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Arabidopsis Transcription Factor WRKY33 Is Involved in Drought by Directly Regulating the Expression of *CesA*8

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ABSTRACT

Arabidopsis (*Arabidopsis thaliana*) WRKY33 is a key transcription factor in pathogen-induced defense signaling, but its function in abiotic stresses remains largely unclear. In this study, we report on the use of a reverse-genetic approach, as well as a yeast (*Saccharomyces cerevisiae*) expression system, to determine the role of WRKY33 in drought. A T-DNA insertion deletion mutant of *WRKY*33 is more sensitive to dehydration. Through genome-wide screening the target genes of WRKY33 in yeast, we identified 23 candidate genes including a drought tolerance gene *CesA*8. Further results revealed that WRKY33 repressed *CesA*8 expression through binding to the W-box elements of *CesA*8 distal promoter region and probably interacting with the transcriptional activator of *CesA*8, MYB46. These findings revealed the primary molecular mechanism underlying the function of WRKY33 in response to drought.

Keywords: WRKY33; Drought; *CesA*8; Target Gene; Inverse Yeast One-Hybrid Assay

1. Introduction

In plants, WRKY transcription factors are one of the largest families of transcriptional regulators with up to 74 representatives in Arabidopsis [1]. WRKY transcription factors are defined by the presence of the highly conserved WRKY domain containing the sequence WRKYGQK and a distinct zinc finger-type motif [1]. They form integral parts of signaling webs that modulate many plant processes including biotic stresses, abiotic stresses, and developments [2]. WRKY transcription factors bind to a specific cis-acting DNA element, W-box (5'-TTGACC/T-3'), within the promoter of target gene [3].

Arabidopsis WRKY33 (At2g38470) is strongly activated by pathogen-induced infection [4,5] and serves as a key transcription factor in pathogens defense signaling [6-8]. WRKY33 is regulated by multiple abiotic stresses including high salinity [9], osmotic stress [10], cold [10], heat shock [10,11], heavy metal stress [12], and UV exposure [5], indicating that WRKY33 may also be involved in abiotic stresses. Recently, WRKY33 has been proved to confer tolerance to salt stress [10] and heat stress [11], but its molecular mechanism in response to abiotic stresses is not well understood.

Considering the facts that WRKY33 confers salt stress and drought may result from a physiological water deficit caused by salt stress, we explored whether WRKY33 also conferred drought. In this study, we showed that WRKY33 was involved in drought through a direct transcriptional regulation of *CesA*8, an Arabidopsis drought tolerance gene. Our findings advanced understanding of the molecular mechanism underlying the role of WRKY33 in response to drought stress.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The *wrky*33-1 mutant (SALK_006603), carrying a T-DNA insertion in At2g38470 of the Col ecotype, was obtained from the Arabidopsis Biological Resource Center. RT-PCR was used to verify homozygous T-DNA insertion for *wrky*33-1 using WRKY33-E1 (5'-GTCACAACAATCCGGAAGAAC-3') and WRKY33-E3 (5'-CAAACTGGCATTGTACACAGC-3'). The seeds of *wrky*33-1 and wild-type were surface-sterilized and sown on 1/2 Murashige and Skoog (MS)-agar plates at 22˚C with 16-h/8-h light/dark cycles.

2.2. Drought Stress Experiments

For dehydration-rehydration assay, rosette leaves were

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detached from 2-week-old plants, weighed immediately on a plate and then slowly dried at room temperature (22˚C). After the rosette leaves lost 60% of their initial fresh weight, the plants were immersed in water and grown in the greenhouse.

For the phenotype tests under drought stress, seedlings grown on 1/2 MS-agar medium for 7 days were transferred to mixed soil (rich soil:vermiculite, 2:1, v/v) and grown for two weeks with sufficient watering. Then the plants were subjected to drought treatment by withholding irrigation. To ensure reproducibility for the phenotype tests, four pots (four seedlings/pots) of plants were grown for wild-type and *wrky*33-1, respectively.

2.3. RNA Isolation and cDNA Synthesis

Total RNA was extracted from *Arabidopsis* seedlings using the RNAprep Pure Plant kit with on-column DNase digestion (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. RNA (2 μg) was used to synthesize the first-strand cDNA with an oligo (dT) primer according to the instruction of PrimeScript™ 1st strand cDNA synthesis kit (Takara, Dalian, China).

