. Moreover, in the reactive oxygen system (ROS)-scavenging system, GST also catalyzes the interaction between GSH and hydrogen peroxide to regulate the perception and tolerance to various abiotic stressors in plants [25–27]. Ectopic overexpression of *GmGSTU4* or *PtGSTF4* resulted in higher catalytic activity towards xenobiotics for detoxification [28, 29]. Four *BcGSTU* genes were significantly induced under Cd stress in pak choi [30], and our previous proteomic data showed that GST proteins were also upregulated under Cu stress condition [17], inferring that GSTs could involve plant response to metal stresses. To our knowledge, however, the regulatory mechanisms of the *GST* gene in response to heavy metal stress, especially for Cu, are still poorly understood.

Transcription factors, which bind to the *cis*-acting elements of the promoters of the target genes and then activate or inhibit their expression, participate in transcriptional regulation processes of heavy metal tolerance in various higher plant species. WRKY transcription factors, which are characterized by diagnostic WRKY domains, specifically recognize and bind with the [(T/C)TGAC(T/C)] sequences (W-box, *cis*-acting elements) on the promoters of its target genes [31]. For instance, AtWRKY47 directly regulates the expression of ELP and XTH17 responsible for cell wall modification under Al stress [32]; and OsWRKY22 positively controls the expression of *OsFRDL4 via* W-box *cis*-acting elements on its promoter under Al stress conditions in rice [33]. Additionally, AtWRKY12 negatively regulates cadmium tolerance by repressing GSH1 expression [34], while AtWRKY13 positively mediates cadmium tolerance by activating the expression of *AtPDR8* [35]. Furthermore, CaWRKY41 coordinates with H_2O_2 in response to excess cadmium stress [36]. However, the regulation mechanism underlying the WRKY-induced tolerance to Cu stress remains unclear.

Wheat is among the most important crops in the world, and it is very sensitive to Cu stress [37]. To circumvent the increasing demand for wheat yields due to the ever-increasing world population, and the decreasing pressure for environmental pollution, it is pivotal to understand the molecular mechanism of Cu stress, and develop wheat cultivars with enhanced Cu tolerance. In this study, we found that the transcripts of *TaGST1* was induced, and one transcription factor-TaWRKY74, which regulated the *TaGST1* expression by binding the W-box *cis*-element sequence on *TaGST1* promoter, was identified via Y1H and LUC assays. BSMV-VIGS experiment demonstrated that TaWRKY74 participated Cu tolerance through regulating *TaGST1* expression, and affecting GST activity and GSH content, whilst exogenous application of GSH reversed the adverse effects of Cu stress; and transiently ectopic overexpression further

suggested the role of *TaWRKY74* in wheat Cu stress response. Our results provided some novel insights into signal transduction pathway on Cu stress in higher plants.

Materials And Methods

Plant materials and treatments

Wheat seeds (*Triticum aestivum* L. cv. Bainong 207) were surface-sterilized with 0.01% $HgCl_2$ and washed thoroughly with the distilled water, and subsequently germinated in the dark at 25 °C for 24 h. Germinated seeds were transferred to glass dishes and cultivated with water in a growth chamber as previously described in our published literature [17]. Afterward, two-week-old wheat seedlings were selected and divided into different groups to be treated with Cu (50 μM) [17], aluminum (Al, 50 μM) [27], and cadmium (Cd, 50 μM) [30] for 3 days. Control wheat seedlings were treated with Hoagland solution only. All solutions were exchanged every 24 h.

RNA isolation and qPCR

Total RNA was extracted with the TRIzol RNA Isolation Reagent (Invitrogen, USA), the extracted RNA was then quantified by ND-1000 NanoDrop spectrophotometry (Wilmington, USA), and cDNA was prepared with the First Strand cDNA synthesis kit (Toyobo, Japan). Afterward, qPCR was performed in a QuantStudio 3 (Thermo, USA) system with the following reaction conditions: one cycle at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 10 s, 58~60 °C for 15 s, and finally 72 °C for 20 s. And the relative expression levels of the genes were calculated by using the $2^{-\Delta\Delta CT}$ method [38]. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as endogenous control gene. All used primers are listed in Table S1.

Y1H assay

Genomic DNA was isolated from wheat leaves by using the TRIzol reagent as described in our previous method [33]. Then, the promoter sequence, 1,511 bp upstream of the translational start codon of the *TaGST1* gene (AJ414697.1), was retrieved from the International Wheat Genome Sequencing Consortium (IWGSC) database and used to design the primer pairs (Table S1). The cDNA library was generated from leaves of 3-days-Cu-stressed wheat seedlings by using SMART cDNA synthesis technology (TaKaRa Biotechnology, China). The *TaGST1* promoter sequence was inserted into the pAbAi vector (Clontech, USA) to construct a bait vector (pAbAi-TaGST1 promoter, pAbAi-P1), which was then linearized and integrated into the genome of the Y1HGold yeast strain. Suitable bait strain colonies, which were identified using specific primers (Table S1), were transformed with the Cu-stressed leaf cDNA library (1 μg) according to the manufacturer's instructions. Next, to isolate the interacting proteins with the pAbAi-TaGST1 promoter bait vector in the Y1H screening, the yeast colonies from the combined bait vector

cDNA library was amplified via PCR with the specific primers listed in Table S1 and then sequenced. The resulting sequences of yeast colonies were searched in the wheat genome database to identify their homologies via the BLAST algorithm.

Another Y1H assay was performed to further identify the interaction between the screened proteins and bait vectors. CDS sequence of TaWRKY74 was cloned and ligated into the pGADT7 vector to produce the pGADT7-TaWRKY74 prey vector. Five fragments (P1, 1,511 bp; P2, 989 bp; P3, 569 bp; P4, 246 bp; P5, 191 bp) of the TaGST1 promoter, which contained different numbers of W-box elements, were separately linked into the pAbAi vector to form different bait vectors. Finally, a cyclic depsipeptide antibiotic (aureobasidin A, AbA), which acted as a selection marker, was used for our Y1H assay.

Transactivation activity assay

The CDS of TaWRKY74 was cloned and inserted into the pCAMBIA1300 vector, which driven by the 35S promoter, to construct the 35S-TaWRKY74 effector vector. The *TaGST1* promoter was amplified and linked into the pGreenII0800-LUC vector, which encodes both reniferase (REN) and luciferase (LUC)[39], to form the reporter vector (LUC-TaGST1 promoter). Then, the various strains, which contained the effector or reporter vectors, were injected into tobacco leaf as described by Ding and his colleagues [39]. The Dual-Luciferase reporter assay system (Promega, USA) was used to determine the activity of LUC, according to the manufacturer's instructions.

Phylogenetic tree construction

The NLS region of TaWRKY74 was predicted by using ScanProsite (http://prosite.expasy.org/scanprosite/). The TaWRKY74 phylogenetic tree was constructed with the rice and *Triticum* WRKY proteins by using MEGA 5.0 software (MEGA Inc., USA).

Subcellular localization and transcription activation

The subcellular localization and transcriptional activation of TaWRKY74 were determined as previously described [33]. The CDS of TaWRKY74, which did not contain the stop codon, was isolated and linked to the upstream of green fluorescent protein (GFP) gene, its coding region was located on the vector of pBluescript,to construct a TaWRKY74-GFP fusion protein. Afterward, the TaWRKY74 fusion protein was transformed into *Agrobacterium* (GV3101) through the positive strains, and then injected into the leaf epidermal cells of tobacco seedlings. TaWRKY74 localization was observed via confocal laser scanning LSM710 microscopy (Karl Zeiss, Germany). TaWRKY74 was also inserted into the pGBKT7 vector to construct a fusion protein (pGBKT7-TaWRKY74). Then, the recombinant vector was transformed into the Y2HGold yeast strain and grown in tryptophan-enriched synthetic defined (SD/−Trp) selective medium with X-α-gal, at 30 °C for 2–4 days; an empty vector (pGBKT7) was served as negative control.

