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# The Cotton *GhWRKY91* Gene Negatively Regulates Root Elongation in Overexpressed Transgenic *Arabidopsis thaliana*

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## ABSTRACT

WRKY transcription factors play important roles in plant growth, development, and stress responses. Our previous research has shown that the *GhWRKY91* gene can delay age-, abscisic acid (ABA)-, and drought-induced leaf senescence when overexpressed in transgenic *Arabidopsis* plants. To explore in more depth the biological functions of the *GhWRKY91* gene, we further observed the root growth of overexpressing transgenic *Arabidopsis thaliana* under ABA and drought treatment. In this study, we transplanted the germinated seeds of wild-type (WT) and three transgenic lines (OE-12, OE-13 and OE-20) to 1/2 MS solid medium containing ABA and different concentrations of mannitol (simulated drought treatment) for culturing. The results showed that the transgenic plants had dark green leaves and short root lengths when no stress treatment was added. After ABA and mannitol treatment, the root growth of the WT and transgenic *Arabidopsis* was inhibited to varying degrees, and the root length downregulation of the transgenic plants was higher than that of the WT, indicating that they were more sensitive to ABA and drought. A bimolecular fluorescence complementation (BiFC) assay showed that the *GhWRKY91* and *GhWRKY3* proteins interact and emit yellow fluorescence in tobacco leaf cells. These results indicate that the *GhWRKY91* gene negatively regulates root elongation in transgenic *Arabidopsis* and provide a basis for further research on the molecular mechanism of its involvement in regulating cotton root development.

## KEYWORDS

Cotton; *GhWRKY91* gene; root elongation; stress treatment; BiFC

## 1 Introduction

WRKY transcription factors constitute one of the largest transcription factor families in higher plants. The WRKY gene family contains a WRKY domain of approximately 60 amino acids, which contains a conserved WRKYGQK sequence and a zinc finger domain [1]. According to the number of WRKY domains and the characteristics of the zinc finger structure, the WRKY gene family is mainly divided into subfamilies I, II (IIa, IIb, IIc, IId and IIe) and III [1]. Since the first WRKY transcription factor SPF1 was identified from sweet potato [2], an increasing number of WRKY transcription factors have been identified from plants as the cost of genome sequencing has decreased. In recent years, 64 *liWRKY* genes have been identified from *Isatis indigotica* [3], 62 *PdWRKY* genes from *Prunus dulcis* [4], 72 *TkWRKY* genes from *Taraxacum kok saghyz* Rodin [5], and 138 *CIWRKY* genes from *Chrysanthemum*



*lavandulifolium* [6]. Clearly, the WRKY transcription factor is increasingly being extensively studied, laying the foundation for the further exploration of the function of the WRKY genes.

The orderly expression of plant genes is the foundation of plant growth and development. Numerous studies have shown that WRKY transcription factors play an important role in various physiological processes, such as plant growth, fruit ripening, aging, and stress response. For example, MdWRKY9 directly inhibits the transcription of the brassinosteroid (BR) restricted synthase *MdDWF4*, reduces the production of BRs, and positively regulates plant dwarfism [7]. Chinese flowering cabbage BrWRKY6 binds to the promoter of senescence-related genes *BrSAG12*, *BrNYC1* and *BrSGR1* through W-box *cis*-elements, inhibits the expression of gibberellin (GA) biosynthetic genes *BrKAO2* and *BrGA20ox2*, and accelerates leaf senescence [8]. The *AcWRKY40* gene responds to ethylene treatment during the postharvest ripening of kiwifruit and may participate in the postharvest ripening of kiwifruit by regulating the expression of genes related to ethylene biosynthesis [9]. In *Iris germanica*, the expression of *IgWRKY50* and *IgWRKY32* can be induced by PEG6000, enhancing the drought resistance of overexpressing transgenic *Arabidopsis* through the abscisic acid (ABA) signaling pathway [10].

