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Identification and Analysis of the EIN3/EIL Gene Family in *Populus* × *xiaohei* T. S. Hwang et Liang: Expression Profiling during Stress

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Abstract: The ethylene-insensitive 3-like (EIN3/EIL) gene family, as a transcriptional activator in plants, not only plays an important role in the ethylene-signaling pathway in regulating plant growth and development but also participates in the defense against various biotic and abiotic stresses. However, there are few studies on the functions of EIN3/EIL genes in woody plants. *Populus* × *xiaohei* is a kind of tree species with strong drought resistance and salt-alkali tolerance and, thus, is an ideal subject for studying abiotic stress mechanisms in trees. Eight EIN3/EIL genes were cloned from *Populus* × *xiaohei*. Bioinformatic analysis showed that the *PsnEIN3/EIL* gene contained a highly conserved EIN3 domain, N-terminal sites rich in proline and glutamine, and other EIN3/EIL family structural characteristics. The results of a multi-species phylogenetic analysis showed that the family EIN3/EIL proteins were divided into three groups (A, B, and C). *EIL3* and *EIL4* belonged to groups A and B, while *EIL2* and *EIN3* generally belonged to group C. Analysis of tissue expression characteristics showed that *PsnEIN3/EIL* was expressed in different tissues and was involved in the development of stem nodes and leaves. The response analysis of the expression of *PsnEIN3/EIL* under abscisic acid (ABA) and abiotic stresses (salts, heavy metals, alkaline conditions, and drought) showed changes in expression, suggesting that *PsnEIN3/EIL* may be involved in the processes of plant hormone responses to salts, heavy metals, alkaline conditions, and drought. This study provides a foundation for further elucidation of the functions of EIN3/EIL genes in forest growth and development and abiotic stress responses.

Keywords: *Populus* × *xiaohei*; T. S. Hwang et Liang; PsnEIN3/EIL family; abiotic stress; expression analysis

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1. Introduction

Ethylene (ET), an endogenous hormone widely present in plants, is abundant in meristems, germinating seeds, senescent flowers, and fruits during ripening. ET not only plays an indispensable role in the whole growth and development of plants [1–9], but is also involved in the response to biotic and abiotic stresses such as mechanical damage, environmental adversity, and pathogens [10–14].

The ET-insensitive protein Ethylene-insensitive3/EIN3-like (EIN3/EIL) is a plant-specific protein that plays a key role in the ET signaling pathway [15]. As an important transcriptional activator, *EIN3/EIL1* regulates ET-mediated transcriptional cascades through its DNA binding [16]. Research on EIN3/EIL began in 1993, with the isolation of the *ein3* mutant and the cloning of *EIN3* and its homologs *EIN3-Like1* (*EIL1*) and *EIL2* in *Arabidopsis thaliana* [17].

As research progressed, *EIN3* and *EIL* were shown to share similar amino acid sequences, including a conserved structural domain-EIN3 domain and function [17], and

they may play a role in the form of homologous or heterologous dimers or multimers [17]. In higher plants, EIN3/EIL is a small transcription factor family with different numbers of members in different species, with nine, nine, and seven members identified in *A. thaliana*, *Oryza sativa*, and *Zea mays*, respectively [17–25].

In *A. thaliana* and other annual plants, the EIN3/EIL gene family has been shown to play important roles in bud formation, leaf senescence, root growth, flower development, and morphogenesis. Seed germination of the *A. thaliana* double mutant *ein3eil* was significantly inhibited, and the proportion of leaf albinism increased, while *EIN3* overexpression enhanced seed germination [26]. In leaves, *A. thaliana* plants with overexpression of *EIN3* showed accelerated leaf senescence, while plants with loss of *EIL1* function had delayed leaf senescence [17,27]. EIN3/EIL transcription factors can also affect root development. The number of crown roots in mutant *ein2* and *eil1* plants was significantly reduced, while overexpression of *ein2* and *eil1* resulted in more crown roots than in the wild type [28].

