

# Cloning and characterization of the *DIR1* promoter from *Eucommia ulmoides* Oliv and its response to hormonal and abiotic stress.

Li Ziyun

Guizhou University

Li Biao

Guizhou University

Zhao yichen (✉ [yczhao@gzu.edu.cn](mailto:yczhao@gzu.edu.cn))

Guizhou University <https://orcid.org/0000-0002-7445-0130>

Zhao Degang

Guizhou University

---

## Research Article

**Keywords:** Abiotic stress, Genetic transformation, lignan biosynthesis, Hormone, *Eucommia ulmoides*

**Posted Date:** February 19th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-162863/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on April 7th, 2021. See the published version at <https://doi.org/10.1007/s11240-021-02070-x>.

# Abstract

The lignans of *Eucommia ulmoides* have been extensively studied and shown to have a dual mechanism of regulating blood pressure. Studies have shown that *DIR1* is a key gene in the biosynthetic pathway of lignans in *Eucommia ulmoides* Oliv. In this study, a 2000 bp upstream promoter sequence was cloned, and part of the sequence (1495 bp) and its 5'-end truncated segment were constructed into the pCAMBIA1391Z expression plasmid upstream of  $\beta$ -glucuronidase (*GUS*). *Agrobacterium*-mediated genetic transformation produced stable transgenic tobacco lines. The results showed that although both full-length and truncated promoters could initiate *GUS* expression, their levels were influenced by the degree of deletion at the 5' end. *GUS* histochemical staining showed that the core promoter region was located in the region containing the transcription initiation site (TIS) within 212 bp. In addition, the *DIR1* promoter responded to environmental and hormonal stressors, such as jasmonic acid (MeJA), abscisic acid (ABA), D-mannitol (drought mimic), and high concentrations of NaCl. In transgenic tobacco seedlings, MeJA, D-mannitol, and ABA could activate the *DIR1* promoter, whereas high concentrations of NaCl could inhibit it. In *E. ulmoides* Oliv seedlings, MeJA, NaCl, and D-mannitol activated the *DIR1* promoter, whereas ABA had an inhibitory effect. In summary, our findings provide a theoretical basis for the use of the *DIR1* promoter in plant genetic engineering, indicating its potential. Our study also presents novel insights for lignan biosynthesis and sheds light on the mechanisms of *E. ulmoides* Oliv in response to stress.

## Key Message

We successfully cloned the *DIR1* gene promoter and studied its function in regulating the expression of the *DIR1* gene.

## Introduction

*Eucommia ulmoides* Oliver (*E. ulmoides*), a deciduous tree belonging to the Eucommiaceae family, is a traditional and valuable source of Chinese medicine (Luo et al. 2020). *E. ulmoides* is also a rich source of collagen and has attracted considerable attention (Nakazawa and Toda 1995; Zhao 2019). Over 200 compounds have been isolated from *E. ulmoides*, including lignin, cycloolefin ethers, and terpenes, as well as pharmacologically active phenylpropanoids, iridoids, and pinoresinol diglycoside (Wang et al. 2019; Zou et al. 2019; Liu et al. 2020). Pinoresinol diglycoside is a lignan from *E. ulmoides* that is known to have medicinal properties (Zou et al. 2019). E-coniferyl alcohol is a general precursor for the synthesis of lignans, and pinoresinol is formed by the dimerization of two molecules of E-coniferyl alcohol. This dimer, which is a lignan, can combine with additional lignan monomers to form a lignan polymer (Davin et al. 2000). The *DIR1* coding gene plays a key role in the biosynthesis of lignans, including pinoresinol diglycoside (Wang et al. 2018). DIRIGENT (DIR) proteins promote the coupling of monolignol rosinol (+) and pinoresinol (-), the first intermediates of the lignan biosynthetic pathway (Kim et al. 2015; Gasper et al. 2016). Studies have shown that DIR proteins are encoded by heterologous genes and that they are involved in the synthesis of lignans in several plant species (Lv and Zhang 2017; Nobile et al. 2017;

Uchida et al. 2017). In addition, the DIR proteins of higher plants, such as pear (Cheng et al. 2018), soybean GmDIR22 (Li et al. 2017), Cucumber (Liu et al. 2018) and alfalfa (Behr et al. 2015) have been isolated.

The promoter region of a gene controls the intensity, location, and pattern of gene expression (Tang and Tu 2015; Zhang et al. 2004). Although constitutive promoters are usually used to drive plant transgene expression (Srivastava et al. 2015), they are associated with a few drawbacks. For instance, they sometimes cannot achieve time-controlled gene expression (Gurr and Rushton 2005; Zheng et al. 2007). Therefore, researchers are focusing on the use of core and gene upstream promoter elements to achieve time-controlled gene expression (Franssen et al. 2017; Yu et al. 2019). In this study, the *DIR1* gene promoter was cloned, bioinformatics analysis was performed, and the response elements were analyzed for different promoter fragments deleted at the 5' end.

## Materials And Methods

### Biological samples and reagents

*E. ulmoides* seeds were collected from the Agricultural Biotechnology Agricultural Base of Guizhou University (Guizhou, China). *Escherichia coli* strain, DH5 $\alpha$ ; *Agrobacterium tumefaciens* strain, LBA4404; *Nicotiana tabacum* (Xanthi); the plant expression vector, pCAMBIA1391Z; and the subcloning vector, pClone007; were used as available in our laboratory. KOD-Plus-Neo high-fidelity DNA polymerase (TOYOBO, Shanghai, China) and EZNA TM Gel Extraction Kits (Omega, Norcross Georgia, USA), RNA pure Kit and RNA extraction kit from Kangwei Century (Beijing, China), High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Waltham Massachusetts, USA), and SYBR® Select Master Mix from NovoProtein (Suzhou, China).