Some WRKY genes can affect root structure by influencing plant hormone signals or gene expression. Compared with the wild-type (WT), overexpression of *OsWRKY31* in rice leads to reduced and shortened lateral roots, which may be achieved by interfering with the response or transport of auxin [11]. Ethylene (ETH) induces the formation of root hairs and adventitious roots during root development. In the wheat experiment, the *1-amino cyclopropane 1-carboxylate synthase* gene for ethylene synthesis was downregulated in the *TaWRKY51*-OE overexpression lines and upregulated in the *TaWRKY51*-RNAi silenced lines. Further research found that *TaWRKY51* is a negative regulator of ETH synthesis. *TaWRKY51* inhibits the expression of the ETH synthesis gene ACS by combining with the W-box *cis*-element in the promoter region and coordinates ethylene synthesis and lateral root formation in wheat [12]. Soybean *GmWRKY46* has a strong inducing effect under phosphate (Pi) starvation conditions, especially in soybean roots. Overexpression of *GmWRKY46* significantly enhances the tolerance of transgenic *Arabidopsis* to Pi starvation and lateral root development [13]. These research results indicate that WRKY transcription factors play important roles in regulating plant root development.

Cotton is an important cash crop in China and the main source of natural fiber. WRKY transcription factors play an important role in the growth and development of plants and their response to stress. Previous studies have found that the *GhWRKY91* gene can delay leaf senescence in transgenic *Arabidopsis* [14], but further research is needed to determine whether this gene has other functions. In this study, overexpression of the *GhWRKY91* gene in *Arabidopsis* affected root development and increased sensitivity to ABA and drought stress. This provides a basis for further research on the molecular mechanism by which *GhWRKY91* participates in regulating cotton root development and is important for stress resistance breeding in transgenic cotton.

## 2 Materials and Methods

### 2.1 Planting and Stress Management of *GhWRKY91* Transgenic *Arabidopsis*

In a previous study, we constructed an overexpression vector of the *GhWRKY91* gene driven by the 35S promoter and obtained three transgenic *Arabidopsis thaliana* positive lines, OE-12, OE-13 and OE-20, by floral dipping [14]. In this study, we further observed the phenotype of transgenic *Arabidopsis* under ABA and drought stress treatment. *A. thaliana* seeds of WT, OE-12, OE-13 and OE-20 were sterilized in 1.5 mL clean centrifuge tubes, planted on 1/2 MS solid medium (0.22 g 1/2 Murashige & Skoog medium, 3 g sucrose, 0.8 g agar powder, sterilized at 121°C for 15 min), and placed in a refrigerator at 4°C for 3 days for vernalization. Then, the solid plates were placed in a light incubator, the temperature was 22°C, and the photoperiod was 16 h of light/8 h of darkness until the seeds germinated. The germinated seeds of WT, OE-12, OE-13 and OE-20 with healthy and consistent growth were transplanted

to 1/2 MS solid medium containing ABA (0.5  $\mu$ M) and mannitol at different concentrations (50, 150 and 200 mM) and cultured in a light incubator. In the control group, the germinated seeds of WT, OE-12, OE-13 and OE-20 were planted on 1/2 solid medium without any stress treatment. The phenotypes were observed after approximately 12 days of cultivation. Three repetitions were carried out for each treatment.

## 2.2 Construction of BiFC Vector

Using WRKY91-F/R and WRKY31-F/R primers, the *GhWRKY91* and *GhWRKY3* genes were cloned from the cDNA of CCRI10 cotton variety leaves, respectively. After the PCR product was purified and recovered, the purified product was connected to the pDONR221 entry vector by Gateway BP Clonase II Enzyme Mix (Invitrogen), and the recombinant plasmids pDONR221-*WRKY91* and pDONR221-*WRKY3* were transformed into TOP10 competent *Escherichia coli* cells. Positive clones were identified by pDONR221-F/R universal primer PCR and sent to the company for sequencing. The pDONR221-*WRKY91* and pDONR221-*WRKY3* plasmids with correct sequencing were extracted, and *GhWRKY91* and *GhWRKY3* were connected to the pEarleyGate201-YN and pEarleyGate202-YC vectors by Gateway LR Clonase II enzyme mix (Invitrogen), respectively. The connected products *WRKY91*-YN and *WRKY3*-YC were transformed into TOP10 competent *E. coli* cells. The positive clones were identified by universal primers YNC-F/R of pEarleyGate201-YN and pEarleyGate202-YC vectors using PCR. The empty vector pEarleyGate201-YN and pEarleyGate202-YC plasmids and the positive clones *WRKY91*-YN and *WRKY3*-YC plasmids were transformed into *Agrobacterium* GV3101 receptor cells and identified using YNC-F/R primers. All primers are listed in Table 1.

