Yellow nutsedge WRI3/4-like gene improves drought tolerance in Arabidopsis thaliana by promoting cuticular wax biosynthesis

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Abstract
In this study we cloned a WRI1-like gene from yellow nutsedge. Conserved domain and phylogenetic analyses indicated it to be a WRI3/4-like gene. Arabidopsis plants transformed with WRI3/4-like gene showed significantly improved tolerance to both PEG-simulated drought stress and real dehydration compared with the wild type. Quantitative RT-PCR indicated that, under unstressed conditions, the expressions of key genes involved in fatty acid biosynthesis was not significantly different between wild type (WT) and transgenic lines, while the expressions of genes involved in cuticular wax biosynthesis was significantly higher in transgenic lines compared with the wild type. The PEG treatment slightly decreased the expression of above mentioned genes in WT plants while it was significantly increased in transgenic lines compared with their respective unstressed control. Without PEG treatment, the expression of TAG1, the gene involved in triacylglycerol (TAG) accumulation, was 10-40% lower in the transgenic lines than that in the wild type. However, after PEG treatment, the expression of TAG1 was slightly decreased in the wild type, while in the transgenic lines its expression was decreased by 20-70% compared with unstressed transgenic lines and was highly significantly lower than that in the wild type. The cuticular wax content in Arabidopsis leaves was significantly higher in the transgenic lines than that in the wild type, while the oil content was not significantly different.

Background
Cyperus esculentus (yellow nutsedge) was considered to be native to areas in Africa and tropical Asia [1-3], or tropical and subtropical regions including Africa, Asia, Europe, and North America [4, 5]. It is usually considered a weed as its tubers and seeds can be easily dispersed and contaminate crop seeds [6-8]. In recent years it is being developed as a new oil crop as its tubers contain substantial amounts of starch, oil, sugar, protein, fiber [9, 10] and high levels of phosphorus, iron, potassium, vitamins E and C [11].

The AP2 (APETALA2)/EREBP (Ethylene Responsive Element Binding Protein) transcription factors contain either a single or two AP2/ERF domains and are associated with responses to various biotic and abiotic stresses [12]. The AP2/EREBP genes could be classified into five groups, i.e., AP2
subfamily with two AP2/ERF domains, DREB and ERF subfamilies with one AP2/ERF domain, RAV subfamily with one AP2/ERF domain and one B3 DNA-binding motif, and others [13-15]. The AP2 subfamily was further divided into two monophyletic groups, i.e., AP2 and ANT [16].

Cernac and Benning (2010) cloned WRI1 from Arabidopsis. Since it contained two AP2/EREB domains it was placed into the AP2 subfamily [17]. The WRI1-like group, composed of WRI1 (At3g54320), WRI2 (At2g41710), WRI3 (At1g16060) and WRI4 (At1g79700), was either considered as part of the ANT-like group [18] or as a monophyletic group of the AP2 subfamily [19]. WRI1, WRI3 and WRI4 were shown to be involved in activation of fatty acid biosynthesis [17, 19] while WRI4 was also shown to control cuticular wax biosynthesis in Arabidopsis stems and thus involved in drought tolerance [20].

In this study we isolated a WRI3/4-like gene with two AP2/ERF domains from yellow nutsedge. Phylogentic analysis indicated that the its encoded peptide was more closely related to WRI3 and WRI4. The nutsedge WRI3/4-like gene was introduced into Arabidopsis under the control of constitutive CaMV35S promoter. Transgenic Arabidopsis plants showed significantly improved drought tolerance compared with the wild type.

Results

Cloning, expression and phylogenetic analyses of WRI1-like gene from yellow nutsedge

A 490 bp fragment corresponding to the conserved region of nutsedge WRI1-like gene was amplified by using degenerated primers (Additional file 1). Subsequently, a 1423bp full length fragment was obtained by using primers based on the 5’- and 3’-RACE results (Additional file 1). This gene contained an ORF of 1098 bp, while the sequences before the start codon and after the termination codon being the 5’UTR and 3’UTR, respectively (Fig. 1).