### Bioinformatics analysis and cloning of the *DIR1* promoter

Based on the known *DIR1* gene sequence, Primer Premier 5.0 and oligo 7.0 were used to design primers specific for the promoter, and a *Sal*I and *Eco*R I restriction site was introduced at the 5' and 3' ends (Table 1). The 2000 bp promoter sequence was amplified and cloned by BGI (Beijing, China). Briefly, genomic DNA was extracted from *E. ulmoides* using the CTAB method, and amplified using KOD high-fidelity enzyme with the following reaction parameters: pre-denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 15 s, annealing at 61°C for 30 s, and extension at 68°C for 1 min, and a final extension of 10 min at 68°C. The polymerase chain reaction (PCR) products were electrophoresed using a 1.2% gel, and the target fragment was cut and purified using an E.Z.N.A. Gel Extraction Kit. The cloned 2000 bp promoter sequence was ligated to the pClone007 vector and sequenced using BLAST against the genomic DNA.

The 2000 bp *DIR1* promoter sequence obtained by cloning and sequencing was submitted to BDGP to predict the transcription start site. The PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al. 2002) online website was

used to analyze and predict the cis-acting regulatory elements in the promoter region and for the initial determination of the core region of the promoter.

### **Construction of expression vectors and genetic transformation of tobacco**

To study the functional regions of the *DIR1* gene promoter, we constructed several 5'-end deletion mutants of *EuDIR1p* in this study. The following expression vectors were constructed: 1) *pCAMBIA1391Z-EuDIR1p-1::GUS* (1495 bp) containing CGTCA-motif and osmotic pressure response element, STRE, etc.; 2) *pCAMBIA1391Z-EuDIR1p-2::GUS* (804 bp), including the putative drought-responsive MYB element; 3) *pCAMBIA1391Z-EuDIR1p-3::GUS* (549 bp), including Myb; and 4) *pCAMBIA1391Z-EuDIR1p-4::GUS* (212 bp), including the transcription start site. The core area was predicted using the TATA-box and CAAT-box. The primer sequences are listed in Table 1.

The respective *EuDIR1p* fragments were cut using *SalI* and *EcoRI* and ligated to the double-digested *pCAMBIA1391Z* expression vector (0.4  $\mu$ L *SalI*, 0.4  $\mu$ L *EcoRI*, 4  $\mu$ L plasma, 2  $\mu$ L of 10x buffer H, made up with ddH<sub>2</sub>O to a total volume of 13.2  $\mu$ L at 37°C for 6–12 h) with T4-ligase overnight. Competent *E. coli* DH5 $\alpha$  cells were transformed with the *EuDIR1p-pCAMBIA1391Z* constructs using the heat shock method, and the respective plasmids were harvested using the alkaline cleavage method, verified by *SalI* and *EcoRI* digestion, and then sequenced. Next, the verified plasmids were used to transform *Agrobacterium* LBA4404 competent cells using the heat shock method. Finally, transgenic *Nicotiana tabacum* explants were generated by incubating the leaf discs with the genetically engineered *Agrobacterium*.

### **GUS staining verification of genetically modified tobacco**

The integration of *EuDIR1p* sequences into the tobacco genome was verified using PCR using the primers specific for *EuDIR1p-4*. Wild-type tobacco DNA was used as a negative control, whereas *pCAMBIA1391Z-pEuDIR1-4::GUS* plasmid served as a positive control. The *in situ* expression of *GUS* was determined using immunohistochemical staining. Briefly, the buds, roots, stems, and leaves of mature plants were cut into small pieces (2–3 cm) after 20–40 days of rooting and incubated overnight with the *GUS* staining solution at 37°C. The solution was discarded and the leaves were decolorized using 75% alcohol at 15–25°C to completely remove the chlorophyll and visualize the coloring effect of the *GUS* dye solution.

### **Stress induction**

Seedlings of *E. ulmoides* and different transgenic tobacco were grown at 28 and 25°C, respectively (12 h day-night culture), and 70% relative humidity, and seedlings with similar growth rates were selected for subsequent experiments. Appropriate pressure induction chemical treatments were performed on the same batch of plants using distilled water treatment as a control. Each experiment used three biological and technical replications. To induce hormonal stress in *E. ulmoides*, 100 mmol/L each of methyl jasmonate (MeJA) and abscisic acid (ABA) were sprayed on the leaves. To induce drought stress and osmotic stress, the seedlings were grown in 100 $\pm$ 0.37 g of uniform high-quality soil. When the soil moisture was 4.8 $\pm$ 0.2%, 300 mmol/L mannitol and 10 g/L NaCl were used for irrigation treatment. To

induce stress in tobacco seedlings, the samples were treated with the same concentration of chemical reagents used for the induction of stress in *E. ulmoides*. *EuDIR1p-1* tobacco was treated with MeJA and NaCl, *EuDIR1p-2* tobacco was treated with mannitol, and the *EuDIR1p-3* tobacco was treated with ABA. Tobacco seedlings were sampled at 0, 3, 6, 12, 24, and 48 h, and the RNA was extracted and reverse-transcribed for *GUS* expression analysis. *DIR1* gene expression was analyzed in *E. ulmoides* seedlings at 0, 3, 6, 12, and 24 h after RNA extraction and reverse transcription (Singh et al. 2020; Zhou et al. 2020).

### **Real-time fluorescence quantitative analysis**

To compare the relative expression level of *GUS* in transgenic tobacco with *DIR1* in *E. ulmoides* to better verify the cis-acting element function of different promoter fragments, real-time fluorescence quantitative analysis was performed. Apart from *GUS* histochemical staining, fluorescence quantitative analysis is also used to monitor the relative expression of *GUS*. RNA was extracted from the *E. ulmoides* and tobacco seedlings using the RNA Pure kit and then reversed transcribed to cDNA. The relative expression levels of *EuDIR1p* and *GUS* were analyzed using RT-PCR with *EuActin* (Ye et al. 2018) and  $\beta$ -actin as the respective housekeeping genes for *E. ulmoides* and tobacco. The reaction parameters were as follows: pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing at 60°C. The primers are listed in Table 1. Three biological and three technical replicates were tested per sample, and the gene expression levels were calculated as previously described (Hellemans et al. 2007), using SPSS 22.0 and GraphPad Prism 8.3.0.

