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

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# Abstract

Using RACE PCR, full length WRI1-like gene was amplified from yellow nutsedge. Conserved domain and phylogenetic analyses suggested it as WRI3/4-like gene. Tissue-specific expression data showed the highest expression in leaves, followed by roots while the lowest expression was detected in tuber. Transgenic Arabidopsis plants expressing nutsedge WRI3/4-like gene showed significantly improved tolerance to both PEG-simulated drought stress and real dehydration, compared with the wild type (WT). Under normal growth conditions, the expressions of key fatty acid biosynthesis genes was not significantly different between WT and transgenic lines, while the expressions of genes involved in cuticular wax biosynthesis was significantly higher in transgenic lines compared with the WT. The PEG-simulated drought stress did not induce any significant change in the expression of fatty acid and wax biosynthesis genes in WT plants, while the expression of fatty acid and wax biosynthesis genes was significantly increased in transgenic lines compared with WT as well as unstressed transgenic control. The expression of TAG1, the gene involved in triacylglycerol (TAG) accumulation, was significantly lower in the transgenic lines than that in the WT in normal growth conditions. Drought stress slightly decreased the expression of TAG1 in the WT, but significantly lowered it in transgenic lines compared with its unstressed transgenic control and WT. Consistent with gene expression data, the cuticular wax content in Arabidopsis leaves was significantly higher in the transgenic lines than in the WT, while the oil content was not significantly different. Our results indicated that WRI3/4-like gene from Cyperus esculentus improves drought tolerance in Arabidopsis probably by promoting cuticular wax biosynthesis and, hence, could be a valuable target for improving drought tolerance in crops through recombinant DNA technology.


# Background

The aerial surfaces of terrestrial plants are covered with a cuticular wax layer that protects plants from environmental stresses such as desiccation and UV radiation [1]. Attempts have been made to improve drought tolerance by increasing the amount of cuticular wax in plant organs [2-8].

*Cyperus esculentus* (yellow nutsedge) is considered to be native to areas in Africa and tropical Asia [9-11], or tropical and subtropical regions including Africa, Asia, Europe, and North America [12, 13]. It is usually considered a weed as its tubers and seeds can be easily dispersed and contaminate crop seeds [14-16]. In recent years it is being developed as a new oil crop as its tubers contain substantial amounts of starch, oil, sugar, protein, fiber [17, 18] and high levels of phosphorus, iron, potassium, vitamins E and C [19].

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 [20]. 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 [21-23]. The AP2 subfamily is further divided into two monophyletic groups, i.e., AP2 and ANT [24]. Cernac and Benning cloned WRI1 from Arabidopsis. Since it contained two AP2/EREB domains it was placed into the AP2 subfamily [25]. 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 [26] or as a monophyletic group of the AP2 subfamily [27]. WRI1, WRI3 and WRI4 are involved in activation of fatty acid biosynthesis [25, 27] while WRI4 has been shown to control cuticular wax biosynthesis in Arabidopsis stems and thus involved in drought tolerance [1].

In this study we isolated a *WRI3/4*-like gene with two AP2/ERF domains from yellow nutsedge. Phylogenetic 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 WT.

# 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 through RT-PCR by using degenerated primers (Additional file 1). Subsequently, a 1423bp full length fragment was obtained by using the 5'- and 3'-RACE technique (Additional file 1). The amplified gene contained an ORF of 1098 bp, 5'UTR and 3'UTR (Fig. 1). Conserved domain analysis (https://www.ncbi.nlm.nih.gov/Structure/cdd/) indicated that this WRI1-like gene contained two AP2 domains; the second domain was found to be more conserved than the first one as shown by multiple alignment analysis (data not shown). Phylogenetic data showed that AP2, ANT and WRIs clustered into three major groups (Fig. 2). Since this *WRI1*-like peptide clustered with WRI3 and WRI4, it was named as *Cyperus esculentus WRI3/4* (*CeWRI3/4*).

We then determined the expression of *CeWRI3/4* gene in different organs. The gene was found to show highest expression in leaves, followed by roots, while the lowest expression was found in tuber (Additional file 2).

### *CeWRI3/4* improves drought tolerance in transgenic Arabidopsis

For functional characterization of the cloned gene, *Arabidopsis thaliana* plants were transformed with *CeWRI3/4* gene and the drought tolerance of the transgenic plants was determined by growing on PEG-supplemented growth media and also by applying real dehydration. Seed germination of both WT and transgenic Arabidopsis was higher than 90% under control conditions and PEG-simulated stress conditions (data not shown). We also determined drought tolerance at seedling stage. In control conditions, the growth of 10-day-old WT seedlings was not significantly different from the transgenic lines B1 and K2 (Fig. 3A). However, under PEG-induced drought stress, WT seedlings exhibited severe primary root growth inhibition and much fewer lateral roots as compared with transgenic lines (Fig. 3A). Similarly, whereas 14-days old WT seedlings developed two very small yellow true leaves, the same-aged transgenic seedlings had 4 well developed true leaves (Fig. 3A). The root length and seedling fresh weight

(FW) data also confirmed that the transgenic lines exhibited higher tolerance to PEG-simulated drought stress (Fig. 3B-C).

We also determined the plant drought stress through real dehydration by withholding water. The growth of WT and transgenic Arabidopsis plants was not significantly different under normal growth conditions (Fig. 4). After 15 days of dehydration, the wilting frequency was 63.6-81.8% in the WT compared with 18.2-25% for transgenic lines (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 WT. (Fig. 4-5). Based on these data, it can be concluded that *CeWRI3/4* improves drought stress tolerance in Arabidopsis.