The expression analysis of WRI1-like gene showed the highest expression in the leaves, followed by that in the roots, while the lowest expression was found in tuber (Additional file 2). Conserved domain analysis (https://www.ncbi.nlm.nih.gov/Structure/cdd/) indicated that this WRI1-like gene contained two AP2 domains. Multiple alignment analysis indicated that the second AP2 domain was more conserved than the first one (data not shown). Phylogenetic analyses indicated that AP2, ANT and
WRIs clustered into three major groups and the nutsedge \textit{WRI1}-like peptide was more closely related to \textit{WRI3} and \textit{WRI4} (Fig. 2). Therefore, this gene is named as nutsedge \textit{WRI3/4} (\textit{CeWRI3/4}).

\textbf{CeWRI3/4 improves drought tolerance in transgenic Arabidopsis}

\textbf{Tolerance to PEG-simulated drought stress}

For functional characterization of the cloned gene, Arabidopsis thaliana plants were transformed with \textit{CeWRI3/4} gene and the drought tolerance of the transgenic plants was determined. Seed germination of both wild type and transgenic Arabidopsis was higher than 90\% under non-stress and PEG-simulated stress conditions (data not shown). We also determined drought tolerance at seedling stage. Without PEG stress the growth of 10-day-old wild type plants was not significantly different from the transgenic lines B1 and K2 (Fig. 3A). On the other hand, 14-day-old wild type seedlings grown on PEG-medium had only two very small yellow true leaves and the growth of primary roots was severely inhibited with much fewer lateral roots, while the transgenic seedlings contained 4 much larger true leaves and the primary and lateral roots were not significantly inhibited (Fig. 3A). The root length and seedling fresh weight (FW) data also confirmed that the transgenic lines had better tolerance to PEG-simulated drought stress (Fig. 3B-C).

\textbf{Tolerance to real dehydration}

The growth of wild type and transgenic Arabidopsis plants was not different before the onset of dehydration stress (Fig. 4). After 15 days of dehydration, the wilting frequency reached 63.6-81.8\% in the wild type, while in the transgenic lines it was only 18.2-25\% (Fig. 4-5). After 22 days of dehydration, the stressed plants were re-watered, and the extent of recovery was determined 1 day after resumption of the watering. The transgenic lines showed significantly higher recovery frequency i.e 58.3-63.6\% compared with 18.1-36.3\% in wild type. (Fig. 4-5). Based on these data, it can be concluded that \textit{CeWRI3/4} improves plant drought stress tolerance.

\textbf{Expression of key genes involved in fatty acid and cuticular wax biosynthesis is modulated in transgenic lines}
Keeping in view the role of CeWRI3/4, and to further understand the drought tolerance phenotype, we attempted to explore the expression of key genes involved in fatty acid and cuticular wax biosynthesis. Quantitative RT-PCR data indicated that under unstressed conditions the expression of key genes involved in fatty acid biosynthesis such as PIPK-β1 (At5g52920), BCCP2 (At5g15530) and PDHE1α (At1g01090) [19], was not significantly different between wild type and transgenic Arabidopsis lines. However, 10hr after 5% PEG treatment, the expression of above genes was slightly lower in the wild type compared to their unstressed counterparts. While in the transgenic lines the expression of PIPK-β1, BCCP2 and PDHE1α was increased by 130-230%, 50-100% and 130-220% respectively, compared with that without PEG treatment (Fig. 6A-C).

Without PEG treatment, the expression of TAG1 (At2g19450), a gene involved in TAG accumulation [21], was 10-40% lower in transgenic lines compared with the wild type. After PEG treatment, the expression of TAG1 was only slightly decreased in the wild type, while in the transgenic lines this decrease was 20-70% compared with that without PEG treatment (Fig. 6D) and was significantly lower than wild type.