2.4. Semi-Quantitative Duplex RT-PCR Analysis

The semi-quantitative duplex RT-PCR analysis of *CesA*8 and *TUA*4 was performed at annealing temperature 56˚C for 30 or 33 cycles using the EX Taq™ polymerase (Takara, Dalian, China). The following primers were used: *CesA*8, 5'-TGGAGGTGTCTCAGCTCATCTC-3' and 5'-GAGAAAGGGAAGTCGTATCGG-3'; *TUA*4, 5'-CTCTACCTCCGTTGTTGAGCCTTAC-3' and 5'-CACCCACATACCAGTGAACGAAAG-3'.

2.5. Construction of W-Box Sequences Enriched Genomic Library

Arabidopsis genomic DNA fragments enriched for W-box sequences (5'-TTGAC(T/C)-3') were obtained by PCR using forward primer (5'-CGGAATTCTT-GAC (C/T)-3', *Eco*RI site is underlined) and reverse primer (5'-GGACTAGT(G/A)GTCAA-3', *Spe*I site is underlined). The PCR was carried out in a total volume of 50 μl, containing 50 pmol each primer, 0.5 U EX Taq™ polymerase (Takara, Dalian, China), and 500 ng genomic DNA. The first cycle of amplification is 95˚C 2 min, 30˚C 10 min, 42˚C for 20 min and then another 25 cycles of 94˚C 30 s, 42˚C 30 s, 72˚C 30 s, at last 72˚C for 7 min. PCR products were digested with *Eco*RI/*Spe*I and then inserted to the same restriction sites of pHIS 2.1 (Clontech). The recombined DNA was transformed to *E. coli* Top 10 strain. The transformants grown on LB-ampicillin plates were washed into a flask containing LB medium and then plasmids were isolated using standard procedures. The insert size of library plasmids was determined by PCR with Insert-F (5'-GCCAGGGTTTTCCCAGTCAC-3') and Insert-R (5'-CTTCGTTTATCTTGCCTGCTCA-3') using library plasmids and empty pHIS2.1 plasmid (negative control template), respectively.

2.6. Inverse Yeast One-Hybrid Screen

The primer pairs 5'-CGGAATTCGCTGCTTCTTTTCTTACAATGG-3' (*Eco*RI site is underlined) and 5'-CGGGATCCTCAGGGCATAAACGAATCGAAA-3' (*Bam*HI site is underlined) were used to amplify the coding sequence of WRKY33. The resulting PCR products were cloned into the *Eco*RI/*Bam*HI sites of pGAD424 vector (Clontech) and sequenced to yield bait plasmid AD-WRKY33. AD-WRKY33 plasmids were transferred to yeast strain Y187 (Clontech) by the standard LiAc-PEG-mediated transformation procedure.

The library plasmids were transferred to Y187 containing AD-WRKY33. Aliquots (100 μl) of each distilled water-diluted transformants were spread onto synthetic defined (SD) medium lacking Trp, Leu, and His and containing 25 mM 3-amino-1,2,4-triazole (3-AT). The library plasmids were rescued from positive yeast clones, amplified in *E. coli* Top 10, and verified by sequencing using Insert-F and Insert-R.

2.7. Yeast Two-Hybrid Analysis

For the construction of prey, the coding sequence of-MYB46 or VND7 was amplified from *Arabidopsis* cDNA. The following primers were used: MYB46, 5'-CGGAATTCGCAGCTAGTACTCACCAAGTAAAG-3' and 5'-AACTGCAGTAAGAATCTTGAGTATAAACCGC-3', VND7, 5'-CGGAATTCGATAATATAATGCAATCGTCAATG C-3' and 5'-AACTGCAGTTACGAGTCAGGGAAGCATCC-3'.

The underlined sequences of above primers indicate the *Eco*RI and *Pst*I sites. The resulting PCR products were then inserted into *Eco*RI/*Pst*I sites of pGBKT7 (Clontech) to fuse to GAL4 BD, designed BD-MYB46 and BD-VND7, respectively. The different combinations of AD-WRKY33/BD-MYB46, AD-WRKY33/BD-VND7, and pGAD424/pGBKT7 (negative control) were co-transformed into yeast strain PJ69-4A. The interaction was evaluated by spot assay and LacZ-filter lift assay.