**Table 1:** Primer sequences for BiFC vector construction

Primer name	Primer sequences (5'-3')
<i>WRKY91</i> -F	GGGGACAAGTTTGTACAAAAAATGGACAGCGGTGGTAGT
<i>WRKY91</i> -R	GGGGACCACTTTGTACAAGAAGCAGATCCCAACCTGTGG
<i>WRKY3</i> -F	GGGGACAAGTTTGTACAAA AAAATGGCAGCAGAGCAAGGA
<i>WRKY3</i> -R	GGGGACCACTTTGTACAAGAACTCTTATTGCTCCTCTTTTAG
pDONR221-F	GTAAAACGACGGCCAG
pDONR221-R	CAGGAAACAGCTATGAC
YNC-F	GGACACGCTCGAGTATAAG
YNC-R	AGGCACCACTTTGTACAAG

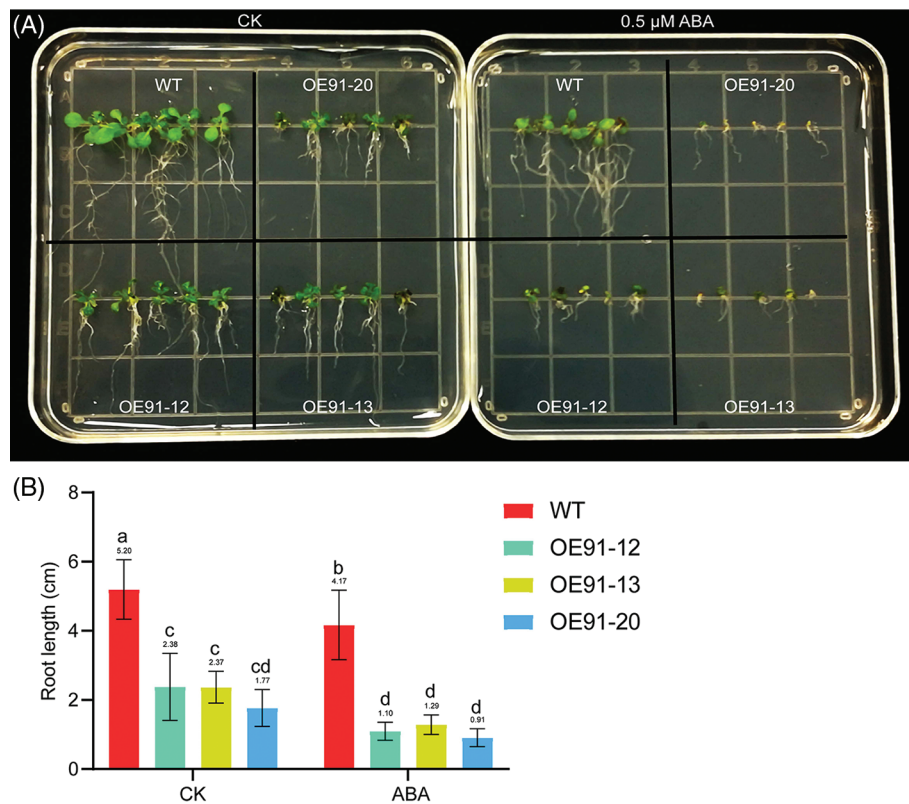
## 2.3 Tobacco Infection and Fluorescence Observation