BSMV-VIGS-mediated gene silencing

One fragment (207 bp, +48 ~ + 255 bp) of TaWRKY74 was amplified and linked into the BSMV-γ vector to form a BSMV-TaWRKY74 vector; BSMV-GFP was used as a control. This experimental procedure to transiently silence TaWRKY74 expression in wheat leaves as previously described in our lab. After inoculation for 8 days, the phenotype of inoculated wheat seedlings was observed, and the leaves were collected for RNA extraction. Afterward, the wheat seedlings inoculated by the BSMV-TaWRKY74 or BSMV-GFP vectors were separately transferred to full-length Hoagland solution (Control, -Cu), Hoagland solution supplemented with 100 μM $CuSO_4$ for 10 days (Cu stress, +Cu), 100 μM $CuSO_4$ with 100 μM exogenous GSH (+Cu + GSH), and 100 μM $CuSO_4$ with exogenous GSH and its inhibitor BSO of 100 μM (B2515, Sigma, USA) (+Cu + GSH+BSO). Leaves and roots were then collected for the determination of several parameters.

Plant growth and Cu content determination

Plant phenotype was monitored and plant biomass was measured as previously described [17]. Sampled leaves and roots were dried, then separately digested, and contents of Cu, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), manganese (Mn), and zinc (Zn) were determined by using Thermo Scientific AAS (ICE 3300, USA)[40].

Measurement of H_2O_2, MDA, and GSH contents, and GST activity

In this study, 0.5% trichloroacetic acid (TCA) was used to homogenize fresh samples (0.2 g) at 4 °C. The homogenized samples were centrifuged at 8,000 g for 10 min at 4 °C. Then, the supernatants were stored at 4 °C and used for MDA and GSH content assays. The contents of MDA and GSH were measured according to our previous study [41]. Briefly, the sample supernatants (1 mL) that were extracted with 0.5% TCA were combined with 0.5% 4 mL TCA. Then, the mixtures were heated at 100 °C for 30 min, and then, immediately cooled on ice. Absorbance of the supernatants was then measured at 450, 532, and 600 nm for MDA content. And for GSH content, 0.5 mL of supernatant extracted from 0.5% TCA was combined with 2.5 mL NaH_2PO_4 (50 mM, PH=7.7) and 0.5 mL of 5'5'-dithiobis-2-nitrobenzoic acid (DTNB, dissolved in PBS buffer). The mixture was then incubated at 30 °C for 5 min, after which the absorbance was measured at 412 nm.

GST activity and H_2O_2 content of wheat plants were determined using GST [42] and H_2O_2 [43] Assay kits (Jiancheng Bioengineering Institute, China), respectively. Briefly, fresh samples (0.2 g) were homogenized with a phosphate buffer solution (PBS, pH=7.4) containing NaH_2PO_4, Na_2HPO_4, KCl, and NaCl, and then incubated in ice for 30 min. The homogenates were centrifuged at 5,000 *g* at 4 °C for 15 min. The supernatants were stored at 4 °C and performed for the assays of the GST activity and H_2O_2 content.

Transient overexpression analysis in tobacco leaf

To confirm the function of TaWRKY74 under Cu tolerance, the transient over-expression experiment was performed as previous study [44]. After infiltrated for 2 days, 50 μM Cu or distilled water (control) was injected into left or right of one tobacco leaf, which harboring TaWRKY74, respectively. Then, the infiltrated leaves were collected for determination of GSH and MDA contents, and gene expression.

Statistical analysis

The mean and standard deviation (SD) of each treatment were calculated from at least three biological replicates. The SPSS software (version 17.0) was used for statistical analyses, and statistically significant differences between the treatments were tested via Duncan's test with a significance threshold of $P < 0.05$.

Results

Expression of *TaGST1* in wheat plants suffering from Cu stress

Transcripts of the *TaGST1* gene have been measured in wheat roots and leaves, and it was observed at higher expression levels in the leaves than in roots (Fig. 1A). Thus, *TaGST1* expression levels were further measured in wheat leaves. Under different Cu concentrations, the transcripts of the *TaGST1* gene were found to be significantly induced at Cu concentrations of 50 or 100 μM (Fig. 1B). It has been reported that induced genes or proteins could play important roles in plant responses to abiotic stresses [17], demonstrating that TaGST1 could function in the Cu stress response, especially at higher Cu concentrations.

Identification of the regulators binding to the *TaGST1* promoter

To explore the regulatory mechanisms of *TaGST1* under Cu stress, its promoter was cloned (Fig. S1), and then was used for Y1H screening (Fig. S2). Upon screening, positive clones were obtained and subsequently sequenced, and 28 putative proteins were identified and functionally annotated (Table S2). Among these regulators, two WRKY transcription factors have been identified. *TaWRKY74* (GenBank accession number: EF368359.1), a WRKY transcription factor, exhibited the higher homology relative to *AetWRKY6* in *Aegilops* (Table S3), and its sequence had a 98.9% similarity to Chinese Spring (CS) (Fig. S3). Thus, the TaWRKY74 transcription factor was used in following analyses.

To confirm the regulatory relationship between TaWRKY74 and TaGST1, the Y1H assay was performed using the pGADT7-TaWRKY74 and pAbAi-TaGST1 promoters. There were eight W-box core elements in the TaGST1 promoter (Fig. S1). Full-length or partially-deleted TaGST1 promoter fragments containing different W-boxes, i.e., P1 (eight W-boxes), P2 (five W-boxes), P3 (three W-boxes), P4 (three W-boxes), and P5 (one W-box) (Fig. 2A), were separately transformed into the Y1HGold yeast strain. Then, four AbA concentrations (0, 100, 150, and 200 ng/mL) were added to test the self-activation of these promoter fragments, thereby identifying positive strains (Fig. 2B). Under different AbA concentrations, the yeast strains harboring the P1, P2, and P3 fragments exhibited similar growth to those harboring the P4 and P5 fragments, which grew worse under 100~200 ng/mL AbA addition (Fig. 2B). This suggested that the former fragments had a stronger self-activation capacity than P4 and P5; therefore, P4 and P5 were better suited for further Y1H assays. Thus, the prey vector pGADT7-TaWRKY74 was transformed into Y1HGold strains containing the P4 and P5 fragments, respectively. In the presence of 100 ng/mL AbA, the P4 and P5 fragment yeast stains were activated by the TaWRKY74 prey vector (Fig. 2C and 2D), suggesting that the TaWRKY74 transcription factor could bind to the TaGST1 promoter.

Binding between TaWRKY74 and TaGST1 promoter via W-box

LUC assay was used to explore whether TaWRKY74 affected the transcriptional activity of the TaGST1 promoter. LUC reporter vectors were constructed using two different TaGST1 promoters, which contained normal or mutant W-box *cis*-element (Fig. 3A and B). In addition, effector vectors of 35S-TaWRKY74-GFP or 35S-GFP were also constructed (Fig. 3B). Compared to the 35S-GFP vector (control), the ratio of LUC/REN in the P5 fragment of the *TaGST1* promoter was upregulated by 2.04-fold, while this ratio was insignificantly changed in the mutated P5 fragment (Fig. 3B and 3C). These suggested that TaWRKY74 interacted with *TaGST1* promoter via the W-box.

Characterization and phylogenetic tree of TaWRKY74

To characterize TaWRKY74 in wheat, a blast search was performed in the IWGSC database (http://www.wheatgenome.org/) and TaWRKY74 was found to possess the highest similarity with TraesCS5D02G190800 (Fig. S4), suggesting that it localizated on 5D chromosome. Sequence analysis showed that TaWRKY74 contained two exons, one intron, and encoded a putative protein with 351-amino acids. It possessed a highly conserved WRKYGQK sequence and a C2HC zinc finger motif (Fig. S5), suggesting that it belonged to group III of the WRKY family. Phylogenetic analyses indicated that *TaWRKY74* had high similarities (≥ 85%) to AetWRKY6, TaWRKY, TaWRKY5, and OsWRKY30 or OsWRKY74 (Fig. S6). However, their functions in responses to metal stresses have not been identified.

Subcellular localization and transcriptional activity of TaWRKY74

In order to measure the TaWRKY74 localization in cells, its CDS was fused with a vector, which driven by a 35S promoter and contained a GFP-encoding gene, to produce TaWRKY74-GFP protein (Fig. 4A). TaWRKY74-GFP was then transiently introduced into leaf cells of tobacco plants. The fluorescence signal of TaWRKY74-GFP was specifically observed in the nucleus compared with the control (GFP protein), which was localized in both the nucleus and cytoplasm (Fig. 4B), suggesting that TaWRKY74 was a nucleus-localized protein.