## Expression of key genes involved in fatty acid and cuticular wax biosynthesis is modulated in transgenic lines expressing *CeWRI3/4*

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 in WT and transgenic plants. 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) [28], was not significantly different between WT and transgenic Arabidopsis lines. In WT plants, PEG-induced drought stress did not significantly alter the expression of above mentioned genes as compared to WT control. In transgenic lines, the expression of *PIPK-β1*, *BCCP2* and *PDHE1α* was significantly induced (130-230%, 50-100% and 130-220% increase, respectively) by PEG treatment as compared with unstressed transgenic lines (Fig. 6A-C).

The expression of *TAG1* (At2g19450), a gene involved in TAG accumulation [29], was significantly lower (10-40% decrease) in transgenic lines compared with the WT under unstressed conditions. PEG-induced drought stress did not cause significant change in expression of *TAG1* in WT, but a significant decrease (20-70%) in expression of this gene was observed in the transgenic lines compared with both WT and transgenic control (Additional file 3).

We also determined the expression of key genes, namely *LACS1* (At2g47240), *WSD1* (At5g37300)、*KCS1* (At1g01120)、*CER1* (At1g02200) and *CER4* (At4g33790), involved in cuticular wax biosynthesis [30-33]. Under control growth conditions, expressions of all the above mentioned genes was significantly higher in the transgenic lines compared with WT. PEG treatment did not cause significant change in the expression of these gene in WT, however, in the transgenic lines the gene expression was significantly induced by PEG as compared to transgenic control (gene expression being 130-300%, 30-80%, 20-80%, 10-110%, 80-140% higher for *LACS1*, *WSD1*、*KCS1*、*CER1* and *CER4,* respectively) (Fig. 6D-H). Over all, these results show that expression of key genes involved in biosynthesis of fatty acid, cuticular wax and oil accumulation is modulated by *CeWRI3/4* in transgenic Arabidopsis lines.

## Soluble sugars, free proline and Malondialdehyde content in WT and transgenic Arabidopsis

To gain further insights into drought tolerance phenotype of transgenic Arabidopsis expressing *CeWRI3/4* we assayed soluble sugars, free proline and Malondialdehyde (MDA) in WT and transgenic lines. After two weeks of real dehydration, the concentration of soluble sugars was significantly lower in all transgenic lines as compared with WT (only 14.3-29.9% of WT) (Fig. 7A). A similar trend was observed for proline and MDA content; in transgenic lines proline and MDA were significantly lower as compared to WT (being 26.9-50.6% and 36.5-45.5% of WT contents, respectively) (Fig. 7B- C).

### Cuticular wax and oil content in the WT and transgenic Arabidopsis lines

The expression data of genes involved in fatty acid and wax biosynthesis prompted us to determine the cuticular wax and oil contents in transgenic lines. Data showed that in both WT 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; C31 being the predominant composition (Fig. 8 A-B). The contents of total cuticular wax, C26, C28 and C30 of primary alcohols, and C27 and C31 of alkanes, were all significantly higher in transgenic lines compared with WT. However, the contents of C33 of alkanes and C32 of primary alcohols, were not significantly different (Fig. 8 A-C). The content of C29 in alkanes was significantly different between WT and transgenic line B1, but not between WT and transgenic line K2 (Fig. 8A). The oil content in Arabidopsis leaves was also assayed, however no significant difference was found between WT and transgenic lines (Additional file 4).

## Discussion

### WRI3/4 nature of the nutsedge gene

AP2/EREB genes are classified into five groups: AP2 subfamily with two AP2/ERF domains, DREB and ERF subfamilies with one AP2/ERF domain, RAV subfamily with one each AP2/ERF domain and B3 DNA-binding motif, and others [21-23]. The AP2 subfamily was further divided into two monophyletic groups namely AP2 and ANT [24]. The WRI1-like group, consisting of WRI1, WRI2, WRI3 and WRI4, was either considered as part of the ANT-like group [26] or as a monophyletic group of the AP2 subfamily [27]. Since the WRI1-like gene reported in this study contained two AP2/EREBP domains we recommend it to be grouped into AP2 subfamily. According to phylogenetic data, 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 found to be more closely related to WRI3s and WRI4s. Therefore, it 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 [1, 33]. Over-expression of genes like *SHN1/WIN1*, *SHINE1*, *MdSHINE2, MYB96* and *MYB94* increased drought tolerance by increasing cuticular wax biosynthesis [2-8]. 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. Furthermore, WRI4 plays role in fatty acid biosynthesis by directly binding

to the promoters of the genes like *BCCP1* and *BCCP2* [1, 27]. Moreover, the disruption of *WRI4* led to down-regulation of above-mentioned genes and other genes such as *PKP1*, *PKP2*, *PDHE1α* and *ENR1* involved in fatty acid biosynthesis [1].

We have shown here that under control conditions the expression of key genes involved in fatty acid biosynthesis (*PIPK-β1*, *BCCP2* and *PDHE1α*) was not significantly different between WT and transgenic Arabidopsis lines. However, after PEG-induced drought stress the expression of these genes was significantly increased in transgenic lines compared with their unstressed control but did not significantly changed in WT (Fig. 6A-C). Regarding wax biosynthesis genes, the expression of *LACS1*, *WSD1*、*KCS1*、*CER1* and *CER4* was significantly higher in transgenic lines than WT in control growth conditions. PEG-induced drought stress did not result in significant change in the expression of these genes in the WT, but significantly higher expression was noted in transgenic lines compared to their respective control (Fig. 6D-H). These findings are consistent with the cuticular wax data. In transgenic lines total cuticular wax content was significantly higher than those in WT (Fig. 8). This might explain why the transgenic lines exhibited significantly improved tolerance to PEG-simulated and real dehydration stresses (Fig. 3-5).