Flower shedding and flower senescence occurred in tomato plants after inhibition of *LeEIL* gene expression [20], suggesting that the EIN3/EIL gene is involved in flower development [29]. *A. thaliana* EIN3/EIL1 directly induces the expression of two key enzymes in the chlorophyll synthesis pathway, protochlorophyllide oxidoreductase A and B (PorA/B), and also works synergistically with the *PIF1* gene of the photosensitive pigment interaction factors (*PIFs*) to prevent photooxidative damage and promote greening of cotyledons [30]. *EIN3* also regulates the pigment-binding protein LHC that is essential for photosynthesis initiation [31] and is involved in the sulfur assimilation pathway [32].

The EIN3/EIL gene plays an important role in regulating plant tolerance to abiotic and biological stresses [25]. *ESE1* is an ET regulatory gene downstream of EIN3/EIL1 that regulates the expression of genes related to the salt response pathway. The expression of *ESE1* was inhibited in *ein2*, *ein3*, *eil1-3*, and *ein3eil1* mutants. In addition, the transcription levels and salt tolerance of salt-responsive genes were increased in *A. thaliana* lines overexpressing *EIN3* [33]. The tolerance to salt and drought stresses was enhanced by overexpression of the mulberry *MnEIL3* gene in *A. thaliana*. In addition, *MnEIL3* also enhanced the activities of *MnACO1* and *MnACS1* promoters in response to salt and drought stress. Therefore, *MnEIL3* may play a role in resistance to salt and drought stress [34]. Drought is a major limiting factor for crop production, and *EIN3* is able to respond to drought stress and actively regulate the growth and morphology of *A. thaliana* under drought stress [35]. In *A. thaliana* seedlings, cadmium inhibits the degradation of EIN3 protein, while *EIN3* can enhance the inhibition of root growth under cadmium stress by regulating the expression of *XTH33* and *LSU1* genes that encode key regulators of cell wall elongation and sulfur metabolic processes. In addition, the *ein4-1/ein3-1/eil1-1* mutants were also found to show stronger resistance to cadmium [36]. The expression of the target gene decreased in rice plants with the trauma-induced inhibition of the *OsEIL1/OsEIL2* gene, suggesting an important role of *EIL* genes in trauma signaling in rice with *EIL* genes [25]. EIN3/EIL transcription factors are also involved in the regulation of pathogenic microbial stresses. In *A. thaliana*, ET and jasmonic acid (JA) act synergistically against necrotizing fungal infections by inducing EIL [37]. *EIN3* and *EIL1* negatively regulated the expression of salicylic acid (SA) and SA synthesis (SA induction deficient 2), thus inhibiting the immune response of plants to living trophic pathogens [38]. The double mutant *ein3eil1* showed reduced susceptibility to the virulent bacteria DC3000 and the non-pathogenic bacteria DC3000 HRP L, suggesting that *ein3eil1* negatively regulates the resistance of *A. thaliana* to both virulent and non-pathogenic bacteria [14].

Functional studies on EIN3/EIL genes have focused on annual plants, while only a few functional studies of EIN3/EIL genes in perennial woody plants have been reported. *Populus* × *xiaohei*, a hybrid of *Populus simonii* Carr and *Populus nigra*, is a greening and timber species occurring in northern China with strong resistance to cold, drought, and salinity [39]. It is an ideal species for studying abiotic stress mechanisms in trees. In this study, eight *EIN3/EIL* genes were cloned, and their sequence characteristics were analyzed. The expression patterns of the EIN3/EIL transcription factor family in the growth and

development of *Populus* × *xiaohei* and abiotic stress response were explored by analyzing tissue-specific expression and expression under abiotic stress. The results provide a basis for understanding the functions of *Populus* × *xiaohei* EIN3/EIL gene.