To detect the transactivation activity of TaWRKY74, its CDS was inserted into the pGBKT7 vector to construct a pGBKT7-TaWRKY74 fusion protein (Fig. 4C). Afterward, the construct was transformed into the Y2HGold yeast strain in SD medium without tryptophan (SD/-Trp). All yeast cells grew well in the SD/-Trp medium at 30 °C for 2~3 days without X-*α*-gal. However, when X-*α*-gal was added in this medium, TaWRKY74 and the positive control (pGBKT7-P53) turned blue, whereas the negative control (pGBKT7-Lam) remained unchanged (Fig. 4D). This indicated that TaWRKY74 possessed the potential of transcriptional activation.

Expression of *TaWRKY74* under metal stresses

To determine whether the *TaWRKY74* gene responded to different metal stresses, transcripts of *TaWRKY74* were determined in leaves of wheat plants suffering from 50 μM Cu, 50 μM Cd, and 50 μM Al. qPCR results indicated that transcripts of *TaWRKY74* were significantly induced by 27.64-, 12.54-, and 11.26-fold under Cu, Cd, and Al stresses, respectively (Fig. 5). Based on these results, it was speculated that the *TaWRKY74* gene could function in metal-induced stresses, especially upon Cu exposure.

Function of TaWRKY74 in response to Cu stress

BSMV-VIGS experiment was used to demonstrate the role of TaWRKY74 for Cu tolerance. A 207 bp cDNA fragment, which shared high similarity (99.7%) among three copies (Fig. S7), was selected and constructed into BSMV-derived vectors to simultaneously silence three copies of the *TaWRKY74* gene in wheat genome. Wheat plants inoculated with the BSMV-TaWRKY74 or BSMV-GFP (control) vectors exhibited visible photobleaching phenotype at 8 days after inoculation (Fig. S8A). Moreover, the TaWRKY74 transcription level remarkably decreased (by 43%) in leaves of BSMVS-TaWRKY74-inoculated wheat plants compared to the controls (Fig. S8B), suggesting that the *TaWRKY74* gene was successfully silenced in wheat plants. The *TaGST1* transcript levels also were decreased by 61% in TaWRKY74-silenced wheat plants (Fig. S8C). These results suggested that the expression of *TaGST1* could be controlled by the TaWRKY74.

Subsequently, BSMV-TaWRKY74- and BSMV-GFP-inoculated wheat plants were separately transferred to Hoagland solutions supplemented with or without 100 μM Cu. After 10-d stress, the BSMV-VIGS-TaWRKY74-inoculated wheat plants exhibited more deleterious phenotypes (e.g., curled and wilted leaves, and stunted growth), compared to the BSMV-GFP-inoculated wheat plants (Fig. 6A). The transcripts of *TaWRKY74* and *TaGST1* were also significantly decreased in BSMV-TaWRKY74-inoculated wheat plants (Fig. 6B and C). Similar to *TaGST1* expression, the GST activity and GSH content were also significantly decreased in BSMV-TaWRKY74- inoculated wheat plants (Fig. 6D and E). The biomass of the BSMV-TaWRKY74-inoculated wheat plants were also decreased, especially at Cu stress conditions (Fig. 7A), whereas both H_2O_2 and MDA contents were significantly increased in these wheat plants (Fig. 7B and C). Additionally, Cu content dramatically increased in root and shoot tissues of the BSMV-VIGS-TaWRKY74-inoculated wheat plants (Fig. 7D). These results showed that TaWRKY74 could be involved in Cu tolerance of wheat plants, by regulating expression of *TaGST1*, which could catalyze the interaction of GSH with H_2O_2.

To further explore the Cu tolerance of BSMV-VIGS-TaWRKY74-inoculated wheat plants, contents of other nutrient elements were also measured in above plants. Similar with Cu content, the translocation factor of Cu, which was calculated using Cu content and plant biomass, was significantly also increased to 1.76-fold in these plants (Fig. S9), suggesting that more Cu accumulation in BSMV-VIGS-TaWRKY74-inoculated plants. And the translocation factors of other nutrients, K (1.59-fold), Mn (1.48-fold), Ca (1.25-fold), and Fe (1.17-fold) were similar with Cu between these two inoculated wheat plants (Fig. S9), whilst B (0.47-fold), Mo (0.89-fold) , and Al (0.71-fold) were opposed with Cu (Fig. S9), speculating that Cu accumulation would lead to the imbalance of other nutrient elements in wheat plants.

Transiently ectopic expression assay was performed to obtain *TaWRKY74* over-expressed tobacco plants, in which this wheat gene was expressed at high level in tobacco plants. Under 50 μM Cu stress, the expression of TaWRKY74 was rapidly induced and peaked at 6 h, and then declined (Fig. 8A); GSH content increased by 22.5% , while MDA content decreased by 23.2 % at 6 h in *TaWRKY74* transient over-expressed plants (Fig. 8B and C). These data further suggested the role of *TaWRKY74* in Cu stress response *via* GSH metabolism.

Effect of exogenous GSH application on Cu tolerance of wheat seedling

To determine the effect of exogenous GSH supplement on Cu toxicity. Both BSMV-inoculated wheat plants were separately transferred to Control, Cu stress, Cu + GSH, and Cu+GSH+BSO mediums. Under Cu stress, exogenous GSH application significantly alleviated Cu-stress-injured morphology in both BSMV-TaWRKY74- and BSMV-GFP-inoculated wheat plants, whereas the above phenotype was broken by exogenous application with BSO (an inhibitor of GSH biosynthesis) under Cu + GSH treatment (Figs. 9A and S10). These phenotypic results were further confirmed by quantitative analysis with the remarkably changed of plants biomass, MDA and H_2O_2 contents (Fig. 9B-D). In addition, the application of

exogenous GSH markedly decreased the GST activity, GSH contents, and Cu contents in both the BSMV-TaWRKY74- and BSMV-GFP-inoculated wheat plants after Cu stress for 10 days (Fig.9E-G).

Discussion

Due to the rapid development of modern industry and agriculture, Cu has caused serious and adverse outcomes not only due to their direct toxic effects on living organisms, but also because this could affect food crop quality and safety [2, 45]. It has been acted as a widespread abiotic stress or typically associated with agriculture and other human activities [46]. Many reports have focused on the physiological characteristics of crops under Cu stress. Excessive Cu inhibits plant growth and induces ROS production, which subsequently damages plant cells [47–50].

Plants have developed antioxidant defense systems to combat ROS accumulation, which include enzymatic and nonenzymatic components such as POD, ASA, and GSH, in response to different stressors. GSTs are known to catalyze the conjugation of xenobiotics with GSH, which is an important mechanism of metal detoxification [45, 51, 52]. It has been reported that GSTs are correlated with Cu stress in several crops, including wheat and rice [17, 20, 30, 47, 48]. Abundance of GST1 proteins has been found to be quickly induced by Cu stress in our previous proteomic research [17]. In this study, the expression of *TaGST1* was also significantly induced at transcription level under Cu stress in wheat (Fig. 1). The rapid changes in stress-tolerant genes (proteins) are fundamental for the interpretation of the molecular mechanisms related to different stress tolerances [1, 19]. Therefore, it has been speculated that *TaGST1* could be a strong mediator of Cu tolerance in wheat. Plant stress responses are modulated not only by the stress-related functional genes (proteins) but also by their upstream regulators. Transcriptional regulation largely from transcription factors, which specifically regulate expression of the targeted gene by binding to *cis*-elements preferentially located in the promoter region of functional genes, has the vital roles in signaling pathways [53]. To our knowledge, nevertheless, the transcriptional regulatory mechanisms of GSTs in Cu tolerance in higher plants have been explored rarely.