The expression of *TAG1* gene was significantly lower in the transgenic lines compared with WT under normal growth conditions (Additional file 3). This could be the possible reason for no significant difference in oil content between WT and transgenic lines, even though the expression of genes involved in fatty acid biosynthesis was significantly higher in transgenic lines than WT.

### 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 [34-36]. 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 [37, 38]. Many plants change their osmotic adjustment abilities to resist drought stress via accumulating proline and soluble sugars, which participate in osmotic protection [39, 40]. In current study, the concentration of soluble sugars, free proline and MDA were all significantly lower in transgenic lines compared with WT (Fig. 7 A-C) suggesting that probably transgenic lines had undergone less lipid peroxidation and membrane damage.

## Conclusions

*WRI3/4*-like gene from *Cyperus esculentus* could improve drought tolerance probably by promoting cuticular wax biosynthesis and could be a valuable target for improving drought tolerance in crops through recombinant DNA technology.

## Methods

### Plant materials and treatments

*Arabidopsis thaliana* ecotype Columbia and tubers of *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. The leaves and roots were collected and flash frozen in liquid nitrogen and stored at -80°C for further assays. 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 *CeWRI1*-like gene fragment 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 length cDNA of *CeWRI3/4*.

### 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 through MEGA 7.0 software using the neighbor joining method with 1,000 bootstrap replicates [41].

### 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 [42] to construct vector pCambia 2300-35S-*CeWRI3/4*-nos. Sequencing was performed to ensure that the construct was correct.

WT Arabidopsis 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⁄$m^2$⁄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⁄$m^2$⁄s) at 22°C. The pCambia 2300-35S-*CeWRI3/4*-nos vector was introduced into *Agrobacterium tumefaciens* EHA105 which was then used to transform Arabidopsis

by floral-dip method[43]. 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, WT and transgenic Arabidopsis seeds were sown on 1/2 MS agar medium with/without 5% PEG 6000. Germination rate of the WT and transgenic Arabidopsis were scored 6 days after seed sowing. The length of the primary roots was recorded on 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 after 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.

After 15 days of dehydration, leaves from WT and transgenic lines were harvested for physiological indices. Leaf MDA content was measured using the thiobarbituric acid method described by Dhindsa and Matowe [44]. Free proline and soluble sugars were determined from plant leaves as described by Troll and Lindsley [45] and Flood and Priestley [46], respectively.

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

24-day-old WT and transgenic Arabidopsis plants were treated with 5% PEG-6000 for 10 hrs. The leaves were harvested and flash frozen in liquid nitrogen and stored at -80°C. Control plants were watered without addition of PEG. 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 and 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 WT 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 were dissolved in a mixture of 40 μl of heptane and 40 μl of toluene, 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 [1].

### 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; RACE: Rapid amplification of cDNA ends; TAG: Triacylglycerol; UTR: Untranslated region

## Declarations

### Ethics approval and consent to participate

Not Applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The data sets supporting the results of this study are included in the manuscript and additional supporting files.

### Competing interests

The authors declare that they have no competing interests.

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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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# Additional File Legends

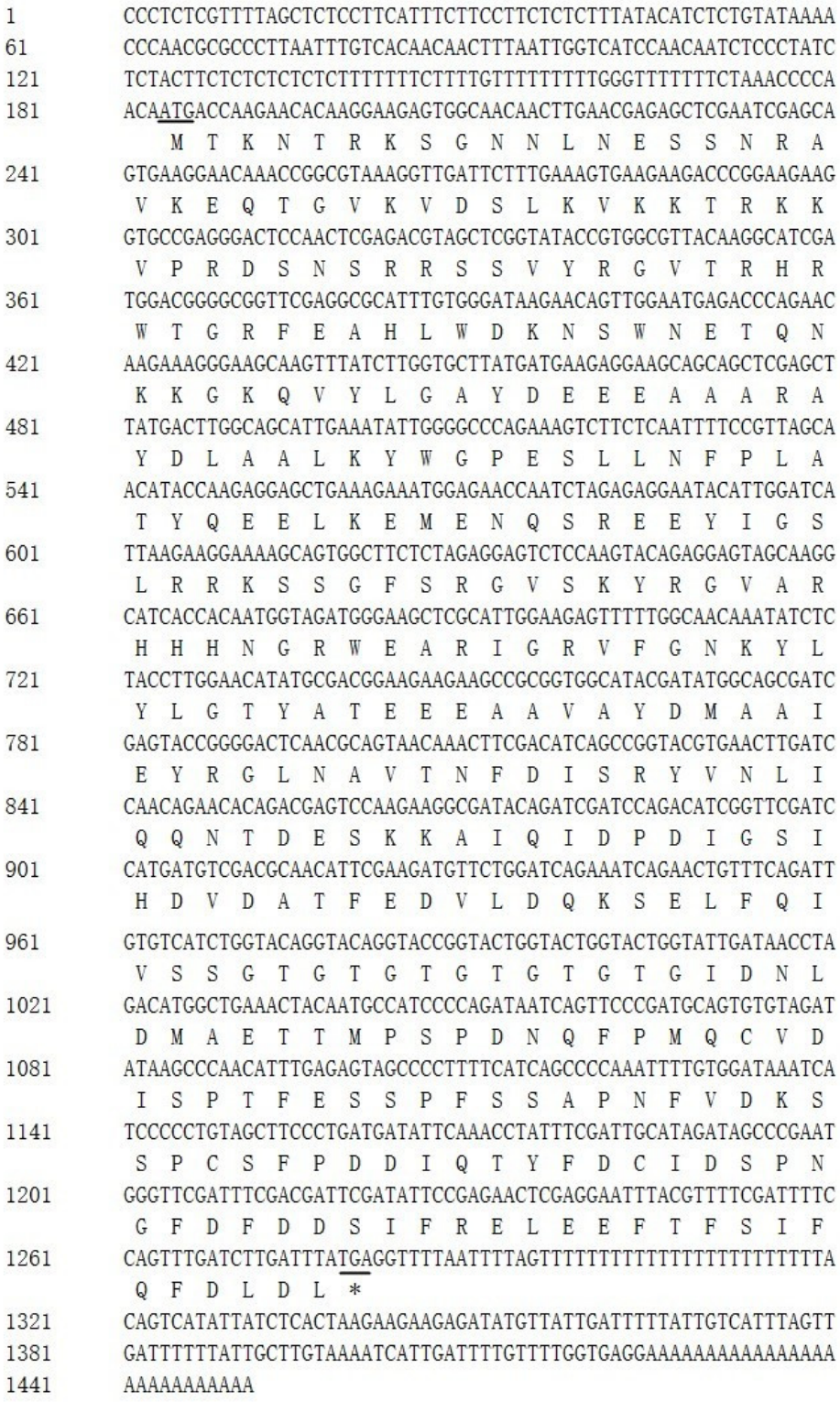
Additional file 1: Table S1 Primers for PCR and qRT-PCR