2. Materials and Methods

2.1. Plant Material

The current year shoots of *Populus* × *xiaohei* asexual plants were cut into spikes with 1–2 axillary buds, and cuttings were inserted into a container with the same weight of mixed substrate (peat soil: vermiculite: perlite = 2:1:1). The culture conditions in the greenhouse were as follows: 16 h/d of light, 60–70% relative humidity, and 25 °C. After 50 days of growth, plants with uniform size and free from diseases and pests were selected as the subsequent research materials.

Specific roots, xylem, phloem, third leaves, and terminal buds of the asexual poplar plants were selected to analyze the expression of the EIN3/EIL genes in these tissue parts. The 1st–14th leaves and 1–11th stem segments of the whole plant, from the first young leaves at the top to the senescent leaves at the bottom, were used to study the expression of the EIN3/EIL genes during the development of leaves and stem segments.

The seedlings of *Populus* × *xiaohei* were irrigated (100 mL/d) with 0.4 mol/L NaCl, 150 µmol/L $CdCl_2$, 0.3 mol/L $NaHCO_3$, and 20% PEG solution to simulate abiotic stress. The leaves were sprayed with 100 µmol/L ABA daily to simulate exogenous hormone treatment. At 0, 6, 12, 24, 48, and 72 h of treatment, the roots and the third leaves were taken as research materials to analyze the responses of the EIN3/EIL genes to abiotic stress.

Three groups of samples were collected for each material as three biological replicates and were stored in a refrigerator at −80 °C for subsequent analysis.

2.2. Cloning of PsnEIN3/EIL Genes of Populus × xiaohei

Total RNA was extracted from the above parts of *Populus* × *xiaohei* using a plant RNA Extraction Kit (Bioteke Corporation, Beijing, China). The total RNA obtained was then reverse transcribed into cDNA using a Primescript MRT reagent kit (Toyobo (Shanghai) Biotech CO. Ltd., Shanghai, China), diluted 10 times, and used as a PCR template.

Specific primers were designed for cloning the full-length ORF of the EIN3/EIL genes of *Populus* × *xiaohei* with reference to the *Populus trichocarpa* genome (https://phytozome-next.jgi.doe.gov/pz/portal.html (accessed on 3 November 2021)) and the genome of *Populus* × *xiaohei* (unpublished) (Table 1). The cDNA of different tissue parts was used as the template for PCR amplification. The reaction system was as follows: 1.7 µL of 10 × KOD buffer, 1.7 µL of 2 mmol/L dNTPs, 0.8 µL of $MgSO_4$, 1 µL of cDNA template, 0.6 µL of 10 mmol/L upstream and downstream primers, 0.4 µL of KOD Plus, and ddH_2O supplemented to 20 µL. The PCR amplification was performed at 94 °C for 2 min, followed by 94 °C for 45 s, 58 °C for 45 s, and 68 °C for 2 min for 35 cycles, with a final extension at 68 °C for 10 min. The PCR products were detected by 1.0% agarose gel electrophoresis and purified products were linked to a pMD-18T vector, and the accuracy of the sequences was verified by PCR and sequencing.

Table 1. Features of the eight *PsnEIN3/EIL* genes.

Gene	Chromosome	ORF Length	Protein Length /Amino Acids	PI	Molecular Weight (Da)	Subcellular Localization
PsnEIN3.1	Chr04	1854	617	5.68	70,025.84	Nucleus
PsnEIL2.1	Chr10	1539	556	5.97	62,939.99	Nucleus
PsnEIL2.2	Chr10	1980	659	5.49	74,463.65	Nucleus
PsnEIL2.3	Chr08	1989	662	5.28	74,991.26	Nucleus
PsnEIL2.4	Chr08	1989	662	5.29	74,934.21	Nucleus
PsnEIL2.5	Chr08	1962	653	5.38	73,785.89	Nucleus
PsnEIL4.1	Chr01	1197	398	8.24	44,013.25	Nucleus
PsnEIL4.2	Chr03	1788	595	5.60	66,742.84	Nucleus

2.3. Bioinformatic Analysis

The ExPasy Proteomics Server (http://expasy.org/ (accessed on 10 December 2021)) was used to predict the molecular weights, isoelectric points and lengths of the cloned EIN3/EIL family member protein sequences [40]. Multiple sequence alignment was performed on EIN3/EIL protein sequences of *Populus* × *xiaohei* and *A.thaliana* by DNAMAN using default parameters. ClustalW2 in BioEdit was used to align the amino acid sequences [41].