## **Results**

### **Cloning and bioinformatics analysis of *DIR1* promoter**

We successfully cloned a promoter sequence of 2000 bp upstream of the *DIR* gene. The sequenced 2000 bp promoter sequence was submitted to the PlantCARE database for bioinformatics analysis. As shown in Fig. 1, a transcription initiation site TIS (+ 1) was revealed 50 bp upstream of the *DIR1* start codon, TATA-box, and CAAT-box core components at -25 ~ -35 bp, together with three ARE (-65 bp, -1381 bp, 1587 bp), one ATCT (-925 bp), one CGTCA (-1468 bp), two MYB (-677 bp, 1569 bp), one MYC(-1450 bp), one MYb (-490 bp), one O2-site (-839 bp), and one STRE (-1296 bp) element.

Based on the results of bioinformatics analysis, the sequence containing the important cis-acting elements were selected and cloned into expression vectors for further research. As seen in Online Resource 1, the sequence of 1495 bp upstream of the *DIR1* gene was successfully cloned using PCR. The putative positions and functions of the cis-acting elements in *EuDIR1p-1* and its 5'-end truncated constructs are shown in Table 2.

### ***GUS* staining verification of genetically modified tobacco**

Different amplified *EuDIR1p* fragments were cloned into expression vectors and transformed into tobacco seedlings using the *Agrobacterium*-mediated leaf disc method (Online Resources 2 and 3). The

transgenic plants were screened using PCR (Online Resource 4). The transgenic tobacco with different truncated fragments was stained using GUS staining solution and the staining results are shown in Fig. 2. All *EuDIR1p-1*, -2, -3, and -4 transgenic tobacco plants exhibited positive GUS immunostaining. It can be seen that the core elements present in *EuDIR1p-1* and truncated promoters promoted *GUS* expression. However, unlike the truncated promoter, stronger staining intensity was observed in plants expressing *EuDIR1p-1*.

### **Different *DIR1* promoter fragments stimulate *GUS* expression under hormonal and abiotic stress**

The *EuDIR1p-1* transgenic tobacco plantlets harboring the CGTCA promoter element were stimulated with 100  $\mu$ M MeJA. Compared to the baseline level, *GUS* gene expression increased significantly by 5.065-fold in a time-dependent manner at 3 h. Then, the expression of the *GUS* gene gradually decreased and showed an upward trend after 24 h; however, after 48 h, the expression was inhibited (Fig. 3A). At 3 h after ABA treatment, *GUS* in *EuDIR1p-3* transgene was significantly upregulated by 3.1 times, and recovered in about 24 h (Fig. 3B). In this study, we exposed the *EuDIR1p-1* transgenic plants to NaCl (10 g/L), which markedly suppressed *GUS* levels in a time-dependent manner. Compared to the baseline expression, *GUS* was downregulated 0.104-fold and 0.258-fold after 12 and 24 h of NaCl irrigation, respectively (Fig. 3C). Mannitol initially increased the expression of *GUS* in *EuDIR1p-3* transgenic tobacco by 2.071 times at 6 h, but downregulated *GUS* expression within 12–24 h (Fig. 3D).

### ***DIR1* promoter stimulates *DIR1* gene expression in *E. ulmoides* seedlings under hormonal and abiotic stress**

Bioinformatics analysis indicated the presence of stress-related response elements including CGTCA-motif, MYB, Myb, and MYC in *EuDIR1p*. Consistent with this observation, *E. ulmoides* seedlings treated with MeJA or ABA showed time-dependent changes in *DIR1* expression. After 3 h of MeJA treatment, *DIR1* levels increased 7.56 times compared to that at the baseline, slowly decreased to the baseline level, and was followed by an upward trend after 24 h (Fig. 4A). The observations were similar to those observed in genetically modified tobacco seedlings. On the other hand, 100  $\mu$ M ABA downregulated *DIR1* 0.061 times relative to the baseline levels after 6 h, which then recovered after 24 h (Fig. 4B). These findings were the opposite of those seen in genetically modified tobacco. To determine the effect of abiotic stresses on the *DIR1* promoter, *E. ulmoides* seedlings were treated with 40% mannitol and 10 g/L NaCl to simulate drought and high-salt conditions, respectively. Contrary to the results seen in genetically modified tobacco, it was observed that osmotic stress significantly upregulated the expression of *DIR1* in *E. ulmoides* seedlings; the expression level was 7.98 times that at the baseline level after 6 h, which recovered after 24 h (Fig. 4C). Drought simulation, on the other hand, upregulated *DIR1* within the first 12 h of mannitol exposure, with a maximum increase of 2.98-fold at 12 h, which recovered after 24 h (Fig. 4D). In transgenic tobacco, mannitol also upregulated gene expression, but the difference was that it emerged inhibition after 12 h.

## **Discussion**

The biosynthesis of lignans mediated by the DIR protein is mainly initiated in the cambium of stems, the starting area of ray cells, and the palisade layer of leaves, and is consistent with the appearance of lignans for defense (Burlat et al. 2001). The DIR protein itself does not have a catalytic center and cannot directly catalyze the synthesis of lignans; however, it confers optical rotation to the lignans. It can capture the monomer free radicals of lignans in the presence of oxidases, such as laccase, resulting in the biosynthesis of structure-specific dimers and macromolecular lignans (Davin et al. 2001; Borges et al. 2013; Seneviratne et al. 2015). Flax (*Linum usitatissimum* L.) and isatis (*Isatis indigotica* Fort.) are two important model plants for studying lignin biosynthesis. Previous studies have confirmed that *DIR* responds to biotic and abiotic stresses. Li first discovered and analyzed the gene family of the DIR protein in Isatis. They used MeJA to induce stress, and then tested the transcription abundance of *DIRs* and found that some *DIR* genes responded to exogenous MeJA (Li et al. 2014). In flax, Corbin studied the transcriptional response of development-related plant hormones, including naphthaleneacetic acid, abscisic acid (ABA), and MeJA to *DIR* (Corbin et al. 2018). Stress induction using salt and insect feeding has also been carried out in plants such as cotton and spruce. These related studies have shown that *DIR* genes can respond to adversity stress, thereby mediating plants to synthesize lignans against these elements of stress (Ralph et al. 2007; Li et al. 2015).