Without PEG treatment the expression of LACS1 (At2g47240), WSD1 (At5g37300)|KCS1 (At1g01120)|CER1 (At1g02200) and CER4 (At4g33790), the genes involved in cuticular wax biosynthesis [20], was 10-160%, 50-140%, 30-70%, 50-270% and 90-130% higher in the transgenic lines compared with that in the wild type. After PEG treatment the expression of above mentioned genes was slightly decreased compared with that without PEG treatment in the wild type, while in the transgenic lines the expression of LACS1, WSD1KCS1CER1 and CER4 was 130-300%, 30-80%, 20-80%, 10-110%, 80-140% higher than that without PEG treatment (Fig. 6E-I). Over all, these results show that expression of key genes involved in fatty acid and cuticular wax biosynthesis is differentially modulated in transgenic lines.

Soluble sugars, free proline and MDA content in wild type and transgenic Arabidopsis

After two weeks of real dehydration, the concentration of soluble sugars, free proline and MDA in the wild type was found to be 27.1mg/g (Fresh Weight, FW), 69.4 μg/g (FW) and 5.3 mmol/g (FW),
respectively, while in the transgenic lines the soluble sugars, free proline and MDA content was only 14.3-29.9% (Fig. 7A), 26.9-50.6% (Fig. 7B), 36.5-45.5% (Fig. 7C) of the wild type, respectively.

Cuticular wax and oil content in the wild type and transgenic Arabidopsis lines

In this study it was found that in both wild type and transgenic Arabidopsis leaves the cuticular wax was mainly composed of alkanes and primary alcohols. C29 and C31 of the alkanes amounted to about 68% of the total cuticular wax, while for alkanes C31 was the predominant composition (Fig. 8A-B). In transgenic lines the contents of total cuticular wax, C26, C28 and C30 of primary alcohols, and C27 and C31 of alkanes, were all significantly higher than those in the wild type, while the contents of C33 of alkanes and C32 of primary alcohols, were not significantly different (Fig. 8A-C). The content of C29 in alkanes was significantly different between wild type and transgenic line B1, but not between wild type and transgenic line K2 (Fig. 8A). The oil content in Arabidopsis leaves was 6.32-6.51mg/g and there was no significant difference between wild type and transgenic lines (Additional file 3).

Discussion

WRI3/4 nature of the nutsedge gene

AP2/EREB genes could be classified into five groups: AP2 subfamily with two AP2/ERF domains, DREB and ERF subfamilies with one AP2/ERF domain, RAV subfamily with one AP2/ERF domain and one B3 DNA-binding motif, and others [13-15]. The AP2 subfamily was further divided into two monophyletic groups: AP2 and ANT [16]. The WRI1-like group, composed of WRI1, WRI2, WRI3 and WRI4, was either considered as part of the ANT-like group [18] or as a monophyletic group of the AP2 subfamily [19]. Here in this study, it was found that the nutsedge WRI1-like gene contained two AP2/EREBP domains therefore it should belong to the AP2 subfamily. Phylogenetic analyses indicated that the two-AP2-domain-containing genes clustered into three major groups: AP2, ANT and WRI. In the WRI group the WRI1s were clearly separated from WRI2s, WRI3s and WRI4s, and the nutsedge gene was more closely related to WRI3s and WRI4s. Therefore, this nutsedge WRI1-like gene is named as nutsedge WRI 3/4.
Biosynthesis of fatty acids and cuticular wax and drought tolerance

Cuticular wax plays important roles in protecting plants from environmental stresses [25]. Overexpression of genes like SHN1/WIN1, SHINE1, MdSHINE2, MYB96 and MYB94 increased drought tolerance by increasing cuticular wax biosynthesis [26-32]. It was shown that WRI4 interacted with LACS1, KCR1, PAS2, ECR, CER4, WSD1 and MAH, the genes involved in fatty acid elongation of wax precursors and production of wax esters, and the genes like BCCP1 and BCCP2 involved in fatty acid biosynthesis, by direct binding to their promoters [19, 20]. Disruption of WRI4 led to down-regulation of above genes and other genes such as PKP1, PKP2, PDHE1α and ENR1 involved in fatty acid biosynthesis [20].