Tobacco was grown in a greenhouse at 22°C under 16 h of light and 8 h of darkness. Tobacco that was approximately 3 weeks old was used for injection. The successful *Agrobacterium* solution was shaken overnight at 28°C and 200 rpm. pEarleyGate202-*WRKY91*-YN & pEarleyGate201-*WRKY3*-YC, pEarleyGate201-*WRKY91*-YN & pEarleyGate202-YC, pEarleyGate201-YN & pEarleyGate202-*WRKY3*-YC and pEarleyGate202-YC & pEarleyGate201-YN combinations were mixed at a ratio of 1:1. After centrifugation, four kinds of mixed bacterial solutions were resuspended in osmotic solution (10 mM MgCl<sub>2</sub>, 10 mM MES pH 5.7 and 0.2 mM acetosyringone) and adjusted to an OD<sub>600</sub> ranging from 0.01 to 0.1. Before infection, the tobacco was placed under a white fluorescent lamp for 1 h to open its pores. Four kinds of suspensions were aspirated into a 1 mL syringe without the needle, injected into the back of the tobacco leaf and marked as the infected area. The infected tobacco was returned to the culture room, and fluorescence observation was performed by laser confocal scanning microscopy after 2–3 days.

### 3 Results

#### 3.1 *GhWRKY91* Transgenic *Arabidopsis* Root Elongation Is Sensitive to ABA

In a preliminary study, we obtained three transgenic *A. thaliana* lines overexpressing the *GhWRKY91* gene [14]. In this study, we further observed the effect of ABA on the root elongation of *GhWRKY91* transgenic plants. Germinated seeds of WT, OE-12, OE-13, and OE-20 plants were placed on a 1/2 MS solid plate without ABA and with 0.5  $\mu$ M ABA for approximately 12 days. From Fig. 1A, under normal growth conditions, the leaves of the seedlings of the three transgenic plants were darker green than those of WT, with shorter roots and overall smaller plants. After ABA treatment, the root growth of both WT and transgenic plants was inhibited, and the leaves of seedlings of the three transgenic plants showed varying degrees of yellowing. Fig. 1B shows that the root length of the three transgenic lines was consistently shorter than that of WT when ABA treatment was applied or not, which is consistent with the phenotype observation in Fig. 1A. The root length of the three transgenic lines was significantly lower than that of the WT before and after ABA treatment (Fig. 1B). We further analyzed the relative root length of WT and three transgenic strains under 0.5  $\mu$ M ABA treatment relative to normal growth conditions using the data in Fig. 1B. The relative root length was 80.19% (WT), 46.22% (OE91-12), 54.43% (OE91-13) and 51.41% (OE91-20), respectively. The relative root length of the three transgenic lines was shorter than that of WT, indicating that the root length downregulation in the three transgenic lines was higher than that of WT, making them more sensitive to ABA treatment.