There have been related studies that have constructed the promoter as a deletion fragment expression vector, followed by studying the response elements in the promoter (Qin et al. 2019). In the current study, 2000 bp sequences of *DIR1* gene were successfully cloned and the missing fragment was used to identify its response elements. *GUS* staining revealed that stronger staining was observed in transgenic tobacco with the 1495 bp promoter. This result indicates that an enhancer was probably in the region from -1495 bp to -804 bp.

Genes can be either upregulated or downregulated by various biotic and abiotic stresses (Liu et al. 2018; Lv and Zhang 2017). It is known that CGTCA is a putative response element of MeJA, and that MYB is involved in the stress response to droughts, jasmonic acid, and powdery mildew infections (Baumann et al. 2007; Kamiya et al. 2015; Ni et al. 2019). MYC regulates the response to abiotic stresses (such as drought) and hormones (such as ABA) (Yang et al. 2019). The results from our study showed that after treatment with MeJA or D-mannitol, the expression of *GUS* in transgenic tobacco increased significantly, which was consistent with the *DIR1* expression in *E. ulmoides*. The MeJA and D-mannitol response elements in the promoter likely had a positive regulatory role at that time. However, the results obtained following ABA and NaCl treatment in transgenic tobacco and *E. ulmoides* were different, which indicated that stress treatment had certain differences in the activation or inhibition of the *DIR1* promoter in different species. A possible reason is that the expression of genes in *E. ulmoides* is more complicated, and the expression of other genes is inconsistent with the expression of the *GUS* gene in transgenic tobacco after interaction with other genes. In addition, there exists another possibility that in plants, *DIR* genes are part of the defense response to biological stress. Its induction may have evolved in different plant lineages, leading to differences in expression between plants (Ralph et al. 2006). Previous studies have also shown that certain transcription factors have inconsistent effects in different species. Similarly, the functions of MYB may be completely opposite in different species, probably owing to high functional

diversity in the MYB family members. (Fang et al. 2020). In summary, further studies are necessary using similar treatment to verify the reasons for inconsistency in gene regulation and expression in *E. ulmoides* and tobacco.

## Conclusions

In this study, the *DIR1* gene promoter was cloned, constructed as a deletion fragment expression vector, and introduced into tobacco. It was found that in tobacco plants, the promoter could successfully initiate the expression of the *GUS* gene. Using bioinformatics analysis, the transcription start site and the related cis-acting elements of the promoter were predicted. Additionally, we discovered that in the *E. ulmoides* seedlings, the *DIR1* gene promoter could be activated by MeJA, NaCl, and D-mannitol, and inhibited by ABA. However, the results of *GUS* gene expression in tobacco are slightly different from those observed in *E. ulmoides*. These findings indicated that the promoter activates or inhibits gene expression, although with certain differences among species. Overall, our study provides a theoretical basis not only for the use of *DIR1* gene promoter but also sheds light on the stress response mechanism of *E. ulmoides* to adversity stress and in the synthetic regulation pathway of lignan pinoresinol.

## Declarations

**Authors' contributions:** ZYC and ZDG conceived and planned the experiments. LZY and LB carried out the experiments. LZY wrote the manuscript, all authors edited and approved the manuscript.

**Funding:** This work was supported by the National Natural Science Foundation #1 under Grant number 31660076; National Special Project for the Cultivation of New Genetically Modified Varieties #2 under Grant number 2016ZX08010003-009; and Construction Program of Biology First-class Discipline in Guizhou #3 under Grant number GNYL [2017] 009.

**Availability of data and materials:** All authors have ensured that all data, materials, software, and custom codes support the published claims and comply with field standards.

**Compliance with ethical standards:**

Conflicts of interest: The authors declare no conflicts of interest.

## References

- Burlat V, Kwon M, Davin LB, Lewis NG (2001) Dirigent proteins and dirigent sites in lignifying tissues. *Phytochemistry* 57(6):883-897.
- Borges AF, Ferreira RB, Monteiro S (2013) Transcriptomic changes following the compatible interaction *Vitis vinifera*-*Erysiphe necator*. Paving the way towards an enantioselective role in plant defence modulation. *Plant physiology and biochemistry* 68:71-80.

- Baumann K, Perez-Rodriguez M, Bradley D, Venail J, Bailey P, Jin H, Koes R, Roberts K, Martin C (2007) Control of cell and petal morphogenesis by R2R3 MYB transcription factors. *Development* 134:1691-1701.
- Behr M, Legay S, Hausman JF, Guerriero G (2015) Analysis of cell wall-related genes in organs of *Medicago sativa* L. under different abiotic stresses. *Int J Mol Sci* 16:16104-16124.
- Corbin C, Drouet S, Markulin L, Auguin D, Lainé É, Davin LB, Cort JR, Lewis NG, Hano C (2018) A genome-wide analysis of the flax (*Linum usitatissimum* L.) dirigent protein family: from gene identification and evolution to differential regulation. *Plant molecular biology* 97(1-2):73-101.
- Cheng X, Su X, Muhammad A, Li M, Zhang J, Sun Y, Li G, Jin Q, Cai Y, Lin Y (2018) Molecular characterization, evolution, and expression profiling of the *dirigent* (*DIR*) family genes in Chinese white pear (*Pyrusbretschneideri*). *Front Gene* 9:136.
- Davin LB, Lewis NG (2000) Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. *Plant Physiol* 123:453-462.
- Franssen HJ, Kulikova O, Willemsen V, Heidstra R (2017) *Cis*-regulatory PLETHORA promoter elements directing root and nodule expression are conserved between *Arabidopsisthaliana* and *Medicago truncatula*. *Plant Signal Behav* 12:e1278102.
- Fang YY, Yan W, Wu JX, Yin DM, Tang XY (2020) Identification and bioinformatics analysis of peanut MYB transcription factor. *Bioinformatics*:1-15.
- Gasper R, Effenberger I, Kolesinski P, Terlecka B, Hofmann E, Schaller A (2016) Dirigent protein mode of action revealed by the crystal structure of AtDIR6. *Plant Physiol* 172:2165-2175.
- Gurr SJ, Rushton PJ (2005) Engineering plants with increased disease resistance: how are we going to express it? *Trend in Biotechnol* 23:283-290.
- Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 8:R19.
- Kamiya T, Borghi M, Wang P, Danku JM, Kalmbach L, Hosmani PS, Naseer S, Fujiwara T, Geldner N, Salt DE (2015) The MYB36 transcription factor orchestrates Casparian strip formation. *P of the Natl Acad Sci* 112:10533-10538.
- Kim KW, Smith CA, Daily MD, Cort JR, Davin LB, Lewis NG (2015) Trimeric structure of (+)-pinoresinol-forming dirigent protein at 1.95 Å resolution with three isolated active sites. *J Biol Chem* 290:1308-1318.
- Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P, Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter

sequences. *Nucleic Acids Res* 30:325-327.

Li W, Zhao F, Fang W, Xie D, Hou J, Yang X, Zhao Y, Tang Z, Nie L, Lv S (2015) Identification of early salt stress responsive proteins in seedling roots of upland cotton (*Gossypium hirsutum* L.) employing iTRAQ-based proteomic technique. *Frontiers in plant science* 6:732.

Li Q, Chen J, Xiao Y, Di P, Zhang L, Chen W (2014) The dirigent multigene family in *Isatis indigotica*: gene discovery and differential transcript abundance. *BMC genomics* 15(1):388.

Li N, Zhao M, Liu T, Dong L, Cheng Q, Wu J, Wang L, Chen X, Zhang C, Lu W, Xu P, Zhang S (2017) A novel soybean dirigent gene *GmDIR22* contributes to promotion of lignan biosynthesis and enhances resistance to *Phytophthora sojae*. *Front Plant Sci* 8:1185.

Liu C, Qin Z, Zhou X, Xin M, Wang C, Liu D, Li S (2018) Expression and functional analysis of the Propamocarb-related gene *CsDIR16* in cucumbers. *BMC Plant Biol* 18:16.

Lv D, Zhang Y (2017) Isolation and functional analysis of apple MdHMGR1 and MdHMGR4 gene promoters in transgenic *Arabidopsis thaliana*. *Plant Cell Tiss Org* 129:133-143.

Liu C, Guo FF, Xiao JP, Wei JY, Tang LY, Yang HJ (2020) Research advances in chemical constituents and pharmacological activities of different parts of *Eucommia ulmoides*. *China journal of Chinese materia medica* 45:497-512.

Luo X, Wu J, Li Z, Jin W, Zhang F, Sun H, Shi Y (2020) Safety evaluation of *Eucommia ulmoides* extract. *Regulatory toxicology and pharmacology* 118:104811.

Nakazawa Y, Toda Y (1995) *Eucommia ulmoides* Oliv. (Eucommiaceae): In Vitro Culture and the Production of Iridoids, Ligands, and Other Secondary Metabolites. *Medicinal and Aromatic Plants VIII*. Springer Berlin Heidelberg.

Ni J, Zhao Y, Tao R, Yin L, Gao L, Strid Å, Qian M, Li J, Li Y, Shen J, Teng Y, Bai S (2019) Ethylene mediates the branching of the jasmonate-induced flavonoid biosynthesis pathway by suppressing anthocyanin biosynthesis in red Chinese pear fruits. *Plant Biotech J* 18(5):1223-1240.

Nobile PM, Bottcher A, Mayer JL Brito, M. S., Dos Anjos, I. A., Landell, M., Vicentini, R., Creste, S., Riaño-Pachón, D. M., Mazzafera, P (2017) Identification, classification and transcriptional profiles of dirigent domain-containing proteins in sugarcane. *Molecul Genet Genomics* 292:1323-1340.

Qin L, Zhang X, Jiang C, Li L (2019) *Betula platyphylla* BpZFP4 gene promoter cloning and stress response element function analysis. *Plant Res* 39:917-926.

Ralph S, Park JY, Bohlmann J, Mansfield SD (2006) Dirigent proteins in conifer defense: gene discovery, phylogeny, and differential wound- and insect-induced expression of a family of DIR and DIR-like genes in spruce (*Picea* spp.). *Plant molecular biology* 60(1):21-40.

- Ralph SG, Jancsik S, Bohlmann J (2007) Dirigent proteins in conifer defense II: Extended gene discovery, phylogeny, and constitutive and stress-induced gene expression in spruce (*Picea* spp.). *Phytochemistry* 68(14):1975-1991.
- Seneviratne HK, Dalisay DS, Kim KW, Moinuddin SG, Yang H, Hartshorn CM, Davin LB, Lewis NG (2015) Non-host disease resistance response in pea (*Pisum sativum*) pods: Biochemical function of DRR206 and phytoalexin pathway localization. *Phytochemistry* 113:140-148.
- Srivastava VK, Raikwar S, Tuteja N (2014) Cloning and functional characterization of the promoter of *PsSEOF1* gene from *Pisum sativum* under different stress conditions using *Agrobacterium*-mediated transient assay. *Plant Signal Behav* 9:e29626.
- Singh DK, Mehra S, Chatterjee S, Purty RS (2020) In silico identification and validation of miRNA and their DIR specific targets in *Oryza sativa* Indica under abiotic stress. *Non-coding RNA research* 5:167-177.
- Tang F, Tu H (2015) Progress in eukaryotic promoter research. *China Forestry Science and Technology* 29: 7-12.
- Uchida K, Akashi T, Aoki T (2017) The missing link in leguminous pterocarpan biosynthesis is a dirigent domain-containing protein with isoflavanol dehydratase activity. *Plant Cell Physiol* 58: 398-408.
- Wang CY, Tang L, He JW, Li J, Wang YZ (2019) Ethnobotany, phytochemistry and pharmacological properties of *Eucommia ulmoides*: a review. *The American Journal of Chinese Medicine* 47:259-300.
- Wang W, Zhao D, Zhao Y (2018) Cloning and sequence analysis of dirigent protein coding genes from *Eucommia ulmoide*. *Genomics and Applied Biology* 37:1293-1301.
- Yang R, Chen M, Sun JC (2019) Genome-wide analysis of LIM family genes in foxtail millet (*Setaria italica* L.) and characterization of the role of *SiWLIM2b* in drought tolerance. *Int J Mol Sci* 20:1303-1311.
- Ye J, Jin CF, Li N, Liu MH, Fei ZX, Dong LZ, Li L, Li ZQ (2018) Selection of suitable reference genes for qRT-PCR normalisation under different experimental conditions in *Eucommia ulmoides* Oliv. *Sci Rep* 8:15043.
- Yu Y, Guo D, Li G, Yang Y, Zhang G, Li S, Liang Z (2019) The grapevine R2R3-type MYB transcription factor VdMYB1 positively regulates defense responses by activating the *stilbene synthase* gene 2 (*VdSTS2*). *BMC Plant Biol* 19:478.
- Zhang CX, Wang WQ, Jiang XN, Chen XM (2004) Review on plant gene promoters. *Acta Genetica Sinica* 31:1455-1464.
- Zhao DG (2019) Study on cloning and utilization of *Eucommia Ulmoides* antibacterial peptide gene. in *Proceedings of the 2019 Annual Conference of the Chinese Society for Plant Protection*. Chinese Plant Protection Society.