Then, a phylogenetic tree was constructed by the neighbor-joining (NJ) method using MGEA 7.0 with the parameter set to Bootstrap and the test time set to 1000 [42]. Multiple expectation maximization for motif elicitation (MEME, http://meme.nbcr.net/meme/cgi-bin/meme.cgi (accessed on 15 December 2021)) was employed to predict the conserved motifs of the proteins of the EIN3/EIL transcription factor family [43]. Parameter settings were as follows: max motif number, 10; min motif width, 6; and max motif width, 50 aa. The MG2C tool (http://mg2c.iask.in/mg2c_v2.0 (accessed on 23 December 2021)) was used to plot the location information of *PsnEIN3/EIL* gene on the corresponding chromosomes [44]. Using coding sequences, the Multiple Collinear Scanning toolkit (http://chibba.pgml.uga.edu/mcscan2/ (accessed on 23 December 2021)) under the Linux system was used to identify gene duplication events. The non-synonymous (Ka) and synonymous (Ks) substitution rates of the homologs were calculated by DnaSP (version 5.10) [45].

2.4. Semi-Quantitative Reverse Transcription PCR, (sqRT-PCR)

Semi-quantitative reverse transcription and polymerase chain reaction (sqRT-PCR) is a simple and specific method for studying differential regulation of target genes [46–48]. An sqRT-PCR analysis was performed in order to study the tissue expression characteristics and stress responses of the *PsnEIN3/EIL* genes [49,50]. The primers were designed according to the specific sequence of the *PsnEIN3/EIL* genes, and TUA was used as the internal reference gene. The PCR reaction system comprised 10μL 2 × Es Taq Mix, 2 μL cDNA template, 1 μL of 10 mmol/L upstream and downstream primers, and ddH_2O supplemented to 20 μL. The reaction conditions were 94 °C for 2 min, 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 10 s. A total of 40 cycles were performed. A housekeeping gene (an internal reference gene) was used as a reference standard in treatments and controls to observe the expression of the *PsnEIN3/EIL* genes (upregulated or downregulated) during stress [46,51–53]. Tanon Gis software was used to convert the electrophoretic bands into numerical values based on brightness, and Heml was used to draw heat maps [39].

3. Results and Analysis

3.1. Cloning and Identification of EIN3/EIL Gene Sequences of Populus × xiaohei

Using cDNAs from different tissues of *Populus* × *xiaohei* as a template, the specific primers were designed according to the genomic sequences of *Populus* × *xiaohei* and *P. trichocarpa* to obtain their full-length ORF sequences by PCR amplification. The amplification products were subjected to 1.0% agarose gel electrophoresis, and the purified gel products were ligated to a pMD-18T vector for bacterium fluid PCR tests and sequencing (Figure 1). The results showed that the specific sequences obtained were consistent with the expected target sequence size. The sequences were highly homologous to the EIN3/EIL genes of *A.thaliana* (Figure S1). Therefore, following the naming strategy of Li et al. [54], the obtained sequences were named *PsnEIN3.1*, *PsnEIL2.1*, *PsnEIL2.2*, *PsnEIL2.3*, *PsnEIL2.4*, *PsnEIL2.5*, *PsnEIL4.1*, and *PsnEIL4.2*.