Additional file 2: Figure S1 Relative expression of *CeWRI3/4* in different nutsedge tissues.

Additional file 3: Figure S2 qRT-PCR analysis of *TAG1* involved in oil accumulation in WT and transgenic lines A5, A7, B1, K2; **P < 0.01, *P < 0.05.

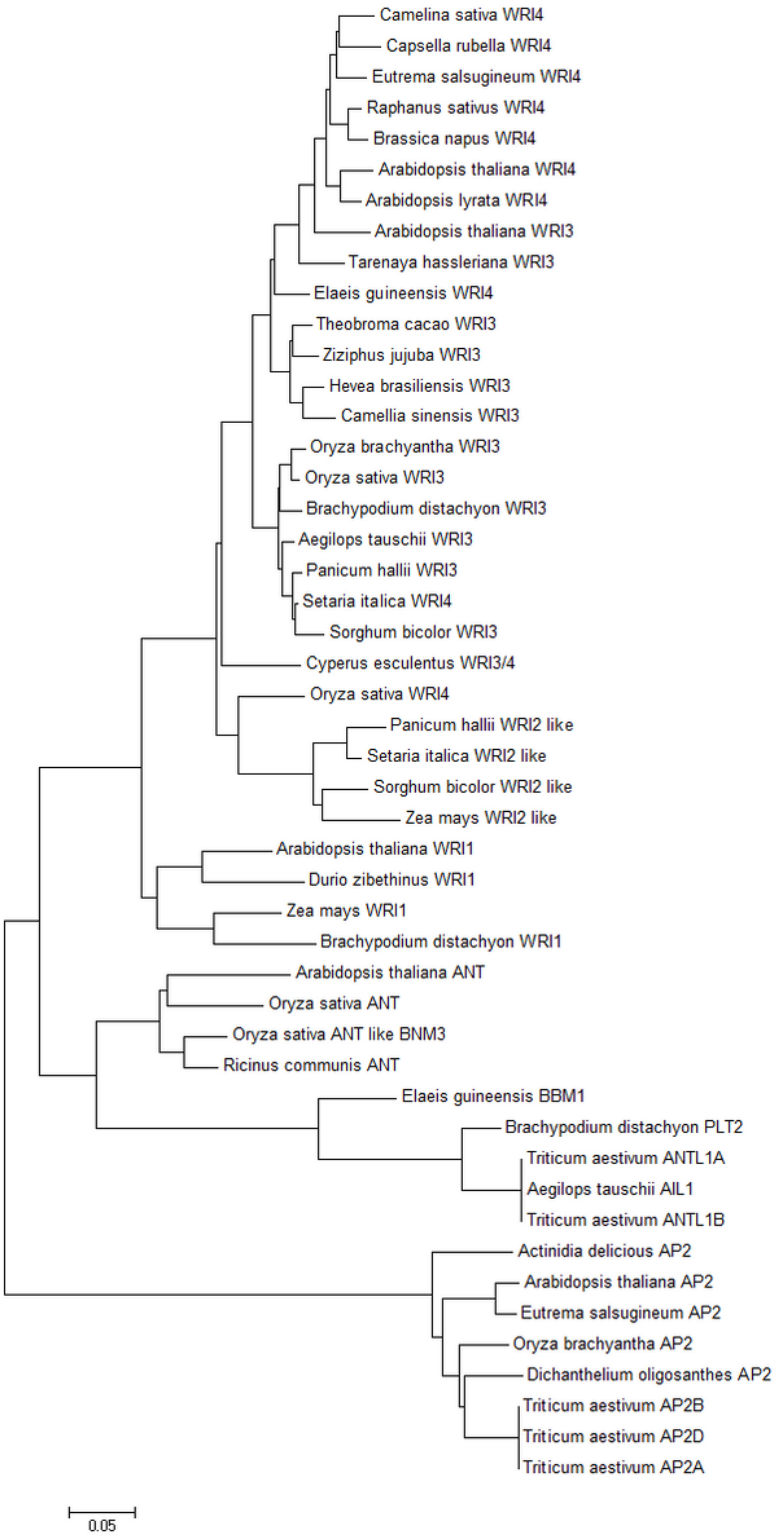
Additional file 4: Figure S3 Oil content in Arabidopsis leaves.