Zheng HQ, Lin SZ, Zhang Q, Zhang ZZ, Zhang ZY, Lei Y, Hou L (2007) Isolation and analysis of a TIR-specific promoter from poplar. *Forestry Studies in China* 9:95-106.

Zhou W, Zhang Q, Sun Y, Yang L, Wang Z (2020) Genome-wide identification and characterization of R2R3-MYB family in *Hypericum perforatum* under diverse abiotic stresses. *Int J Biol Macromol* 145:341-354.

Zou Q, Zhao Y, Zhao D (2019) Prokaryotic expression and protein purification of *Eucommia ulmoides* Dirigent1 gene. *Mol Plant Breed* 17:86-91.

## Tables

**Table 1.** Promoter clones and primers used for qRT-PCR

| Primer                   | Primer sequence (5'-3')*                   | Annealing temperature (°C) |
|--------------------------|--|----------------------------|
| <i>IR1p-1F</i> (-1495bp) | ACGCG <u>TCGAC</u> ACAGGGCTTCTATATCTATGACA | 64.4                       |
| <i>IR1p-2F</i> (-804bp)  | ACGCG <u>TCGACT</u> AGACTCATAACCTTGGCT     | 63                         |
| <i>IR1p-3F</i> (-549bp)  | ACGCG <u>TCGAC</u> GGCCTATAATCTTGGTTCTGA   | 64                         |
| <i>IR1p-4F</i> (-212bp)  | ACGCG <u>TCGAC</u> GGCCCTAATGAAAATGTGAGT   | 63                         |
| <i>IR1p-R</i> (+41bp)    | CCGGA <u>ATTC</u> AATGAGAGAAAATGGGCTTG     |                            |
| <i>ctin-F</i> (qRT-PCR)  | GTGTTATGGTTGGGATGGG                        | 60                         |
| <i>ctin-R</i> (qRT-PCR)  | TGCTGACTATGCCGTGTTCT                       | 60                         |
| <i>IR1p-F</i> (qRT-PCR)  | TGCGACTTCTGCTATTGTAGG                      | 60                         |
| <i>IR1p-R</i> (qRT-PCR)  | AGGTTGTTGTCCAGGGTTATG                      | 60                         |
| -F (qRT-PCR)             | ACTGCTGCTGTCGGCTTTA                        | 60                         |
| -R (qRT-PCR)             | GCACTTGCGGACGGGTAT                         | 60                         |
| <i>tin-F</i> (qRT-PCR)   | GATCTTGCTGGTCGTGATCT                       | 60                         |
| <i>tin-R</i> (qRT-PCR)   | ACTTCCGGACATCTGAACCT                       | 60                         |