In this study it was shown that without PEG treatment the expression of key genes such as PIPK-β1, BCCP2 and PDHE1α involved in fatty acid biosynthesis was not significantly different between wild type and transgenic Arabidopsis line. However after PEG treatment the expression of above genes was slightly lowered in the wild type, while in the transgenic lines the expression of PIPK-β1, BCCP2 and PDHE1α was significantly increased compared with that without PEG treatment (Fig. 6A-C).

Without PEG treatment, the expression of LACS1, WSD1KCS1CER1 and CER4, genes involved in cuticular wax biosynthesis was 10-160%, 50-140%, 30-70%, 50-270% and 90-130% higher than that in the wild type. After PEG treatment the expression of above mentioned genes was only slightly decreased compared with that without PEG treatment in the wild type, while in the transgenic lines the expression of above mentioned genes was 130-300%, 30-80%, 20-80%, 10-110%, 80-140% higher than that without PEG treatment (Fig. 6E-I). These findings are consistent with the cuticular wax data.

In the transgenic lines the contents of total cuticular wax, C26, C28 and C30 of primary alcohols, C27 and C31 of alkanes, were all significantly higher than those in the wild type (Fig. 8). This might explain why the transgenic lines exhibited significantly improved tolerance to PEG-simulated and real dehydration stresses (Fig. 3-5).

Without PEG treatment, the expression of TAG1 [21] was 10-40% lower in the transgenic lines compared with that in the wild type (Fig. 6D). This could be the possible reason for no significant
difference in oil content between wild type and transgenic lines, even though the expression of genes involved in fatty acid biosynthesis (\textit{PIPK-\beta1}, \textit{BCCP2} and \textit{PDHE1\alpha}) was significantly higher in transgenic lines than wild type.

**MDA, free proline, soluble sugars and drought tolerance**

MDA is the product of membrane peroxidation and is usually used as an indicator of lipid peroxidation and membrane damage [33-35]. It has been observed that plants accumulate osmolytes such as soluble sugars and proteins to maintain osmotic equilibrium and the integrity of membranes when they are subjected to drought stress [36, 37]. Many plants change their osmotic adjustment abilities to resist drought stress via accumulating proline and soluble sugars, which participate in osmotic protection [38, 39]. In current study, after two weeks of real dehydration, the concentration of soluble sugars, free proline and MDA in the wild type reached 27.1mg/g (FW), 69.4 \mu g/g (FW) and 5.3mmol/g (FW), respectively, while in the transgenic lines these figures were only 14.3-29.9\% (Fig. 7A), 26.9-50.6\% (Fig. 7B), 36.5-45.5\% (Fig. 7C) of the wild type, respectively, suggesting that the transgenic lines suffered less lipid peroxidation and membrane damage and were more tolerant to drought stress.

**Conclusions**

\textit{WRI3/4}-like gene from \textit{Cyperus esculentus} could improve drought tolerance probably by promoting cuticular wax biosynthesis and might be useful for genetic engineering to improve drought tolerance in crops.

**Methods**

**Plant materials and treatments**

\textit{Arabidopsis thaliana} ecotype Columbia and tubers of \textit{Cyperus esculentus} (yellow nutsedge) cv. Hubu-1 were provided by State Key Laboratory of Biocatalysis, College of Life Sciences, Hubei University, China. The yellow nutsedge tubers were germinated at 22°C under a 16 h light/8 h dark cycle. Leaves and roots were collected and flash frozen in liquid nitrogen and stored at -80°C. Fresh and tender tubers were collected from plants growing in the field, flash frozen in liquid nitrogen and stored at
-80°C for total RNA extraction.