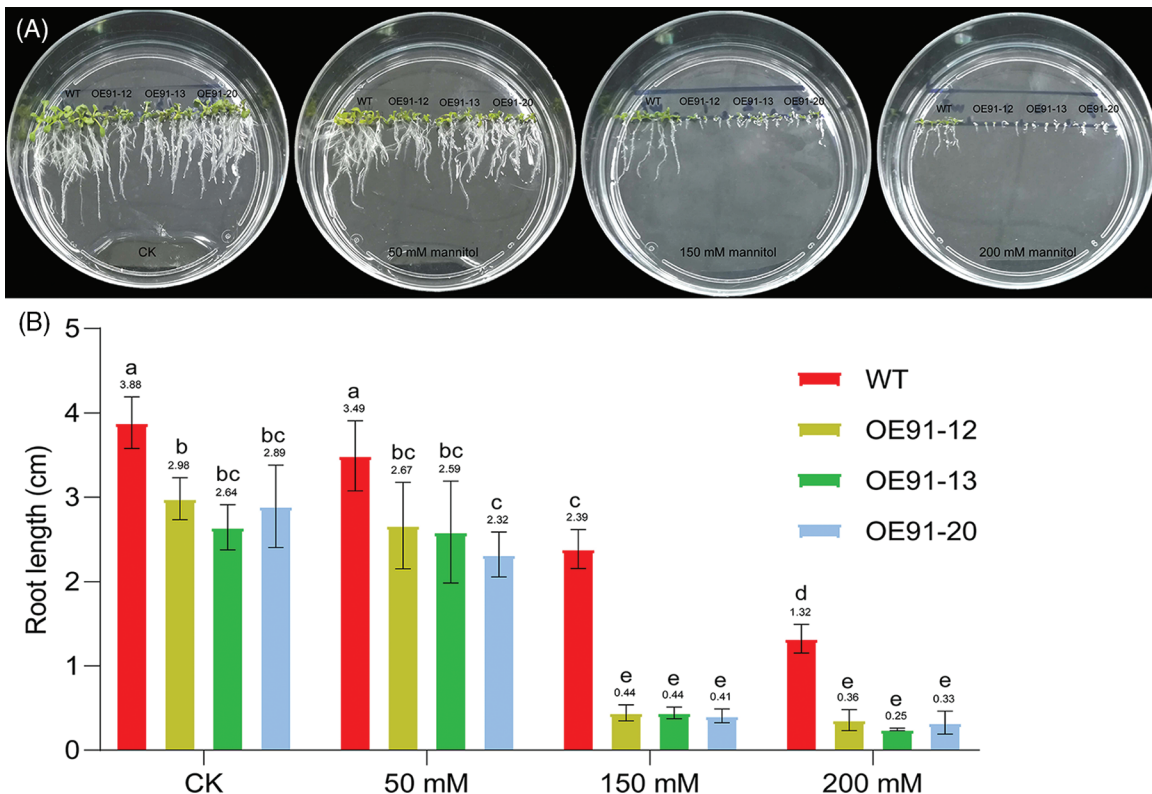


**Figure 1:** *GhWRKY91* transgenic *Arabidopsis* root elongation is sensitive to ABA. (A) Phenotypes of the WT and three transgenic lines treated with 0 and 0.5  $\mu$ M ABA. (B) Root length of the WT and three transgenic lines treated with 0 and 0.5  $\mu$ M ABA