# Figures



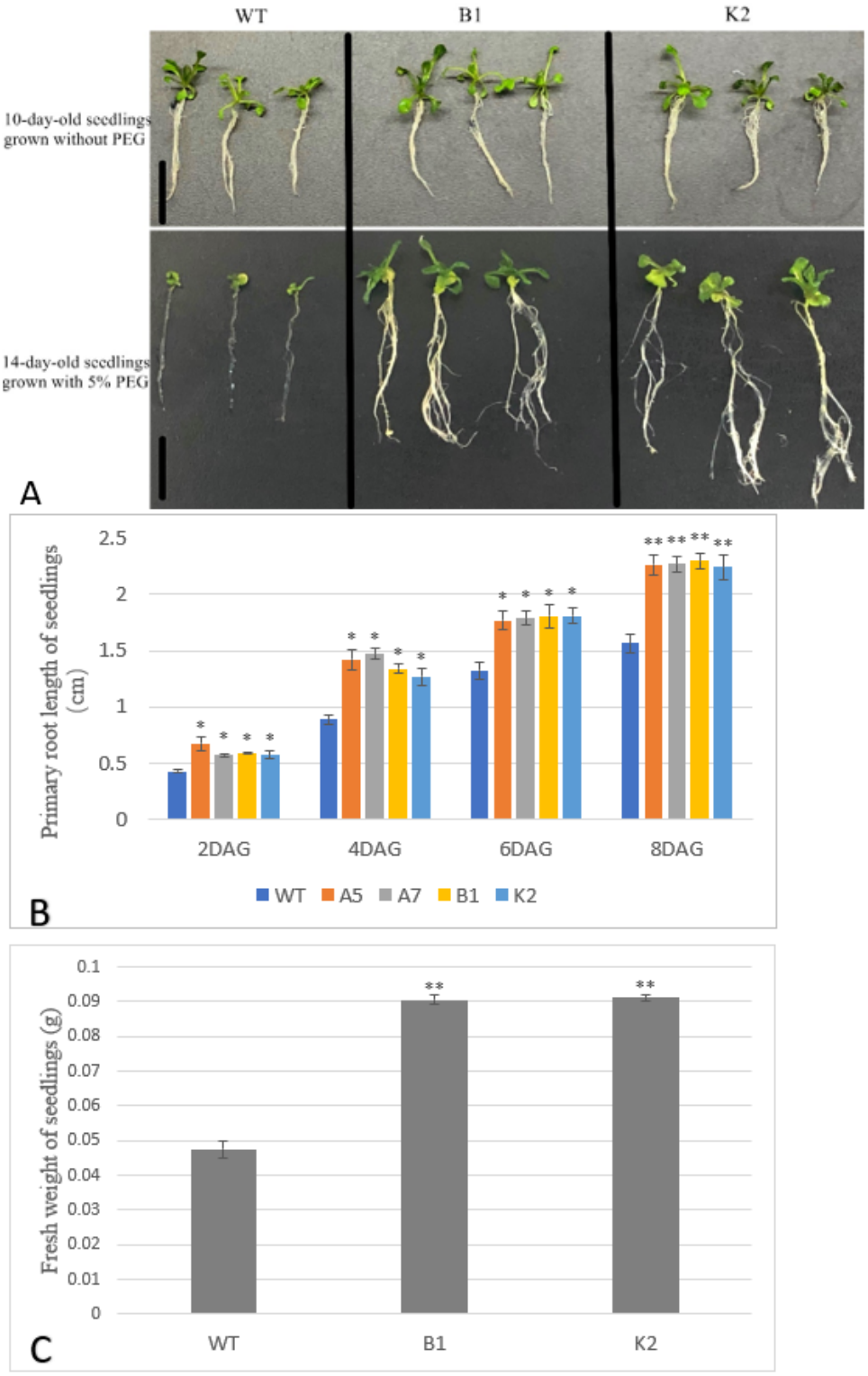

## Figure 1

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



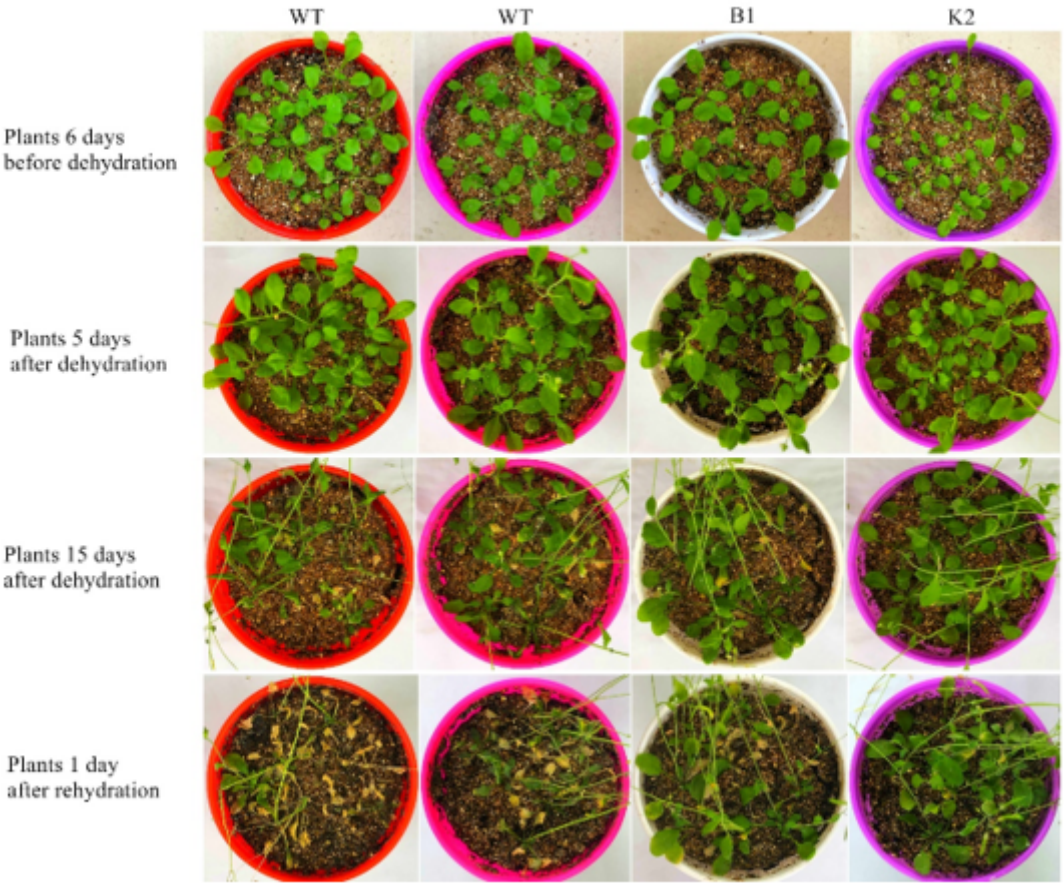

## Figure 2

Phylogenetic tree of the two-AP2-domain-containing genes from different plant species