The bait and prey plasmids were rescued from yeast clones grown on selective medium, amplified in *E. coli* Top 10, and verified by sequencing.

2.8. Transcriptional Activation Activity Assay in Yeast

Transcription activation assay of *CesA*8 promoter and AD-WRKY33 in yeast cells was designed as described previously [13]. A *Xba*I-*Sac*I fragment containing *β*-glucuronidase (GUS) coding region from pBI121 (Clon-

tech) was inserted into the *Xba*I/*Sac*I sites of YEplac181 to construct YEplac181-GUS. The *Xba*I-*Sac*I fragment containing the GUS coding region from YEplac181-GUS was subcloned into pYES to generate pYES-GUS. Two antiparallel oligonucleotides of an internal deleted *CesA*8 promoter fragment (*ProCesA*8) and its mutated version (*ProCesA*8*m*) with overhanging *Hin*dIII and *Xba*I sticky ends were synthesized and cloned to the upstream *Hin*dIII/*Xba*I sites of pYES-GUS to yield *ProCesA*8*:GUS* and *ProCesA*8*m:GUS*, respectively.

The different combinations of AD-WRKY33/*ProCesA*8*:GUS* and AD-WRKY33/*ProCesA*8*m: GUS* were co-transformed into PJ69-4A. The positive transforms were grown in SD/-Leu-Ura liquid medium. The method for measuring GUS activity was adapted from [13]. Briefly, 7.5×10^7 cells of each transformated yeast line were collected by centrifugation at 8000 rpm for 5 min. 100 µl GUS staining buffer were added to the yeast pellet, followed by vortexing and three freeze-thaws in liquid nitrogen. The reaction was incubated at 37˚C for 12 h. Three independent clones for each transformant were analyzed.

3. Results and Discussion

3.1. Mutation in WRKY33 Causes a Moderate Decrease in Drought Tolerance

To explore whether WRKY33 was involved in drought, we analyzed the drought tolerance in a homozygous T-DNA insertion mutant line (*wrky*33-1) from the Arabidopsis Biological Resource Center (**Figure 1(a)**). The primers around the T-DNA insertion site produced a single larger RT-PCR product (783 bp) in *wrky*33-1 compared with the wild type (724 bp) as previously described [10], demonstrating that *wrky*33-1 is a homozygous mutant line (**Figure 1(b)**). By cloning and sequencing of this larger RT-PCR product, we revealed that an extra 59-bp sequence is inserted between the exon2 and the exon 3. This extra sequence T-DNA insertion leads to a frame-shift and premature stop codon within the exon 3. This could result in the production of small truncated WRKY33 protein which has 216 amino acid in length (referred to as S-WRKY33). S-WRKY33 contains a N-terminal region of WRKY33 (residues 1 to 150) and an additional 66 amino acids (residues 151 to 216). Thus, *wrky*33-1 is a deletion mutation line. Because S-WRKY33 lacks two WRKY domains, it cannot act as a WRKY33.

With this *wrky*33-1 deletion mutant line, we performed dehydration-rehydration assay and soil drought experiment. Rehydration for 4 days recovered the dehydration-treated detached rosette leaves of wild-type and *wrky*33-1. After another 6 days of rehydration, most of *wrky*33-1 rosette leaves became bleached and were dead; whereas, wild-type still survived (**Figure 1(c)**). After two-week soil drought treatment, *wrky*33-1 plants showed more severe leaf-wilting phenotype than wild-