**Cloning of WRI3/4 from yellow nutsedge**

WRI1 protein sequences from different plant species (*Solanum tuberosum*, accession No.: AAA66057; *Nicotiana tabacum*, accession No.: ABD60582; *Fragaria x ananassa*, accession No.: AAS00541; *Gladiolus* hybrid cultivar, accession No.: AHN15416; *Triticum aestivum*, accession No.: AAF61173; *Hordeum vulgare*, accession No.: AAU06191; https://www.ncbi.nlm.nih.gov/) were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). One pair of degenerate primers (Additional file 1) was designed based on the highly conserved region to amplify the conserved region of the CeWRI1-like gene using cDNAs from leaf, root, and tuber as templates. 5'- and 3'-RACEs were performed using the SMART RACE Amplification kit (Clontech Laboratories, Mountain View, CA) with gene-specific 5'-RACE and 3'-RACE primers, respectively, in order to clone the upstream and downstream sequences of CeWRI3/4 (Additional file 1). One pair of primers (Additional file 1) was designed based on the 5'- and 3'-RACE sequences to amplify the full cDNA length of CeWRI3/4 gene.

**Sequence and Phylogenetic analyses**

Open reading frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to find the ORFs and to deduce the amino acid sequence. Conserved domains of the deduced CeWRI1-like protein were analyzed using the NCBI CD-Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The phylogenetic tree of the two-AP2-domain-containing genes was constructed using MEGA 7.0 software using the neighbor joining method with 1,000 bootstrap replicates [40].

**Construction of CeWRI3/4 over-expression vector and genetic transformation of Arabidopsis thaliana**

A PCR product of 1423bp containing complete ORF of CeWRI3/4 was digested with PstI and SalI and then placed between CaMV 35S (35S) promoter and nos terminator of pCambia 2300-35S-nos [41] to form vector pCambia 2300-35S-CeWRI3/4-nos and sequencing was performed to insure that the construct was correct.

Arabidopsis wild type seeds were surface sterilized and grown on 1/2 Murashige and Skoog (MS) agar
plates containing 3% sucrose in a growth chamber set to 16 h light/8 h dark (70–80 lm/m²/s) at 22°C after 2 days of stratification at 4 °C. Fifteen-day-old plants were transferred onto soil and grown in a growth chamber at 16 h light/8 h dark (70–80 lm/m²/s) and 22°C. The pCambia 2300-35S-CeWRI3/4-nos vector was introduced into Agrobacterium tumefaciens EHA105 and then transformed into Arabidopsis by floral-dip transformation method [42]. Transgenic plants (T1, T2, T3) were PCR-confirmed by using NPT II and CeWRI3/4 gene specific primers (Additional file 1).

Drought tolerance assessment

For PEG-simulated drought tolerance assessment, wild type and transgenic Arabidopsis seeds were sown on 1/2 MS agar medium with/without 5% PEG 6000. Germination rate of the wild type and transgenic Arabidopsis were scored 6 days after seed sowing. The length of the primary roots was recorded in 2, 4, 6, 8 days after seed germination.

For real dehydration, two-week-old seedlings germinated on 1/2 MS medium were transplanted into trays containing nutrient soil and vermiculite (a mixture of 50% nutrient soil and 50% vermiculite) and grown at 22 °C at a 16-h light/ 8-h dark photoperiod. Before drought stress the plants were watered every 3 days and photographed. 20-day-old Arabidopsis plants growing under normal condition were exposed to dehydration by stopping watering. Wilting frequency was scored after 15 days of dehydration. Seedlings were re-watered after 22 days of dehydration and 1 day later the recovery frequency was scored.

Leaves from wild type and transgenic lines after 15 days of dehydration were harvested for analyses of physiological indexes. Malondialdehyde (MDA) content in the leaves was measured using the thiobarbituric acid method described by Dhindsa and Matowe (1981) [43]. Analyses of contents of free proline and soluble sugars in Arabidopsis leaves were carried out as described by Troll and Lindsley (1955) [44] and Flood and Priestley (2010) [45], respectively.