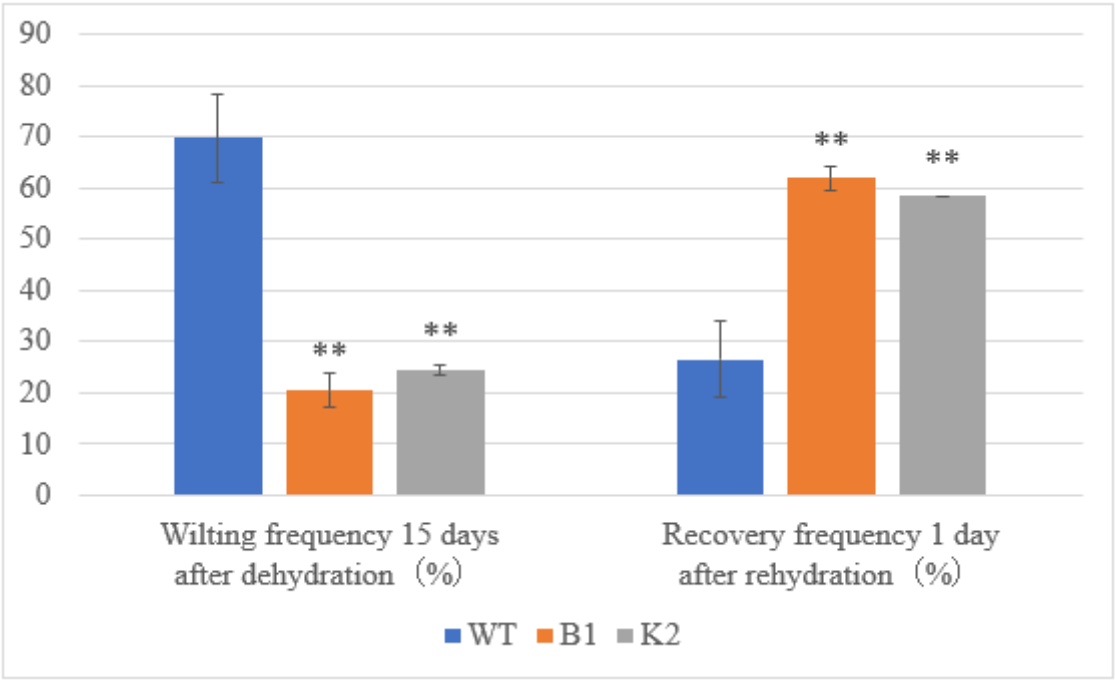

## Figure 3

A: 10-day-old WT and transgenic lines B1 and K2 seedlings without PEG stress (up) and 14-day-old seedlings of 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.