Multiple members of GSTs have been identified in several plants, and their expression levels are induced by various biotic or abiotic stresses and different biological metabolism processes [17, 41, 54, 55]. Such as, tomato *GSTU43* gene is involved in the redox homeostasis of ALA and the synthesis of chlorophyll under low temperature stress [56]; Overexpression of *PtGSTF4* gene in *Arabidopsis* improved the resistance of salt and drought stresses [29]. Moreover, GST genes in strawberry or apple have been identified and regulated by the upstream transcription factor FvMYB10 or MdMYB1, respectively [55]. As well as other plants, 330 GSTs members were detected in wheat [57]. To our knowledge, however, the upstream transcription regulatory mechanism of GSTs in wheat, especially under Cu stress, has not been reported. Through Y1H screening, in this study, TaWRKY74 transcription factor has been identified to directly bind to the *cis*-acting elements (W-boxes) on *TaGST1* promoter, and activate its transcription (Figs. 2, 3, and S5). These findings provided a new understanding of TaGST1, which was regulated by upstream transcription regulatory-TaWRKY74.

The WRKY transcription factors, the largest families of plants transcription factors, have been reported to play crucial roles by regulating the downstream target gene expression in response to various metal stresses. Under Al stress, for instance, OsWRKY22 in rice, WRKY46 and WRKY47 in *Arabidopsis*, were participated in Al tolerance by regulating the related genes expression [32, 33, 39]. Additionally, *ZmWRKY4* in *Zea mays*, *WRKY53* in *Thlaspi caerulescens*, *ThWRKY7* in *Tamarix hispida*, and *WRKY12* and *WRKY13* in *Arabidopsis* have been reported to respond to Cd stress via different pathways[35, 58–60]. To our knowledge, however, the role of TaWRKY74 on participating in metal stresses has not been reported to date. Although it sequence had high similarities to AetWRKY6, TaWRKY, and TaWRKY5 in *Triticum* or OsWRKY30 in rice, their functions in responses to metal stresses have not been identified. Similar to other plant WRKY transcription factors (32, 33, 36, 39), TaWRKY74 was more markedly induced by Cu stress compare to Al and Cd stress (Fig. 5), suggesting that the TaWRKY74 transcription factor plays more key roles in the response to Cu stress in wheat.

To further confirm the role of TaWRKY74 in the wheat Cu-induced stress response, we performed transient silencing or ectopic expression of TaWRKY74 in wheat or tobacco plants. *TaWRKY74* and *TaGST1* expression levels, GST activity, and GSH content significantly decreased, while the contents of $H_2O_{2,}$ MDA, and Cu remarkably increased in BSMV-TaWRKY74-inoculated wheat plants (Figs. 6 and 7). Similarly, contents of GSH and MDA were separately induced or inhibited in transiently over-expression of *TaWRKY74* in tobacco plants during Cu stress (Fig. 8). Different with to Cd stress [61], the Cu tolerance of wheat plants was also negatively correlated with its accumulation, which had higher translocation factors in the BSMV-TaWRKY74-inoculated wheat plants (Fig. S9). And the changes of other nutrient elements could be attributed to further underlying the Cu tolerance (Fig. S9), which had been reported that graphene atred the imbalance of nutrient homeostasis in wheat plants [40]. These results suggested that TaWRKY74 participated in wheat tolerance to Cu stress through GSH-mediated antioxidant system.

It is also known that exogenous GSH was a beneficial regulator involved in response to environmental and abiotic stresses in higher plants [62]. Such as, under Cd stress conditions, exogenous GSH application significantly ameliorated Cd-induced oxidative stress and alleviated Cd toxicity in maize, tomato, and *populus*, respectively [62–64]. In this study, exogenous GSH greatly increased the wheat tolerance to Cu stress in both BSMV-GFP- or BSMV-TaWRKY74-inoculated wheat seedlings (Figs. 9 and S10), suggested that TaWRKY74 played an important role in GSH-mediated Cu detoxification in wheat plants. And these results are similar with the molecular regulatory mechanisms of GSH or GST under Cd stress, low-temperature stress, or in anthocyanin accumulation [34, 45, 55]. However, our results focused on their regulatory mechanisms in response to Cu stress, and these findings improved our understanding of *TaGST1* regulation in higher plants under Cu stress, especially during GSH-mediated Cu detoxification. Therefore, these findings provided new insights into the regulatory mechanism of the GSH pathway in Cu-exposed crops.

Conclusions

In conclusion, this study first isolated the upstream regulator-TaWRKY74 of TaGST1 by Y1H, and the binding between TaWRKY74 and *TaGST1* promoter through the W-boxes was confirmed by another Y1H and LUC assays. Further, BSMV-TaWRKY74-silenced wheat plants showed more sensitivity to Cu stress, and in these plants, *TaGST1* expression and GSH synthesis were inhibited remarkably; And transiently ectopic over-expression of TaWRKY74 affected GSH synthesis. Moreover, exogenous GSH application reversed the functions of transiently TaWRKY74-silenced wheat plants during Cu stress. These results confirmed the GSH-mediated plant Cu detoxification activation, which regulated by TaWRKY74 transcription factor. Based on these results, we proposed a signal transduction pathway involving in TaWRKY74 regulating the *TaGST1* expression in wheat response to Cu stress (Fig. 10). To our knowledge, this is the first study to report the transcriptional regulatory mechanisms of Cu detoxification in higher plants. These results provide new insights into the molecular mechanism in plant response to Cu stress, and will contribute to the sustainable development of agriculture and food security in Cu-polluted areas.

Declarations

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Author’s contributions

This study was designed by G-Z L and G-Z K. Most experiments were performed byY-X Z and S-J C, and the data analyze was was conducted by JL and P-F W. Y-HW and T-CG provided technical assistance to Y-X Z and S-J C; G-Z L and G-Z K wrote the this article. All authors contributed equally to this work.

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Availability of data and materials

All essential data are part of the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

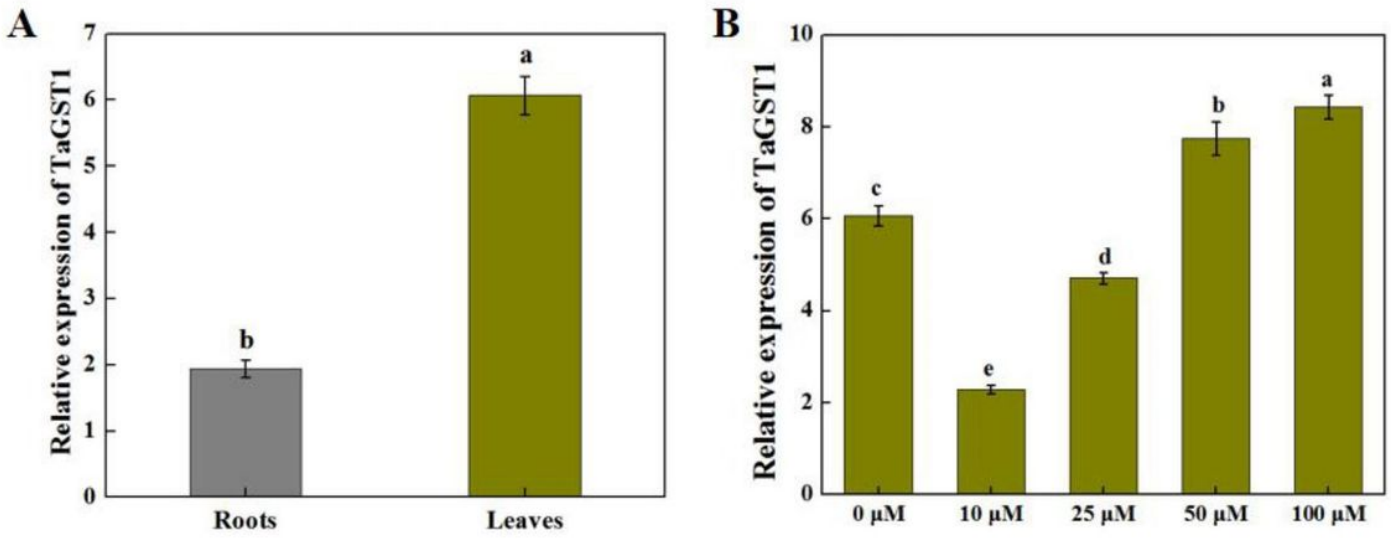

Figure 1

Expression patterns of TaGST1 gene in wheat.