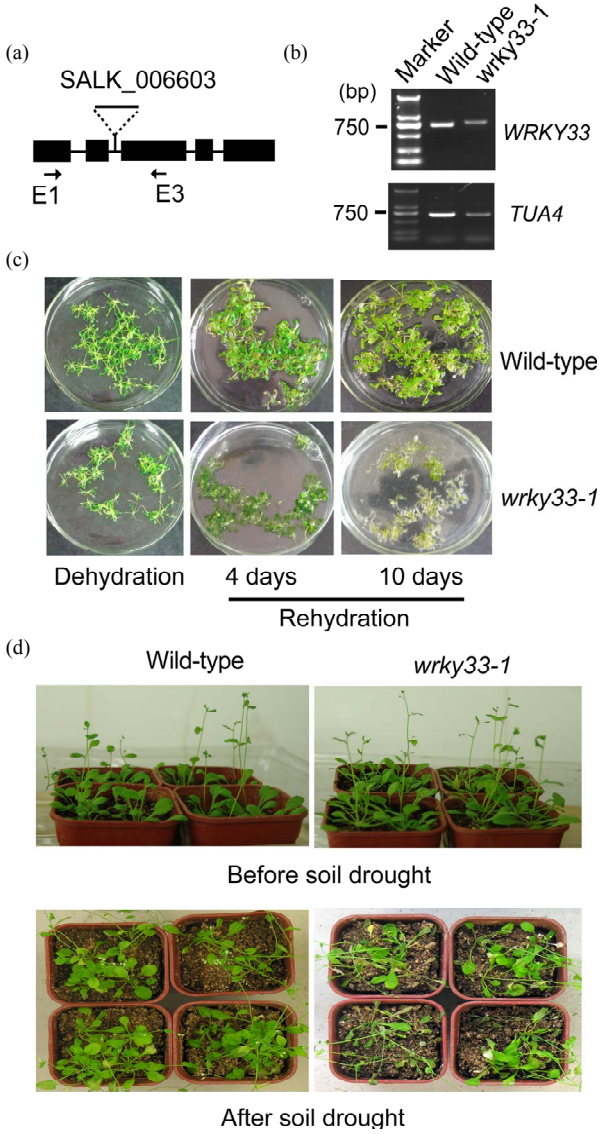

Figure 1. Response of *wrky*33-1 seedlings to drought stress (a) The T-DNA was inserted in the second intron of the WRKY33 genomic DNA. Black boxes and lines denote exons and introns, respectively. Arrows indicated the primers that were used to RT-PCR confirmation of *wrky*33-1 mutant. (b) RT-PCR analysis of wild-type and *wrky*33-1 plants using the exon 1-specific forward primer (E1) and the exon 3-specific reverse primer (E3). *TUA*4 (At1g04820), encoding an α-tubulin isoform, served as a loading control. (c) Dehydration-rehydration assay of detached rosette leaves of wild-type and *wrky*33-1 was performed as described in Materials and methods. The photographs were taken after dehydration and at 4 days or 10 days of rehydration, respectively. (d) Phenotype analysis of three-week-old *wrky*33-1 or wild-type plants under soil drought (without watering). The photographs were taken before and two weeks after water stress, respectively.

type plants (**Figure 1(d)**). These results confirmed that *wrky*33-1 is more sensitive to drought than wild-type. The decrease in drought tolerance caused by *WRKY*33 mutation suggested the involvement of WRKY33 in drought. Moreover, the moderate drought-sensitive phenotype of *wrky*33-1 may reflect WRKY33 functional redundancy with other transcription factors in drought tolerance. Similarly, WRKY33 is involved in thermotolerance interacting with WRKY25 and WRKY26 in Arabidopsis [11].

3.2. Inverse Yeast One-Hybrid Assay Identifies 23 Candidate WRKY33 Target Genes

Identifying the downstream target genes of WRKY33 is crucial in understanding its molecular mechanism in response to drought stress. Zeng *et al.* [14] develop an inverse yeast one-hybrid (Y1H) system which allows rapid and genome-wide identification of transcriptional binding targets. We modified this inverse Y1H system to screen the candidate target genes of WRKY33 (**Figure 2(a)**). In this system, yeast Gal4 activation domain (AD)-WRKY33 fusion protein was used as bait to screen a genomic library enriched with W-box sequences.

W-box enriched genomic fragments were obtained by PCR using primers containing W-box sequences (**Figure 2(b)**) and then cloned upstream of the *HIS*3 reporter gene to construct W-box enriched genomic library. The recombinant ratio and insert size of genomic library plasmids were estimated by PCR using the primers around the insertion sites (**Figure 2(a)**). Most of PCR products amplified from the library plasmids were larger than that from empty plasmid control (**Figure 2(c)**), suggesting that most of library plasmids contained insert DNA. The size of insert DNA varied from approximately 50 bp to 200 bp by subtracting 197 bp representing the empty vector control (**Figure 2(c)**).