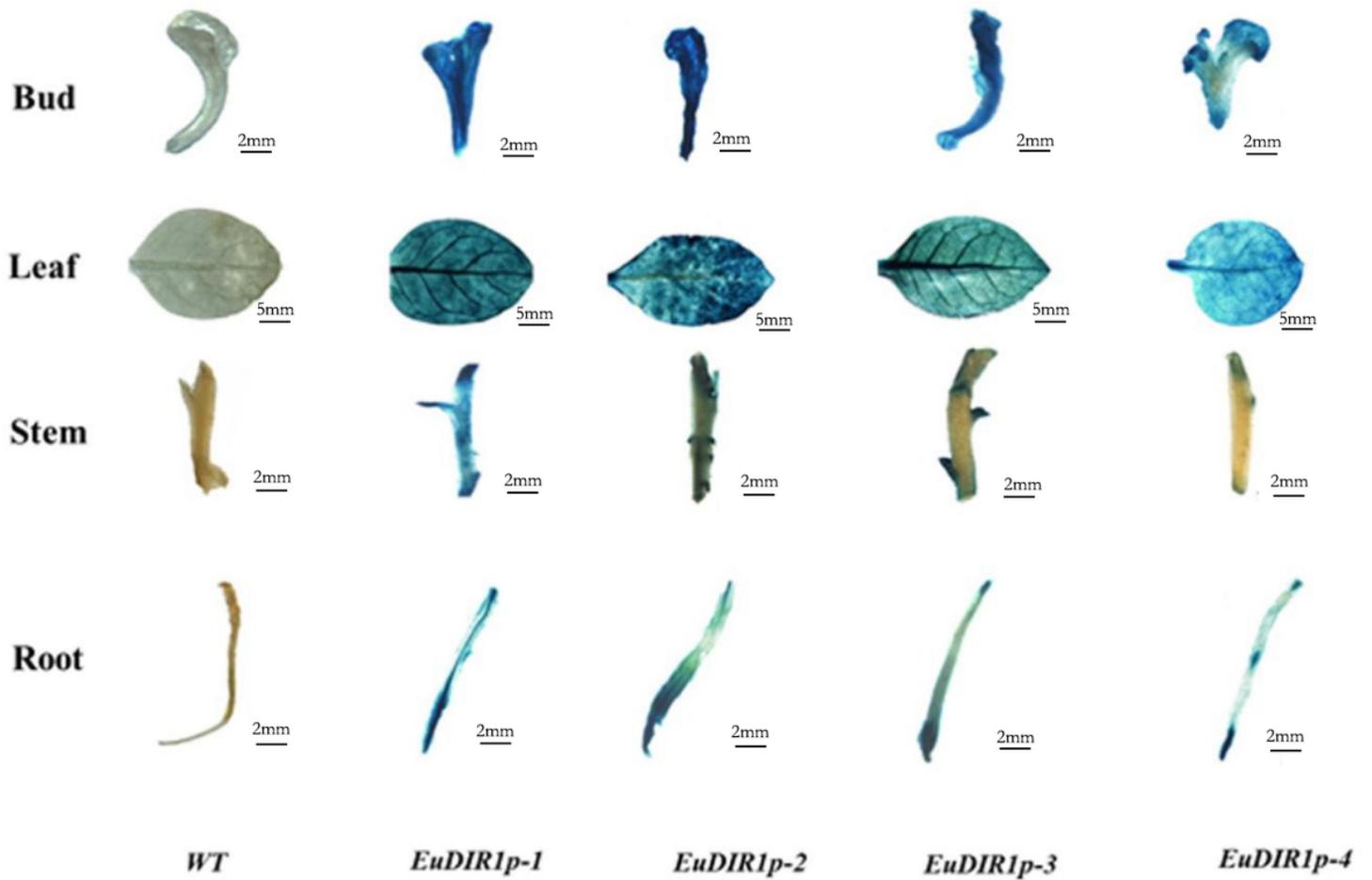
\*Underlined letters indicate the restriction enzyme sites

**Table 2.** Prediction of the cis-acting elements of the *DIR1* gene promoter

| Promoter sequence             | Cis-elements | Position (bp) | Effect   |
|-------------------------------|--------------|---------------|--|
| <i>EuDIR1p-1</i><br>(1495 bp) | CGTCA-motif  | -1468         | Cis-acting regulatory elements involved in the MeJA response |
|                               | STRE         | -1243         | Osmotic stress response element                              |
| <i>EuDIR1p-2</i><br>(804 bp)  | MYB          | -624          | Participates in drought-induced response                     |
| <i>EuDIR1p-3</i><br>(549 bp)  | Myb          | -490          | Participates in plant stress response                        |
| <i>EuDIR1p-4</i><br>(212 bp)  | TIS          | +1            | Transcription initiation site                                |
|                               | TATA-box     | -25           | Promoter core element  |
|                               | CAAT-box     | -51           | Promoter core element  |