### 3.2 *GhWRKY91* Transgenic *Arabidopsis* Root Elongation Is Sensitive to Drought

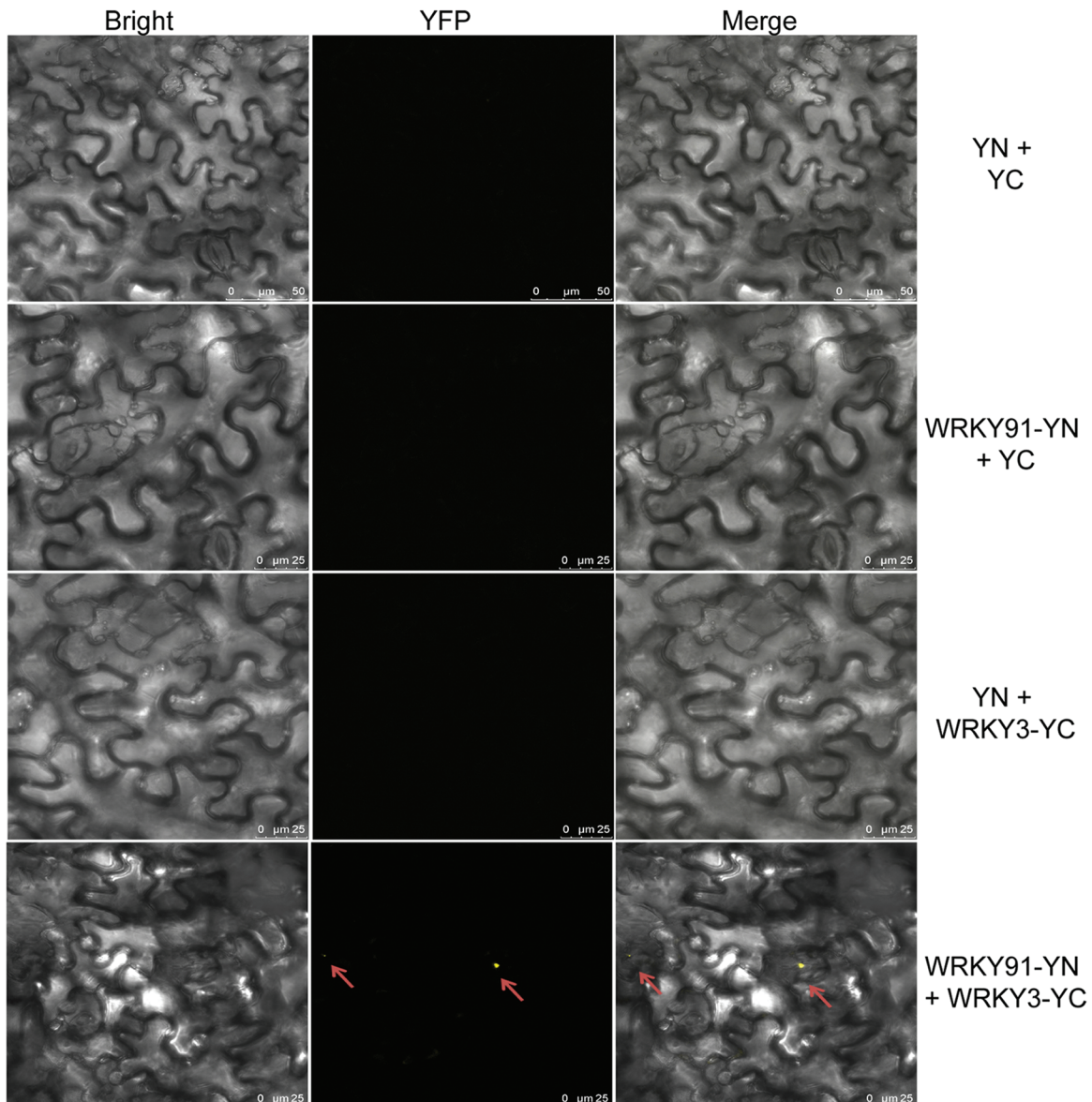
The germinated seeds of WT, OE-12, OE-13 and OE-20 were cultured on 1/2 MS solid plates containing 0, 50, 150 and 200 mM mannitol, and the effects of simulated drought treatment on root development were observed. When mannitol was not added, the leaves of the three transgenic lines were dark green, and the root length was shorter than that of the WT (Fig. 2A). After mannitol treatment, the root length of the WT and the three transgenic plants became shorter to varying degrees, the root length of the three transgenic lines was shorter than that of the WT, and the root length of the three transgenic lines decreased precipitously when treated with 150 mM mannitol (Fig. 2A). The statistical results for root length are consistent with the phenotypic observations of root length (Fig. 2B). The root length downregulation ratio of WT and three transgenic lines treated with mannitol at different concentrations compared with normal growth conditions was further analyzed based on the data in Fig. 2B. There was no significant difference in WT root length between the control and 50 mM mannitol treatment, nor was there any significant difference in the three transgenic lines. However, under 150 and 200 mM mannitol treatment, the WT and the three transgenic lines were significantly different from the untreated lines (Fig. 2B). With the increase in mannitol concentration, the root length of the WT and the three transgenic lines decreased continuously. Under 50 mM mannitol treatment, there was no significant difference in the root length decrease between the three transgenic lines and WT. Under 150 mM mannitol treatment, the root length of WT was decreased by 38.40%, and those of the three transgenic lines were decreased by 85.23%, 83.33% and 85.81%. Under 200 mM mannitol treatment, the root length of WT was decreased by 65.98%, and those of the three transgenic lines were decreased by 87.92%, 90.53% and 88.58%. These results showed that the root length reduction ratio of transgenic lines under drought treatment was higher than that of WT, and they were more sensitive to drought.



**Figure 2:** *GhWRKY91* transgenic *Arabidopsis* root elongation is sensitive to drought. (A) Phenotypes of the WT and three transgenic lines under 0 (CK), 50, 150 and 200 mM mannitol treatment. (B) Root length of the WT and three transgenic lines under different concentrations of mannitol