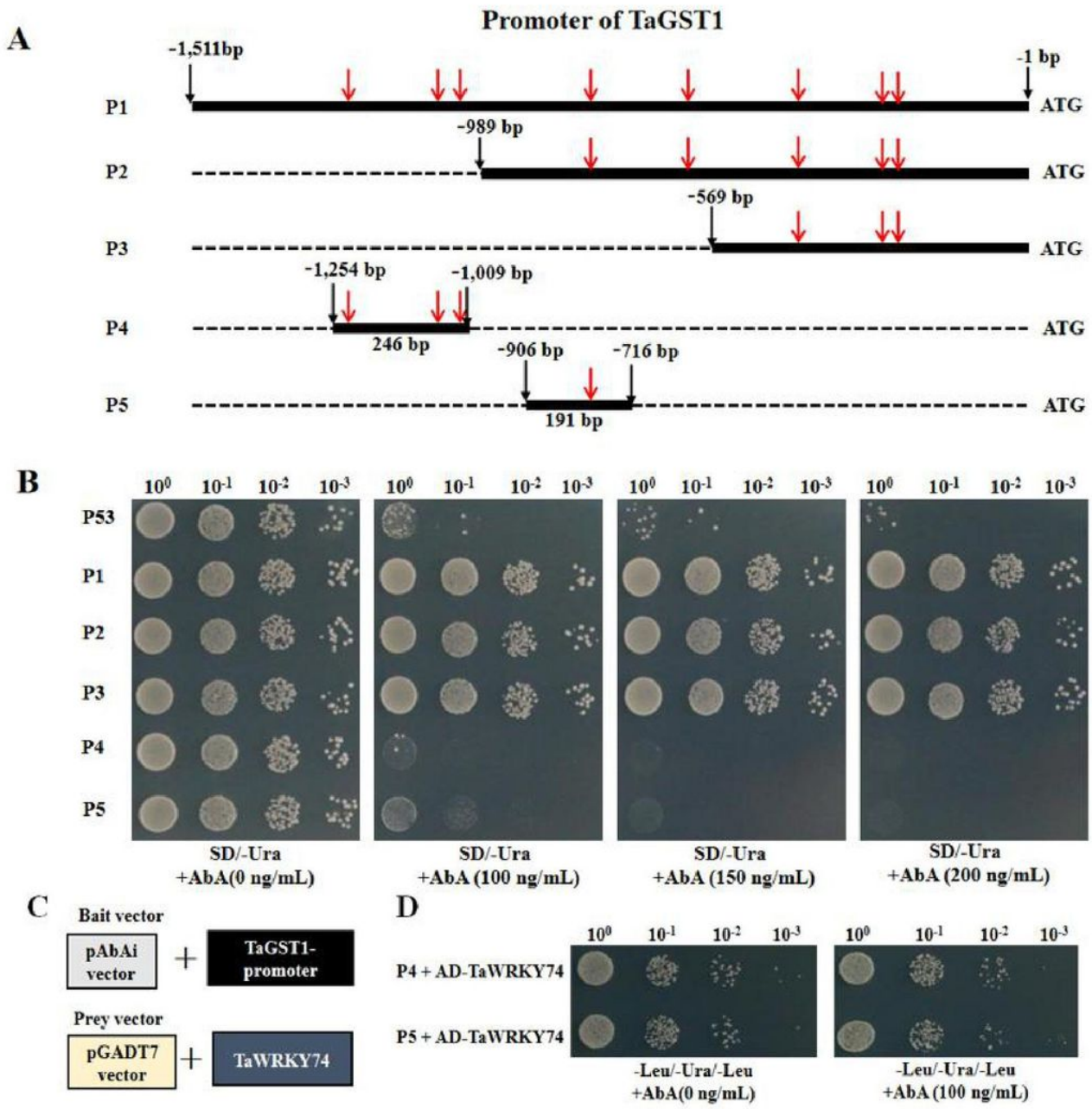

Figure 2

Y1H assays between TaWRKY74 and TaGST1 promoter.

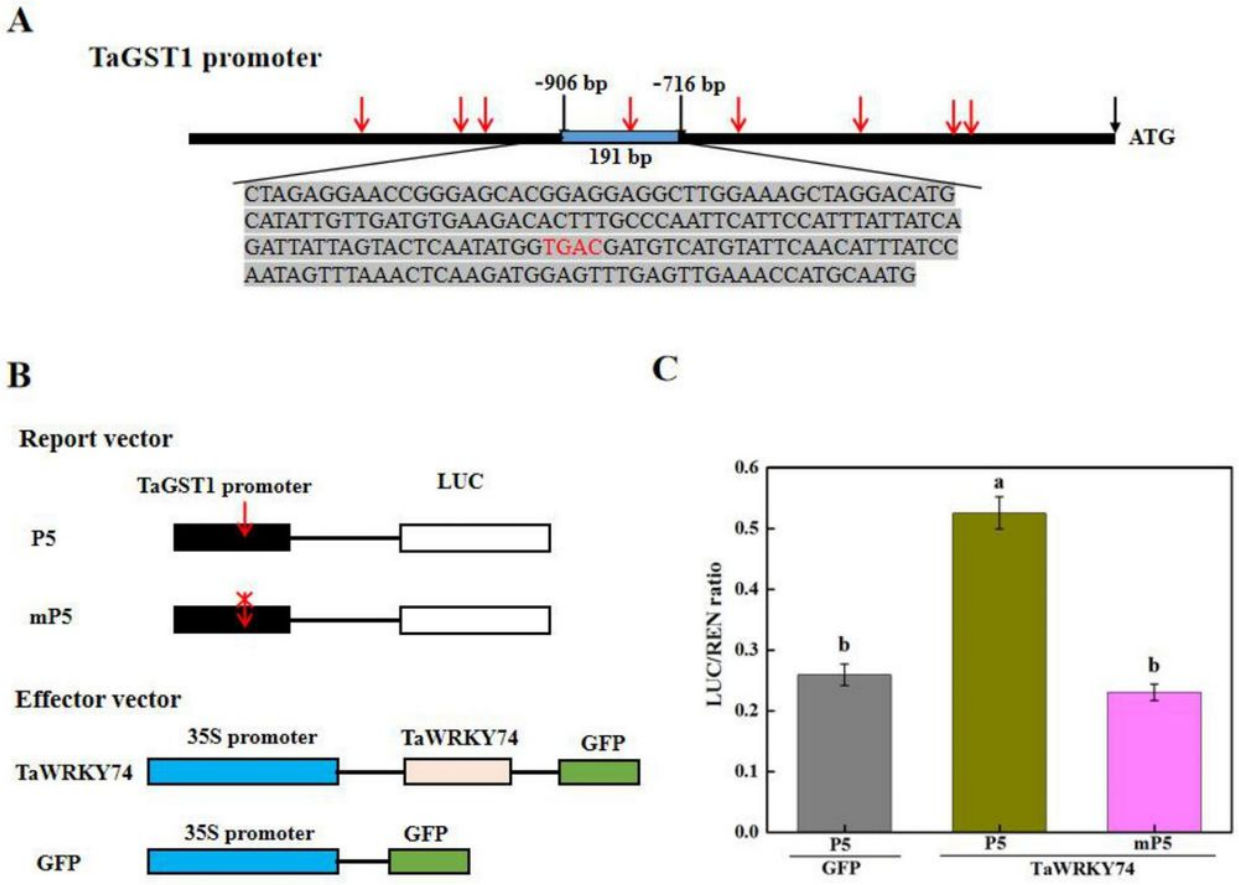

Figure 3

Transient assay analysis of the interaction between TaWRKY74 and the TaGST1 promoter.