After transforming the library plasmids into the yeast containing AD-WRKY33, we got sixty positive clones by using HIS-free selective medium. Using the sequenced insert DNA as queries, 23 candidate target genes were identified by screening 3000 bp of upstream sequence of all known genes in the *Arabidopsis* genome. These putative target genes of WRKY33 encode proteins related with various biological functions (**Table 1**). Three salt stress-related genes (*CesA*8, *HOS*3-1, and *EDGP*) and two defense-related genes (*MED*8 and At4g13580) were included. This result was consistent with the roles of WRKY33 in salt stress [10] and pathogens-induced defense [4,5].

3.3. WRKY33 Binds to the *CesA*8 Promoter in Yeast

Among the 23 candidate genes, *CesA*8 has been reported

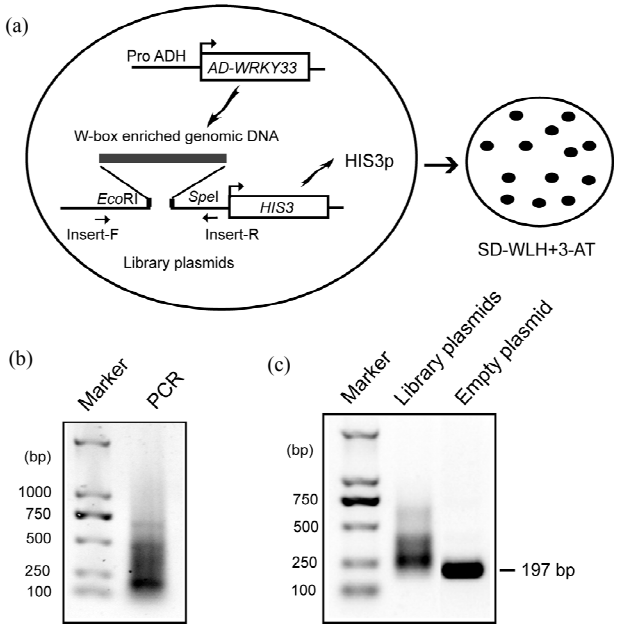

Figure 2. Genome-wide screening target genes of WRKY33 in yeast. (a) Strategy of the modified inverse Y1H screening to identify the WRKY33 target genes. The W-box enriched genomic library plasmids were transformed into the yeast strain Y187 containing AD-WRKY33 bait. AD-WRKY33 binds to the target DNA fragment enriched for W-box sequences to result in generation of HIS3 protein (HIS3p) and permit yeast growth on SD medium lacking Trp, Leu, and His (SD-WLH) and containing 3-amino-1,2,4-triazole (3-AT, a competitive inhibitor of the HIS3p). (b) Agarose gel analysis of PCR products enriched for W-box sequences. (c) Agarose gel analysis of amplicons from library plasmids and empty plasmid using Insert-F/Insert-R (as shown in Figure 2(a)).

to confer drought tolerance. *CesA*8 encodes a member of the cellulose synthase family required for secondary cell wall biosynthesis [15]. Disruption of this gene enhances tolerance to drought stress [16]. Therefore, we asked whether *CesA*8 was involved in WRKY33-mediated drought tolerance.

Results from the inverse Y1H experiment (**Table 1**) suggest that WRKY33 binds to the distal *CesA*8 promoter region (-2988 to -2969) containing two repeat W-box elements (5'-TTGACT-3'). We used the yeast transcriptional activation assay developed by Yuan *et al.* [13] to verify this binding. The WRKY33-binding distal *CesA8* promoter region or its W-box mutated version fused to the minimal *CesA*8 promoter (designated *ProCesA*8 and *ProCesA*8*m*, respectively) were cloned into the upstream of the *GUS* reporter gene to construct two reporter plasmids (*ProCesA*8:*GUS* and *ProCesA*8*m*:*GUS*), respectively (**Figure 3(a)**). As expected, co-expression of AD-WRKY33 and *ProCesA*8:*GUS* in yeast cells resulted in strong blue color, whereas, co-expression of AD-WRKY33 and *ProCesA*8*m*:*GUS* only caused

Table 1. Candidate target genes of WRKY33.