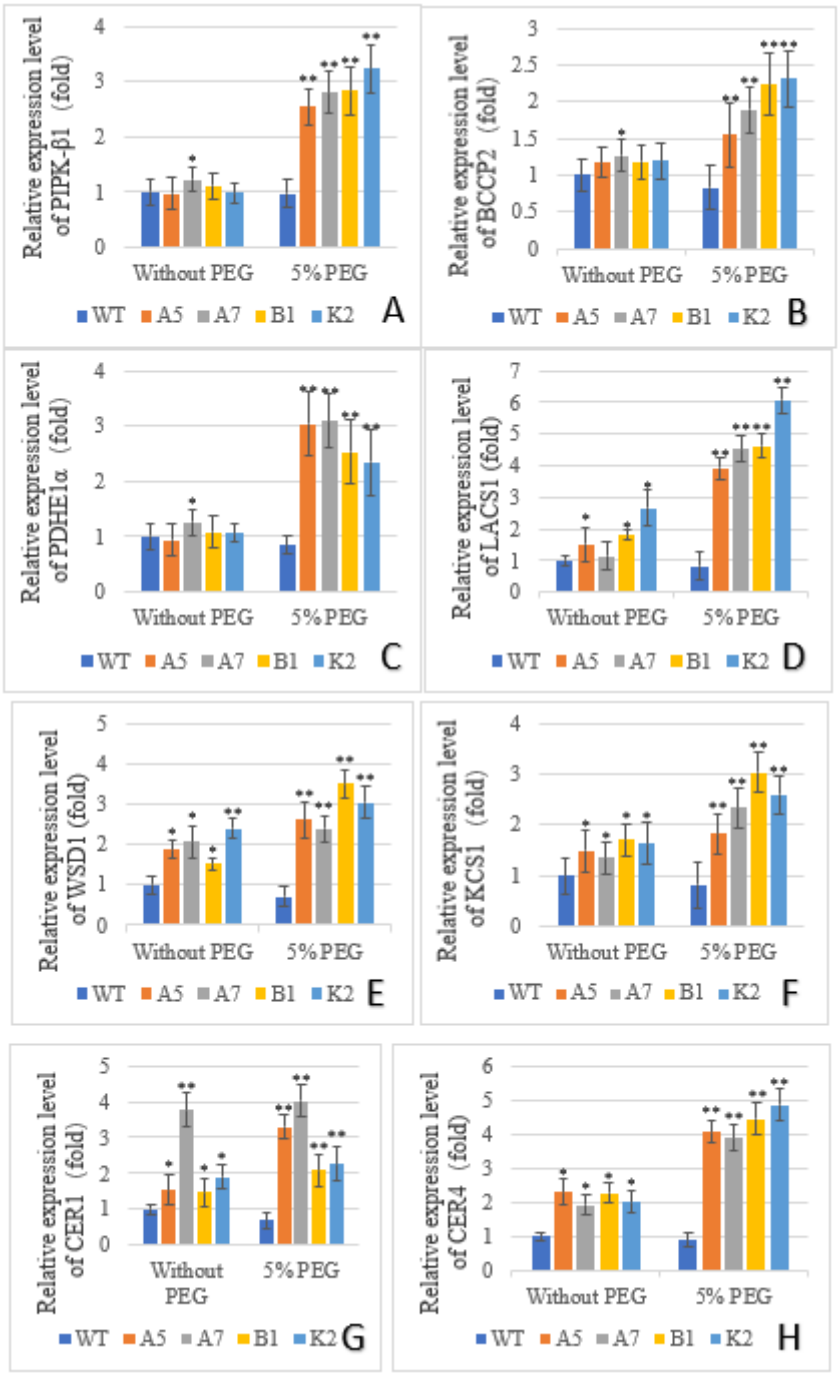

## Figure 4

Morphology of WT and transgenic lines B1 and K2.