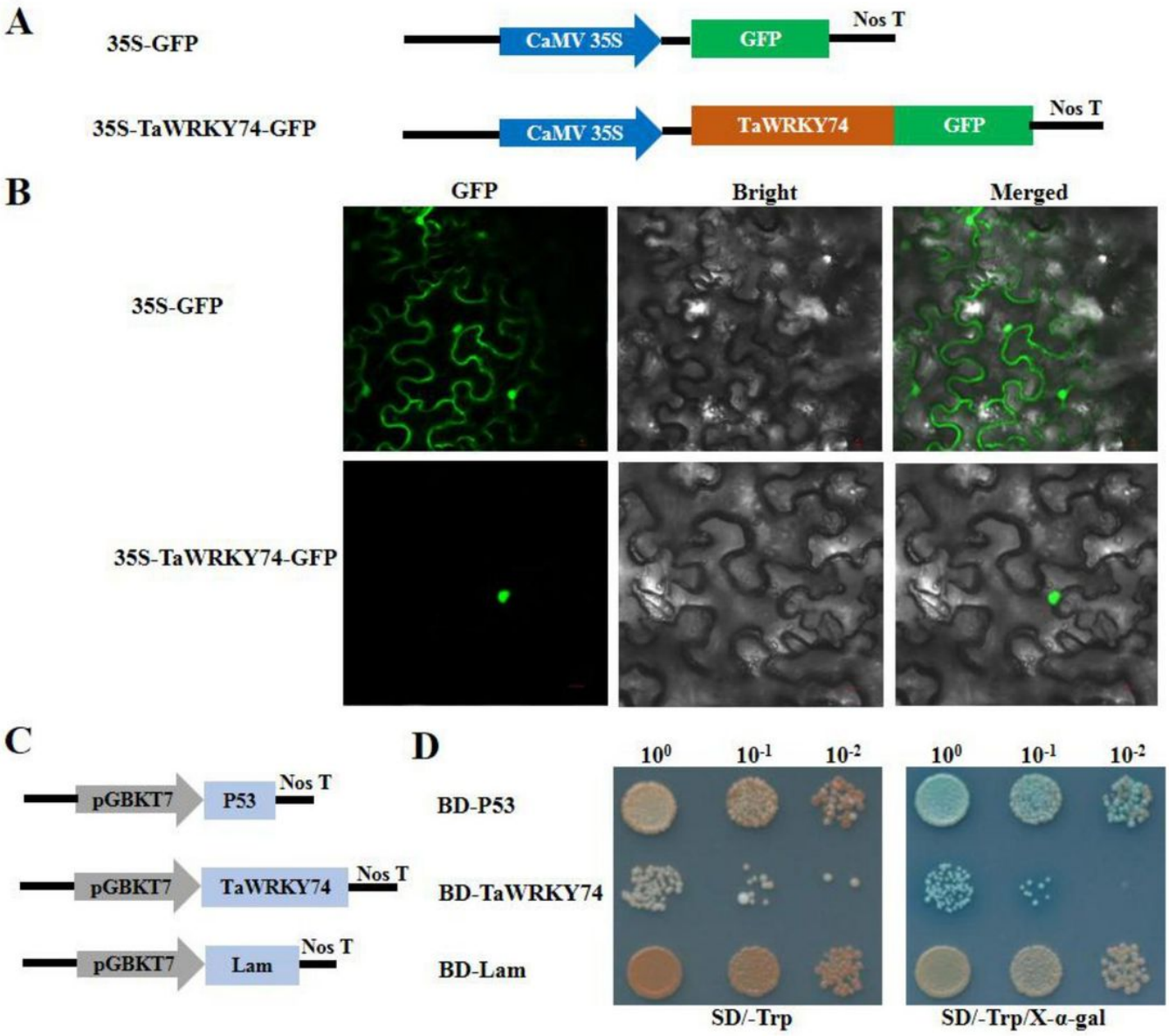

Figure 4

Subcellular localization and transcriptional activity of TaWRKY74.

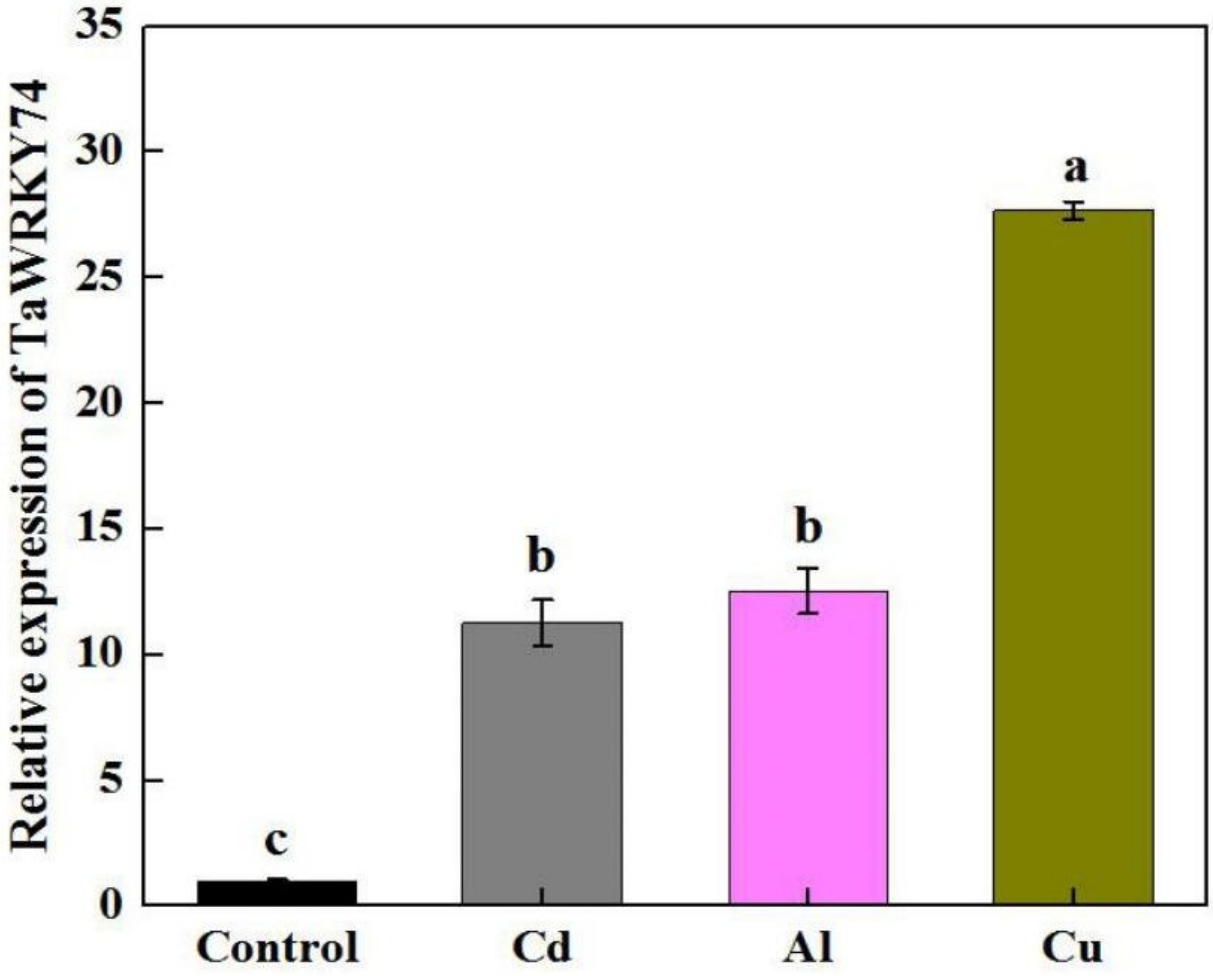

Figure 5

Expression patterns of TaWRKY74 under Cd, Al, and Cu stresses.