Locus	Binding Region[a]	Gene description
At2g03050	−2247 to −2232	A locus involved in embryogenesis (EMB93)
At2g03070	−2439 to −2424	Regulates plant defense and flowering (MED8)
At5g43020	−2615 to −2601	Leucine-rich repeat protein kinase family protein
At5g16330	−694 to −680	NC domain-containing protein-related
At1g45229	−659 to −645	Unknown protein
At1g45246	−1978 to −1964	pre-tRNA; tRNA-Pro (anticodon: TGG)
At1g45242	−484 to −470	pre-tRNA; tRNA-Pro (anticodon: TGG)
At1g45244	−925 to −911	pre-tRNA; tRNA-Pro (anticodon: TGG)
At4g13580	−2762 to −2741	Disease resistance-responsive family protein
At4g36840	−2801 to −2786	Galactose oxidase/kelch repeat superfamily protein
At4g36830	−884 to −869	Response to salt stress (HOS3-1)
At1g59710	−164 to −151	Unknown protein
At1g03230	−839 to −825	Eukaryotic aspartyl protease family protein (EDPG). Response to salt stress
At4g18790	−114 to −100	Member of Nramp2 family (NRAMP5)
At4g18780	−2988 to −2969	Cellulose synthase 8 (CesA8) involved in drought and osmotic stress tolerance
At4g08150	−438 to −424	A member of class I knotted1-like homeobox gene family (KNAT1)
At1g66783	−284 to −270	Encodes a microRNA that targets several SPL family members (MIR157A)
At5g01250	−2943 to −2925	alpha 1,4-glycosyltransferase family protein
At5g51570	−2849 to −2835	SPFH/B and 7/PHB domain-containing mem brane-associated protein family (HIR3)
At5g14011	−581 to −568	A small protein and has either evidence of transcription or purifying selection
At5g42480	−174 to −161	Unknown protein
At4g08810	−668 to −655	Calcium binding protein involved in cryptochrome and phytochrome coactions (SUB1)
At1g59690	−2394 to −2381	F-box associated ubiquitination effector family protein

[a]The WRKY33-binding promoter region containing W-box sequences is defined relative to transcriptional start site (+1) of target gene.

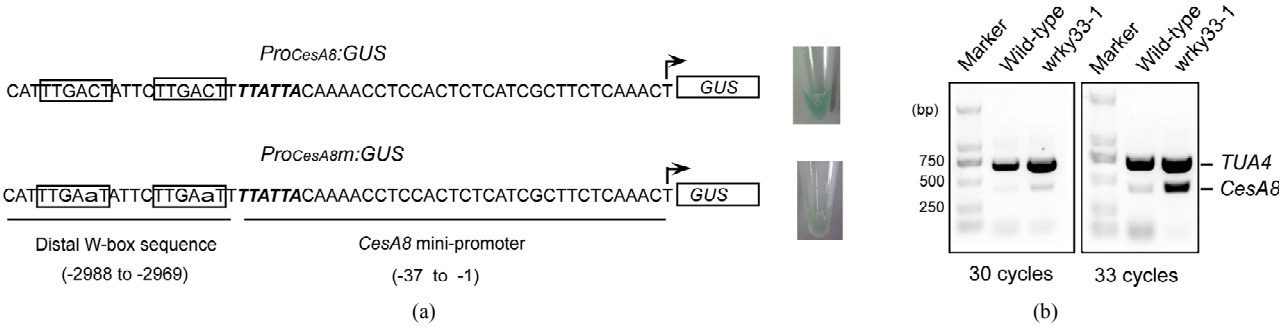

Figure 3. Characterization of WRKY33-mediated *CesA*8 expression. (a) The yeast transcriptional activation assay of WRKY33-mediated *CesA*8 promoter expression. The sequences of the internal deleted *CesA*8 promoter and its W-box mutated (lowercase) version were shown in left panel. The arrow indicated transcriptional start site. The W-box sequence was boxed and TATA-box was shown in italics. The corresponding GUS staining was shown in right panel. Three independent positive clones for each transformant were analyzed and similar results were obtained. (b) Validation of differential expression for *CesA*8 in wild-type and *wrky*33-1 plants by semi-quantitative duplex RT-PCR. *TUA*4 was used as a loading control.

very weak blue color. This may result from the failure of WRKY33 in binding to the mutated W-boxes in *Pro-CesA*8*m*. Thus, WRKY33-mediated *CesA*8 mini-promoter activity required the distal W-boxes in yeast. This result confirmed that WRKY33 bound to the W-box sequences localized in the distal *CesA*8 promoter region in yeast.