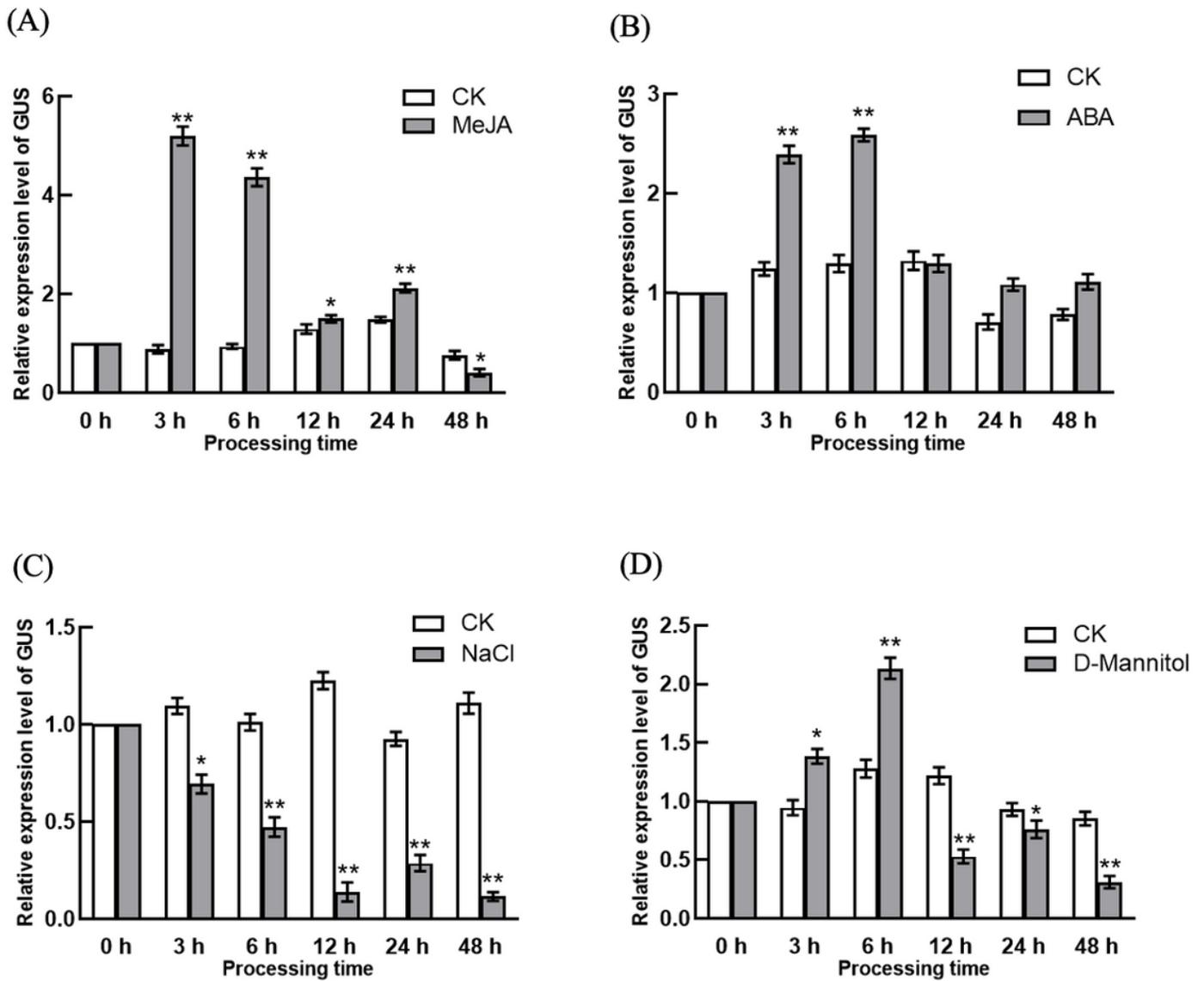
## Figures





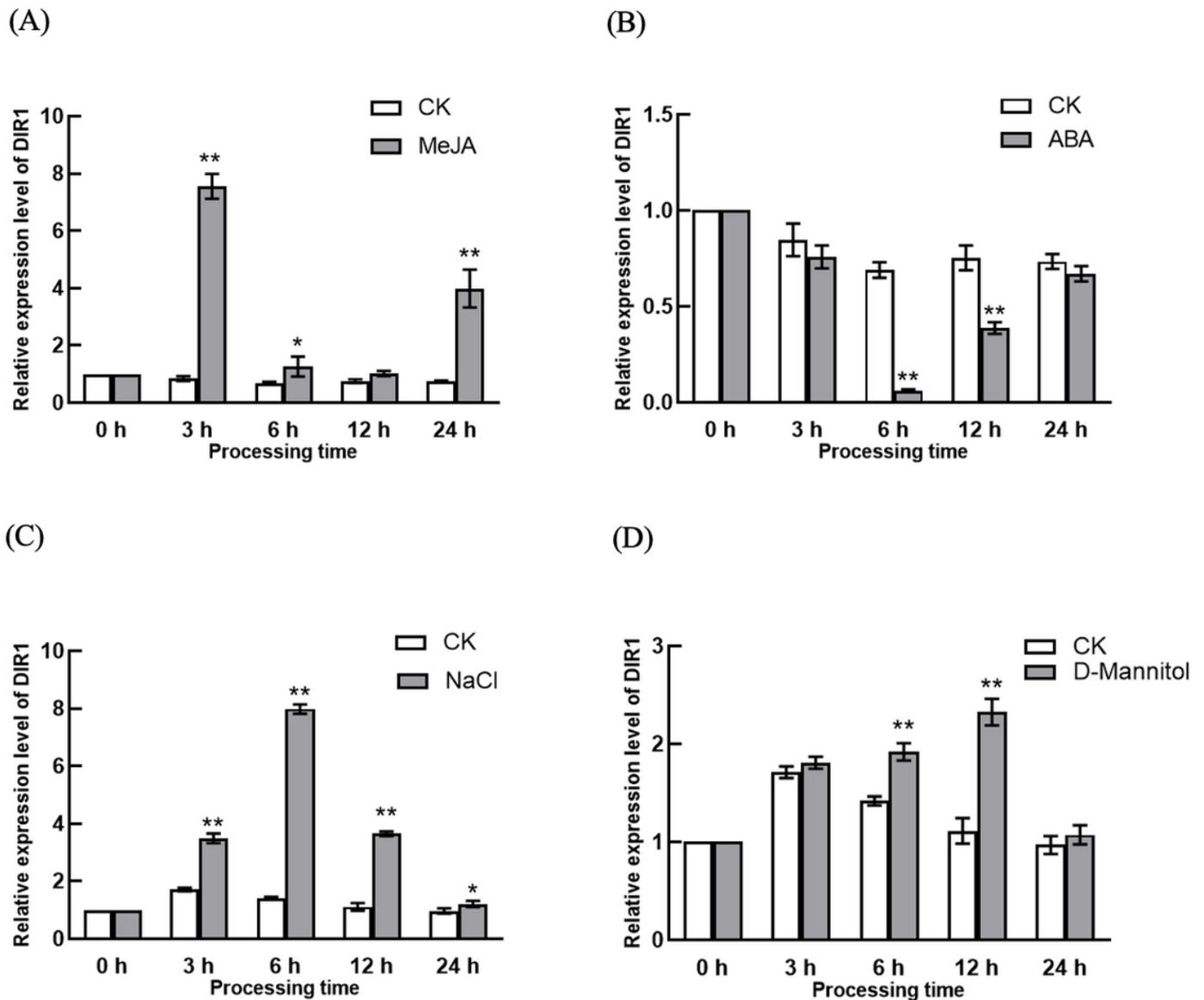
**Figure 2**

GUS fusion protein expression in transgenic tobacco lines. Representative images showing GUS immunostaining of young shoots, roots, stems, and leaves of WT (wild-type), *EuDIR1p-1*, *EuDIR1p-2*, *EuDIR1p-3*, and *EuDIR1p-4* transgenic plants. Scale bar = 2 mm.



**Figure 3**

GUS expression in transgenic tobacco under biotic stress. Relative expression levels of GUS in the transgenic tobacco seedlings at 0, 3, 6, 12, 24 and 48 h after treatment with (A) 100  $\mu$ M MeJA; (B) 100  $\mu$ M ABA; (C) 10 g/L NaCl; (D) 300 mM D-mannitol. CK: negative control. Error bars represent standard deviations of three replicates, \* P < 0.05, \*\* P < 0.01.



**Figure 4**

DIR1 expression in *E. ulmoides* under abiotic stress. Relative expression levels of DIR1 in *E. ulmoides* seedlings at 0, 3, 6, 12 and 24 h after treatment with (A) 100  $\mu$ M MeJA; (B) 100  $\mu$ M ABA; (C) 10 g/L NaCl; (D) 300 mM D-mannitol. CK: negative control. Error bars represent standard deviations of three replicates, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MJ11736SupplementaryDG0106.docx](#)