### 3.3 Interaction between the GhWRKY91 and GhWRKY3 Proteins

Previous studies have shown that *GhWRKY3* belongs to the group I WRKY transcription factor subfamily, is located in the nucleus and is induced by various hormones, biological stress and abiotic stress [15]. In addition, *GhWRKY3* is constitutively expressed in roots [15], and its homolog in *Arabidopsis thaliana*, *AtWRKY3* (NM\_126385), regulates root elongation under stress treatments [16]. Therefore, *GhWRKY3* was selected as a candidate gene to validate the interaction with *GhWRKY91*, in order to preliminarily explore the regulatory mechanism of *GhWRKY91* in regulating root growth. To verify whether the GhWRKY91 transcription factor interacts with GhWRKY3, we conducted BiFC experiments in tobacco leaf cells. The results showed that the three control combinations YN+YC, WRKY91-YN+YC, and YN+WRKY3-YC could not emit yellow fluorescence in tobacco leaf cells, and only WRKY91-YN+WRKY3-YC could emit yellow fluorescence in tobacco leaf cells (Fig. 3). This result indicates that GhWRKY91 and GhWRKY3 can interact and emit yellow fluorescence.



**Figure 3:** Interaction between GhWRKY91 and GhWRKY3 in tobacco leaf cells. The red arrow indicates the position of yellow fluorescence

#### 4 Discussion

In the process of plant growth, adverse environmental conditions such as drought, salinity, high temperature, low temperature, heavy metals and ultraviolet rays are often encountered, which affect the growth and development of plants and even lead to plant death in severe cases [17]. Plants have evolved a series of mechanisms to resist adverse environments after long-term adversity training so that they can better adapt to changes in the external environment [18–20]. As an important factor regulating the expression of stress-related genes, plant WRKY genes play an important role in plant adaptation and resistance to stress [5,21,22]. Cotton is the largest cash crop in China and the source of natural plant fiber. The function of WRKY genes in the cotton response to abiotic stress has been studied. For example, virus-induced *GhWRKY5* gene silencing results in greater salt sensitivity in cotton than in WT plants, while overexpression of *GarWRKY5* positively regulates salt tolerance in *Arabidopsis* seeds during germination and vegetative growth stages [23]. Under drought stress, *GhWRKY33*-overexpressing transgenic *A. thaliana* showed a higher water loss rate, higher wilting degree, and faster decay rate than the WT. *GhWRKY33* may act as a negative regulator to mediate the plant response to drought stress [24]. Therefore, WRKY transcription factors play an important role in the regulation of cotton stress resistance, and it is very important to study the function of WRKY genes in cotton.

ABA is an important plant hormone involved in the stress response that can inhibit root elongation by delaying the initiation of cell elongation. Takatsuka et al. [25] showed that ABA inhibits root cell elongation by inhibiting the cytokinin signaling pathway. Qin et al. [26] showed that ABA can promote the biosynthesis of auxin and inhibit the elongation of primary roots in rice [27]. Drought stress can affect root development. For example, PEG simulation of drought stress can inhibit root hair elongation in *35S:ZmTIP1<sup>Mo17</sup>* and *35S:ZmTIP1<sup>CIMBL55</sup>* transgenic *Arabidopsis* [28]. In this study, root elongation of the WT and three transgenic lines was inhibited after ABA and drought treatment, which is consistent with previous research results. When no stress treatment was applied, the *GhWRKY91* transgenic lines grown on 1/2 MS solid medium had a stronger green leaf color and smaller plant size than the WT, which is consistent with the results of our previous research on *GhWRKY91* transgenic and WT plants in nutrient soil [14]. In addition, the root length of *GhWRKY91* transgenic plants was shorter than that of WT in the absence of stress treatment. Dark green leaves can promote photosynthesis and effectively increase crop yield [29]. Root systems play a crucial role in soil nutrient absorption capacity and largely control plant productivity and adaptation to different soil conditions [30]. A greener leaf color indicates that *GhWRKY91* transgenic plants may have high yield potential; however, yield potential may be limited by shorter root length. Our previous research found that transgenic *Arabidopsis* with *GhWRKY91* can delay ABA- and drought-induced leaf senescence, exhibiting insensitivity to ABA and drought [14]. In this study, after ABA and drought treatment, the root length of *GhWRKY91* transgenic *A. thaliana* decreased more than that of WT, indicating sensitivity to ABA and drought. This result is inconsistent with our previous research results, and there are three possible reasons for this discrepancy. First, different treatment periods were used. In this study, germinated seeds were used, while in the previous study, 3-week seedlings were used; second, the growth environment was different. In this study, plants were grown on 1/2 MS solid medium and cultured in a light incubator. In the previous study, plants were grown in nutrient soil and cultured in a greenhouse; third, different observation sites were observed. In this study, the root system was observed, while in previous studies, leaf senescence was observed. The *GhWRKY91* transgenic seedlings had increased sensitivity to ABA and drought compared to the WT, indicating that the transgenic seedlings may be more susceptible to stress conditions at the seedling stage. However, the effects of ABA and drought treatment on growth and development as well as root elongation in the transgenic plants need to be further confirmed by measuring a series of morphological, physiological and biochemical indicators, including chlorophyll content, stomata, superoxide dismutase, peroxidase, and catalase activity. In