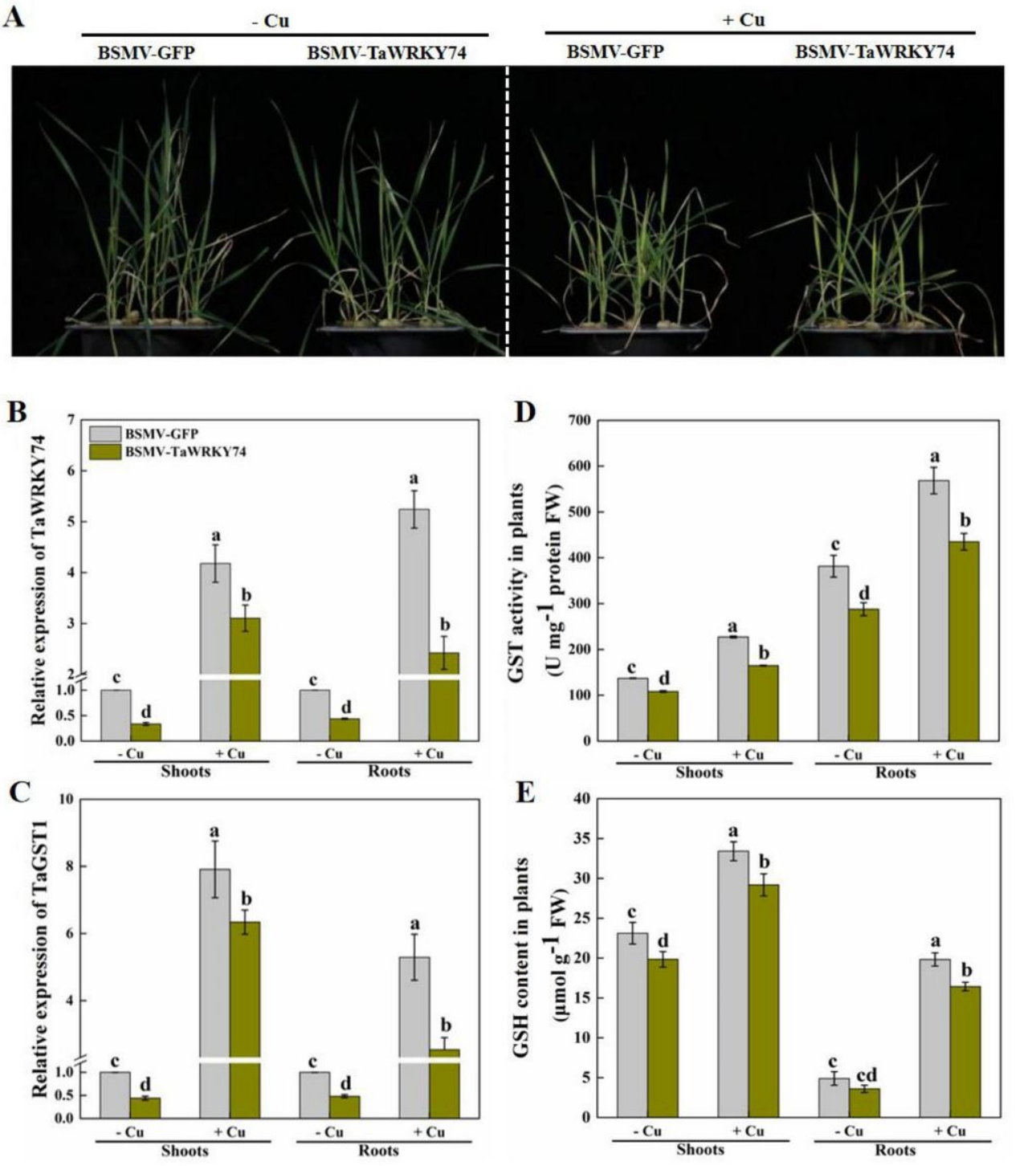

Figure 6

Phenotype of BSMV-TaWRKY74-inoculated wheat plants and their GSH synthesis under Cu stress.

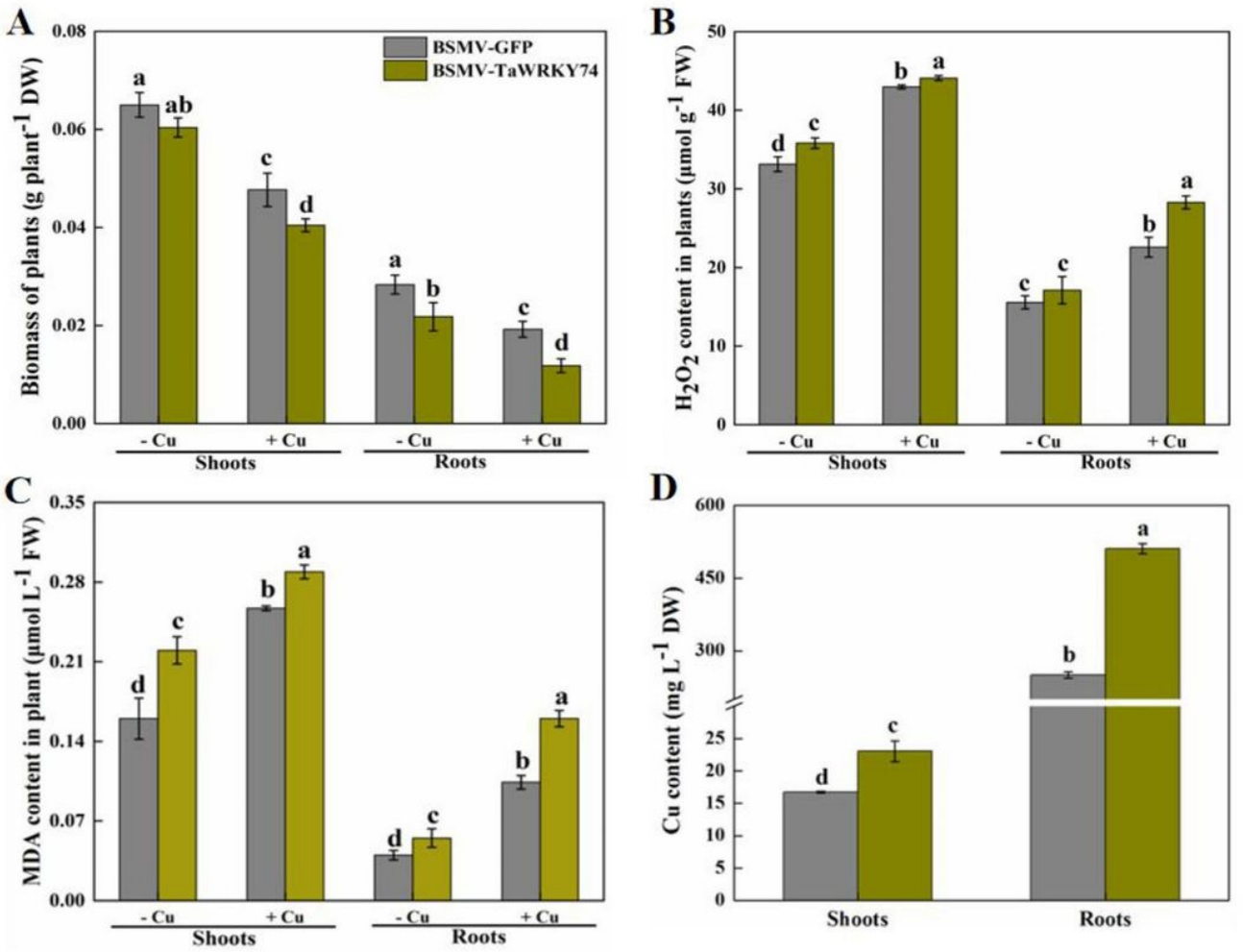

Figure 7

Characteristics of BSMV-TaWRKY74-inoculated wheat plants suffering from Cu stress.

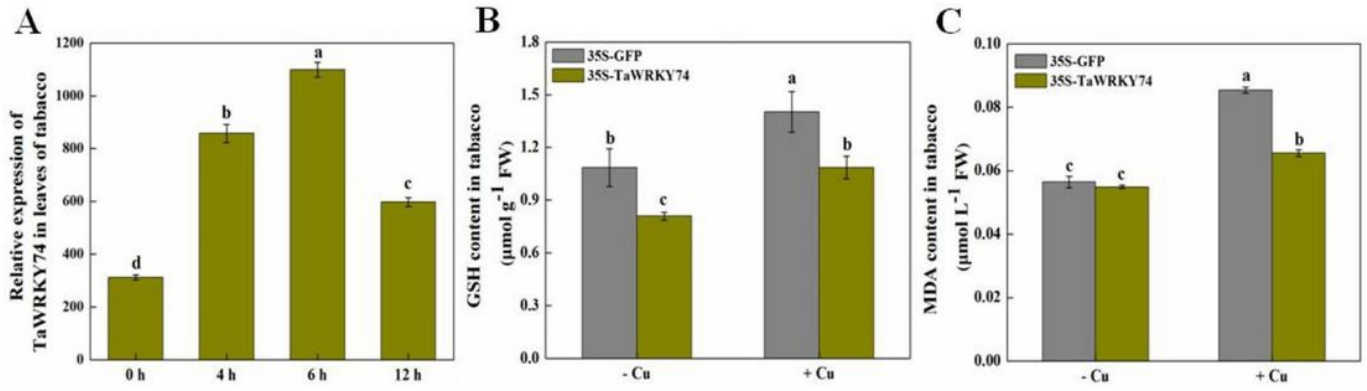

Figure 8

Expression of TaWRKY74 and contents of MDA and GSH in leaves of its transient- overexpression tobacco plants suffering from Cu stress.