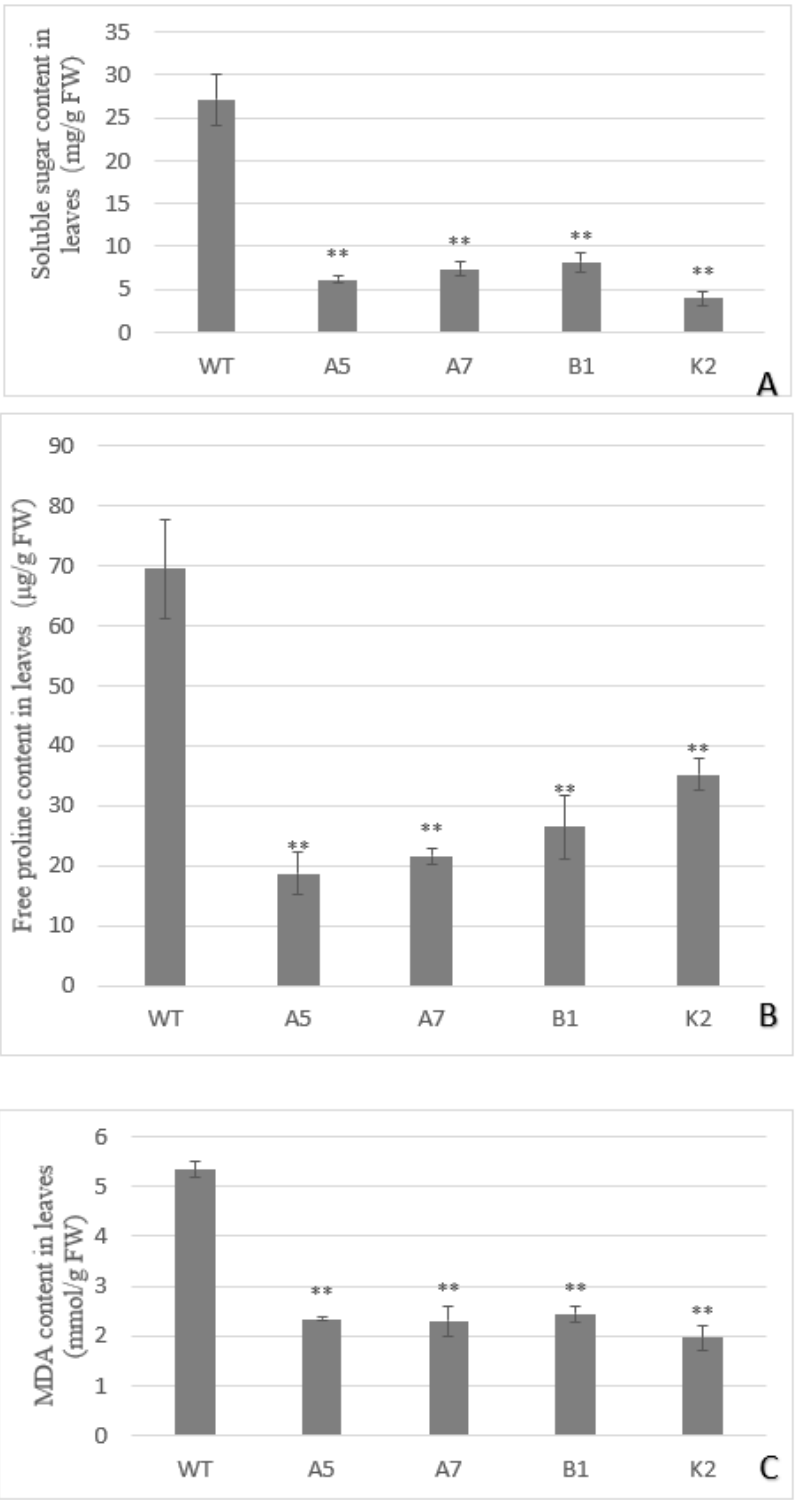

## Figure 5

Wilting frequency after 15 days of dehydration and recovery frequency 1 day after rehydration; **P < 0.01.




## Figure 6

qRT-PCR analysis of genes involved in fatty acids and cuticular wax biosynthesis in WT and transgenic lines A5, A7, B1, K2; **P < 0.01, *P < 0.05.




## Figure 7

Physiological indexes in wild type (WT) and transgenic Arabidopsis lines A5, A7, B1 and K2 with water deprivation. **P < 0.01.

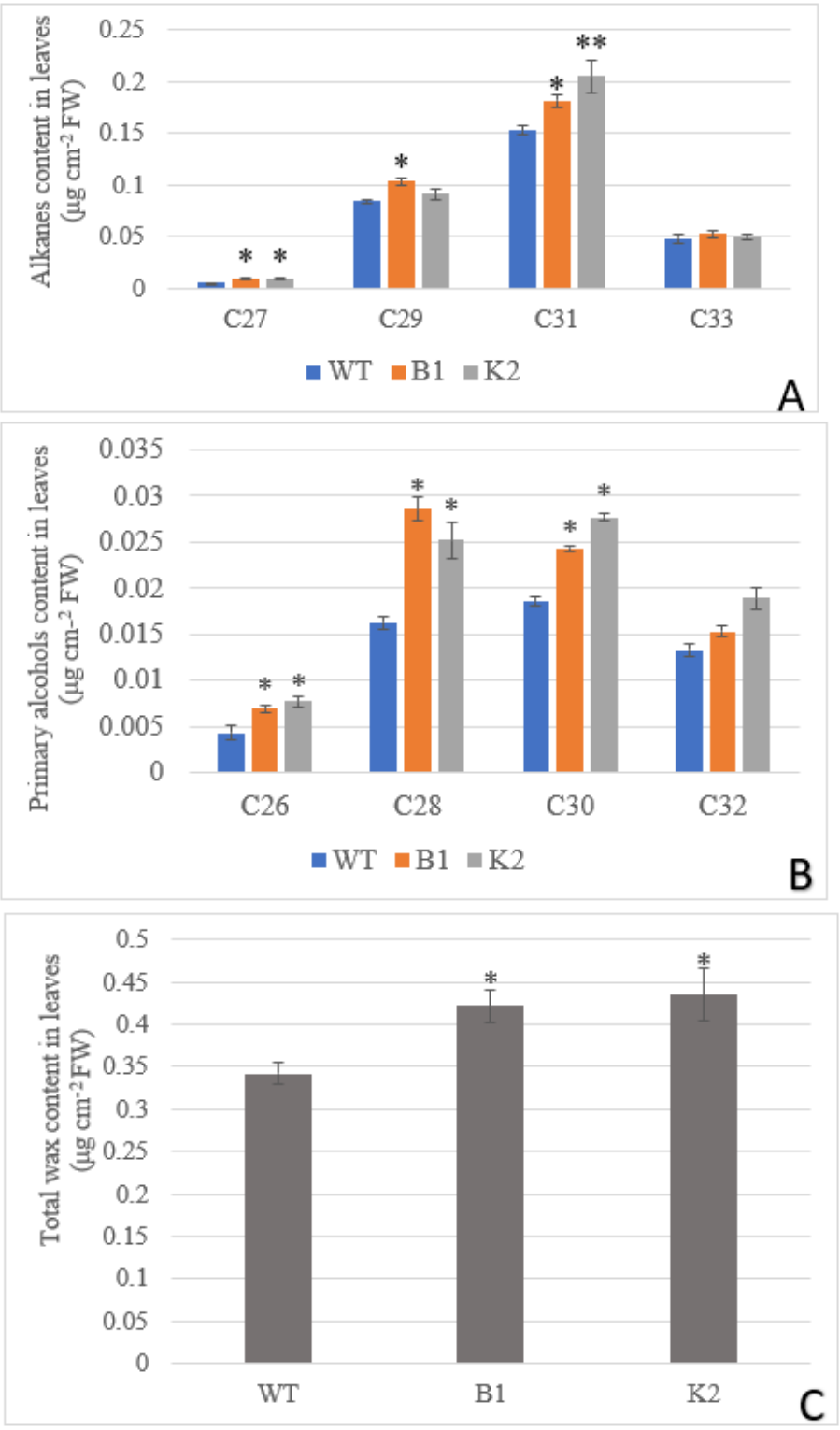



**Figure 8**

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

# Supplementary Files

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