addition, the molecular mechanism by which *GhWRKY91* participates in regulating root elongation still needs further exploration.

BiFC is a technology that intuitively and quickly determines the location and interaction of target proteins in living cells and can utilize the characteristics of fluorescent proteins to study protein interactions [31,32]. For example, Gu et al. [33] conducted a BiFC experiment using *Arabidopsis* protoplasts to demonstrate that GhWRKY27 and GhTT2 interact to jointly regulate the expression of the cytochrome P450 gene *GhCYP94C1* and ripen-related protein *GhRipen2-2* in the process of leaf senescence. Li et al. [34] showed that AcMYB1 and AcbHLH1 of *Hypericum perforatum* interacted in the protoplast of *A. thaliana* and participated in the biosynthesis of anthocyanidin. Therefore, BiFC experiments are also an effective means of analyzing the molecular mechanisms of gene regulation. Previous studies have found that the *GhWRKY3* gene can be transcriptionally upregulated by the plant hormones SA, ABA, MeJA, ET, GA and H<sub>2</sub>O<sub>2</sub> and is also induced by injury and infection by three fungal pathogens, *Rhizoctonia solani*, *Colletotrichum gossypii* and *Fusarium oxysporum f. sp. vasinfectum* but is not induced by 6-BA, NAA, drought, salt and cold [15]. In addition, the *GhWRKY3* gene is constitutively expressed in roots [15], and its homolog in *Arabidopsis thaliana*, *AtWRKY3*, regulates root elongation under stress treatments [16], suggesting that *GhWRKY3* has potential functions in root growth and development. In this study, GhWRKY91 can interact with GhWRKY3, indicating that they may jointly participate in regulating multiple stress responses and root elongation regulation, which provides a preliminary exploration for the mechanism of *GhWRKY91* in regulating root growth. The BiFC assay confirmed that the GhWRKY91 and GhWRKY3 interact in tobacco leaf cells; however, whether they interact in the nucleus requires further verification by DAPI staining. Furthermore, the interaction between GhWRKY91 and GhWRKY3 proteins requires further validation using techniques such as yeast two-hybrid, co-immunoprecipitation, and GST pull down. Whether *GhWRKY3* is involved in the regulation of root growth and development needs to be further verified by obtaining transgenic plants through transgenic technology in the future.

## 5 Conclusion

In summary, using *GhWRKY91* transgenic *A. thaliana* obtained previously, we further studied the effects of ABA and drought on the root growth of transgenic *A. thaliana*. ABA and drought negatively regulated the root elongation of *GhWRKY91* transgenic *A. thaliana*, and GhWRKY91 and GhWRKY3 interacted. In our future research, transgenic cotton with *GhWRKY91* gene overexpression, RNAi, gene editing deletion and gene editing transcription activation needs to be achieved for further functional verification to analyze the effect of *GhWRKY91* on root development in cotton. In addition, we can use yeast one-hybrid, electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation sequencing (ChIP-Seq), dual luciferase assay and other technologies to further identify the downstream target genes of *GhWRKY91* regulation of root development and explore the molecular mechanism of *GhWRKY91* regulation of root development. Our findings provide a basis for the future development of resistant cotton materials.

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**Availability of Data and Materials:** All data generated or analysed during this study are included in this published article.

**Ethics Approval:** Not applicable.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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