RNA extraction and real-time quantitative reverse transcription qRT-PCR

24-day-old seedlings of wild type and transgenic plants were treated with water/5% PEG 6000 for 10
hrs, then fresh leaves were harvested and flash frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the Biospin Plant Total RNA Extraction Kit (Bioflux). Samples were treated with RNase-free Dnase (Bioflux) using on-column DNase digestion. cDNA was synthesized using the PrimeScript RT reagent Kit (TAKARA). Transcript levels were analyzed by qRT-PCR using BioEasy Master Mix SYBR Green (BIOER) and the Stratagene Mx3005P quantitative PCR system (Agilent Technologies). Primers used for qRT-PCRs are listed in Additional file 1. Primer pairs were selected based on 100% efficiency and single peaks. Relative mRNA levels were measured by the comparative threshold cycle method and normalized to ACTIN2 (At3g18780) expression.

Wax extraction and GC analysis
Cuticular waxes were extracted from leaves of 5- to 6-week-old wild type and transgenic Arabidopsis. Each tissue was immersed in 6 mL of chloroform for 30 sec, after which n-octacosane was included as internal standards. The solvent was evaporated under nitrogen gas at 40°C, followed by addition of 100 µL of bis-N, N trimethylsilyl trifluoroacetamide (BSTFA, Sigma, St. Louis, MO, USA) and 100 µL of pyridine. After treatment for 30 min at 90°C, the mixture was concentrated under nitrogen gas at 40°C. The samples dissolved in a mixture of 40 µl of heptane and 40 µl of toluene were separated and quantified by GC equipped with flame ionization detector (GC-2010, Shimazu, Tokyo, Japan). Retention times and temperatures for GC were programmed as described by Lee and Suh (2015) [30].

Statistical analysis
All experiments were carried out with three replicates. Significant differences were detected by t tests using the SPSS software (*P < 0.05; **P < 0.01).

Abbreviations
FW: Fresh weight
GC: Gas Chromatography
MDA: Malondialdehyde
MS: Murashige and Skoog
PEG: Polyethylene glycol
qRT-PCR: Quantitative reverse transcription Polymerase Chain Reaction
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Authors’ contributions

BH, XZ and JH conceived the experiments and finalized the paper, CC performed most of the experiments and prepared the draft manuscript, SH, YH, DX, B-L H and WW helped with material preparation, drought tolerance determination, qRT-PCRs and data analyses. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and/or analyzed in this study are available from the corresponding author on reasonable request.

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**

1. CCCCTCGTTTATAGCTCTCTCCTATTTCTTCTCTCCTTCTCTCCTTCTCTTATACATCCTCTGATATAAAA

61. CCCAACGCGCCCTACATATTGACACAAACCATTTAAATGGGTCATCCAAACAAATCTTCCCTATC

121. TCTACTTCTCTCCTCTCCTTTTTTTTCTTTGTTTTTTGTTTTTTCTAAAAACCCCA
Figure 1

Full-length of yellow nutsedge CeWRI3/4 cDNA sequence and the deduced amino acid sequence. The initiator and stop codons are marked by black bars. Sequences before initiator codon and after stop codon are the 5'-UTR and 3'-UTR, respectively.
Phylogenetic tree of the two-AP2-domain-containing genes from different plant species

Figure 2
Figure 3

A: 10-day-old wild type (WT) and transgenic lines B1 and K2 seedlings without PEG stress (up) and 14-day-old seedlings of wild type (WT) and transgenic lines B1 and K2 with PEG stress (down). Scale bar = 1cm; B: Fresh weight; C: Primary root length; **P < 0.01, *P < 0.05.
Figure 4

Morphology of wild type (WT) and transgenic lines B1 and K2.
Figure 5

Wilting frequency after 15 days of dehydration and recovery frequency 1 day after rehydration; **P < 0.01.
qRT-PCR analysis of genes involved in fatty acids and cuticular wax biosynthesis in wild type
(WT) and transgenic lines A5, A7, B1, K2; **P < 0.01, *P < 0.05.
Physiological indexes in wild type (WT) and transgenic Arabidopsis lines A5, A7, B1 and K2 with water deprivation. **P < 0.01.
Figure 8

Cuticular wax composition and content in leaves of wild type (WT) and transgenic lines B1 and K2. *P < 0.05.