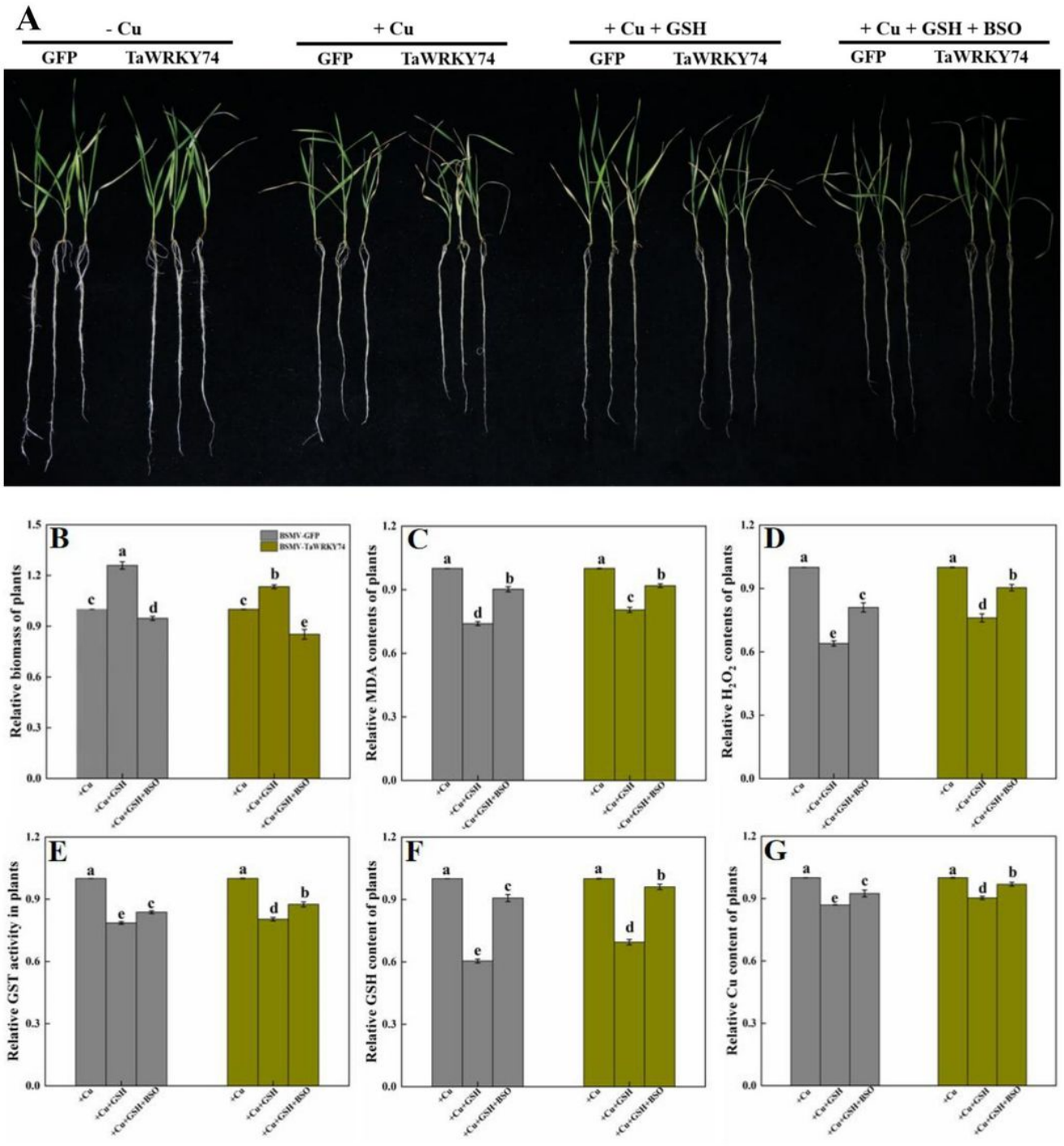

Figure 9

Effect of Cu and GSH supplementation on the growth indexes of BSMV- TaWRKY74-inoculated wheat plants.

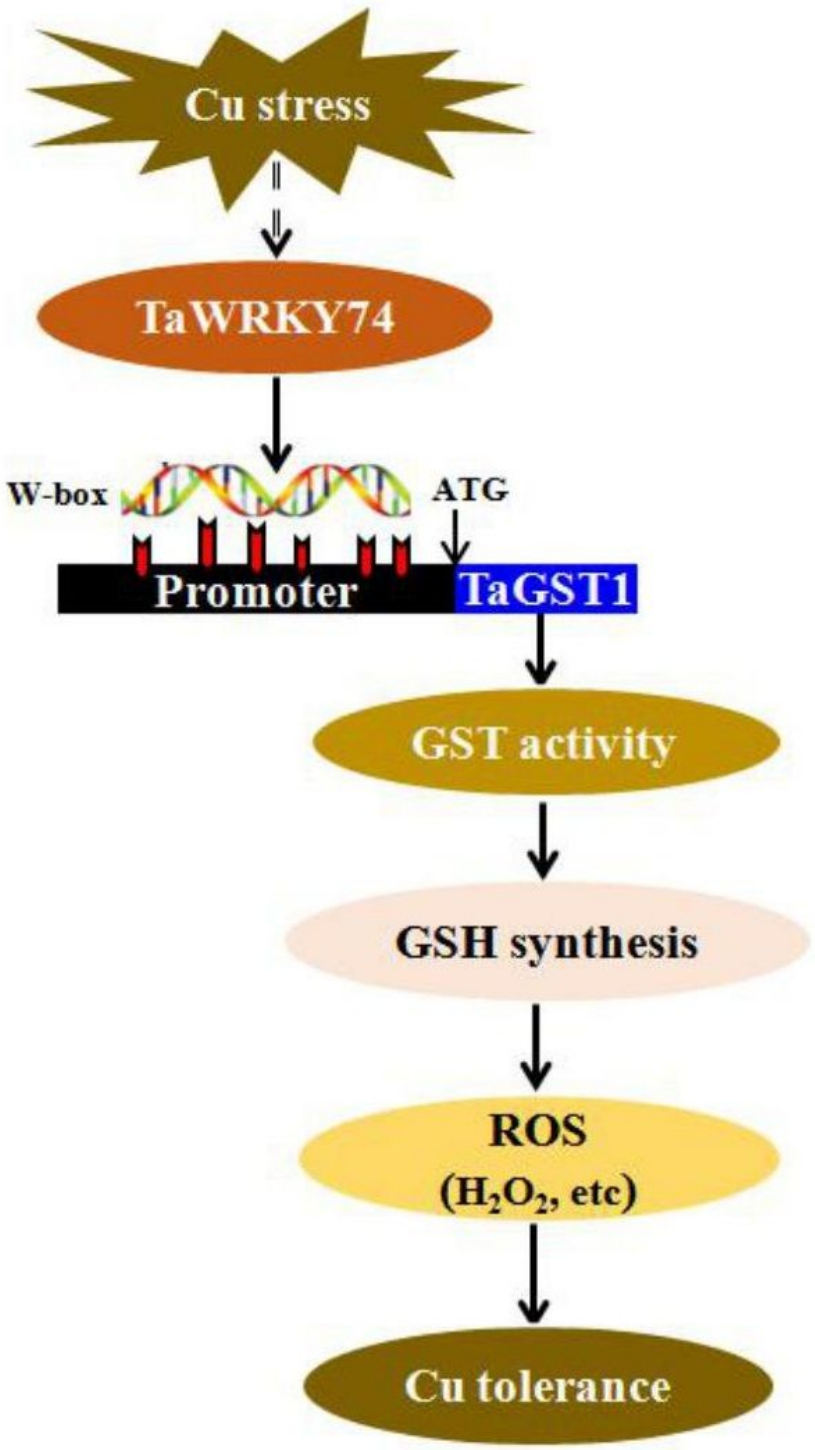

Figure 10

Model of TaWRKY74 participates Cu tolerance in wheat plant.

Supplementary Files

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