3.4 *CesA*8 Is Up-Regulated in *wrky*33-1

To further confirm whether *CeA*8 is a direct target gene of WRKY33 in *Arabidopsis*, we therefore analyzed the expression of *CesA*8 in *wrky*33-1 plants. Transcript abundance of *CesA*8 was determined using semi-quantitative duplex RT-PCR assay which allows amplification of *CesA*8 and an internal control *TUA*4 gene in a single reaction. Result showed that *CesA*8 mRNA accumulation was greatly increased in *wrky*33-1 compared with the wild-type line (**Figure 3(b)**). The down-regulation of *CesA*8 in *wrky*33-1 confirmed that *CesA*8 was a direct target gene of WRKY33. And WRKY33 may serve as a negative transcription regulator of *CesA*8.

3.5. WRKY33 Interacts with MYB46 in Yeast

As a transcriptional repressor of *CesA*8, WRKY33 may interact with other transcriptional factors of *CesA*8 to regulate *CesA*8 expression. Recently, VND7 (At1g71930) and MYB46 (At5g12870) are identified to be the transcriptional activators of *CesA*8 expression [17,18]. We performed yeast two-hybrid (Y2H) assay and showed that WRKY33 could interact with MYB46 but not VND7 to activate the expression of both *HIS*3 and *LacZ* reporter genes (**Figure 4**). The interaction between WRKY33 and MYB46 indicates that WRKY33 may regulate the down target genes of MYB46 including *CesA*8.

3.6. *CesA*8 Connects WRKY33 with Its Role in Drought Stress

Collectively, the results from altering expression of *CesA*8 in *wrky*33-1 and the *CesA*8 promoter-binding ability of WRKY33 suggested that *CesA*8 was a direct target gene of WRKY33. WRKY33 negatively regulated *CesA*8 expression through binding to the W-box elements of *CesA*8 distal promoter region and probably through forming a heterodimer with MYB46. Above conclusion is mainly based on a yeast heterologous system and thus poses a limitation in our study. Therefore, we need to be further verified these in *Arabidopsis* plants.

Because mutation in *CesA*8 leads to enhanced tolerance to drought stress, salt stress, and pathogen resistance [16,19], the increased accumulation level of *CesA*8 mRNA in *wrky*33-1 plants can partly explain its drought- or salt stress-sensitive phenotype [10] and enhanced pathogen susceptibility [4,5]. Therefore *CesA*8 links

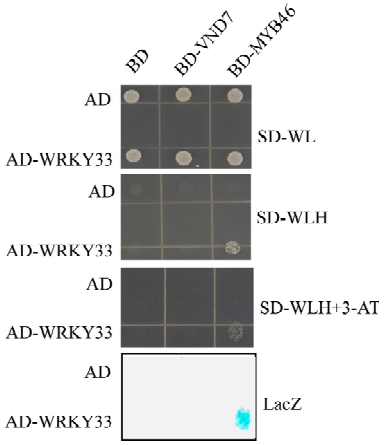

Figure 4. Y2H interaction assay of WRKY33 with MYB46 or VND7. The yeast strain PJ69-4A was co-transformed with bait (AD-WRKY33) and prey (BD-MYB46 or BD-VND7) construct. The empty bait (AD) or prey (BD) vector was used as a negative control. Protein-protein interaction was examined by colony filter-lift assay (*LacZ*) and by cell growth on plates with SD-WLH medium or with same medium containing 5 mM 3-AT. The experiments were performed three times with consistent results.

WRKY33 with its roles in response to drought, salt stress [10] and pathogens deference [4,5].

Besides *CesA*8, five genes (At3g60120, At4g16260, At5g24540, At5g24550, and At5g24090) encoding glycosyl hydrolase, which play a role in cell wall remodeling, are identified as the target genes of WRKY33 [10]. These data as well as our results in this study indicate that WRKY33 may be involved in transcriptional regulation of cell wall synthesis- and remodeling-related genes.

In conclusion, our results revealed that WRKY33 was involved in drought partly through a direct transcriptional repression of *CesA*8. These findings enriched our understanding of the function and molecular mechanism of WRKY33 in drought stress. Further examination of drought-related genes resulting from high *CesA*8 mRNA accumulation will be an interesting line to provide more insights into the molecular mechanism of WRKY33 in drought stress signaling.

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