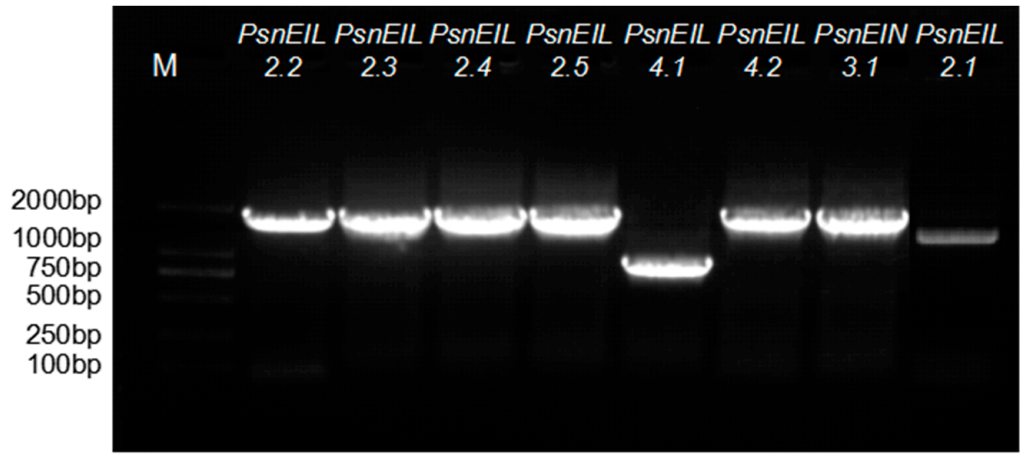


Figure 1. The products of PCR amplification of *PsnEIN3/EIL* gene. The arrow on the left is the band size of the electrophoresis marker. Electrophoresis bands from left to right: DL2000Marker(M); gel electrophoresis imaging bands of *PsnEIL2.2*, *PsnEIL2.3*, *PsnEIL2.4*, *PsnEIL2.5*, *PsnEIL4.1*, *PsnEIL4.2*, *PsnEIN3.1*, and *PsnEIL2.1*. The lengths of the *PsnEIN3/EIL* genes were 1980, 1989, 1989, 1962, 1197, 1788, 1854, and 1539 bp.

3.2. Bioinformatic Analysis of the EIN3/EIL Gene of Populus × xiaohei

3.2.1. Analysis of the Physicochemical Properties of PsnEIN3/EIL Gene in *Populus × xiaohei*

The physicochemical property analysis revealed that the length of the ORFs of the eight *PsnEIN3/EIL* genes ranged from 1197 to 1989, and the amino acid lengths of the members differed somewhat, ranging from 398 to 650 aa. The amino acid sequences of *PsnEIL2.3* and *PsnEIL2.4* proteins were the longest, both containing 662 aa; the amino acid sequence of *PsnEIL2.1* protein was the shortest, containing 556 aa. The molecular weight of the proteins ranged from 44013.25 to 74934.21 Da. The theoretical isoelectric points ranged from 5.28 to 8.24, and most were acidic proteins. In addition, subcellular localization analysis demonstrated that all members were located in the nucleus (Table 1).

3.2.2. Multiple Sequence Alignment of PsnEIN3/EIL Proteins

The EIN3/EIL family is a class of specific proteins localized in the nucleus in higher plants. These proteins have highly conservative N-terminal sequences and contain acidic N-terminal amino acids, basic amino acid clusters and a proline-rich domain [23]. Conserved amino acid and characteristic analyses of the 14 EIN3/EIL proteins of *Populus × xiaohei* and *A. thaliana* were performed using BioEdit software to produce the complete alignment (Figure 2). In this study, *PsnEIN3/EIL* protein sequences exhibited some structural features of the EIN3/EIL family protein, with a completely conserved EIN3 domain at the N-terminus, an acidic amino acid enriched region (AD), five conserved basic amino acid enriched regions (BDI–V), and a proline-rich region (PR) (Figure 2). It has been shown that regions rich in the acidic amino acids proline and glutamate are common transcriptional activation domains in some plants [17,55,56]. Therefore, it has been speculated that AD, BDI–V, and PR are transcriptional activation domains and functional regions of the *PsnEIN3/EIL* transcription factor family. Meanwhile, amino acid residues 300–600 at the C-terminal sequences did not show any significant similarity, suggesting that these sequences may be crucial for the functional divergence of EIN3/EIL members.

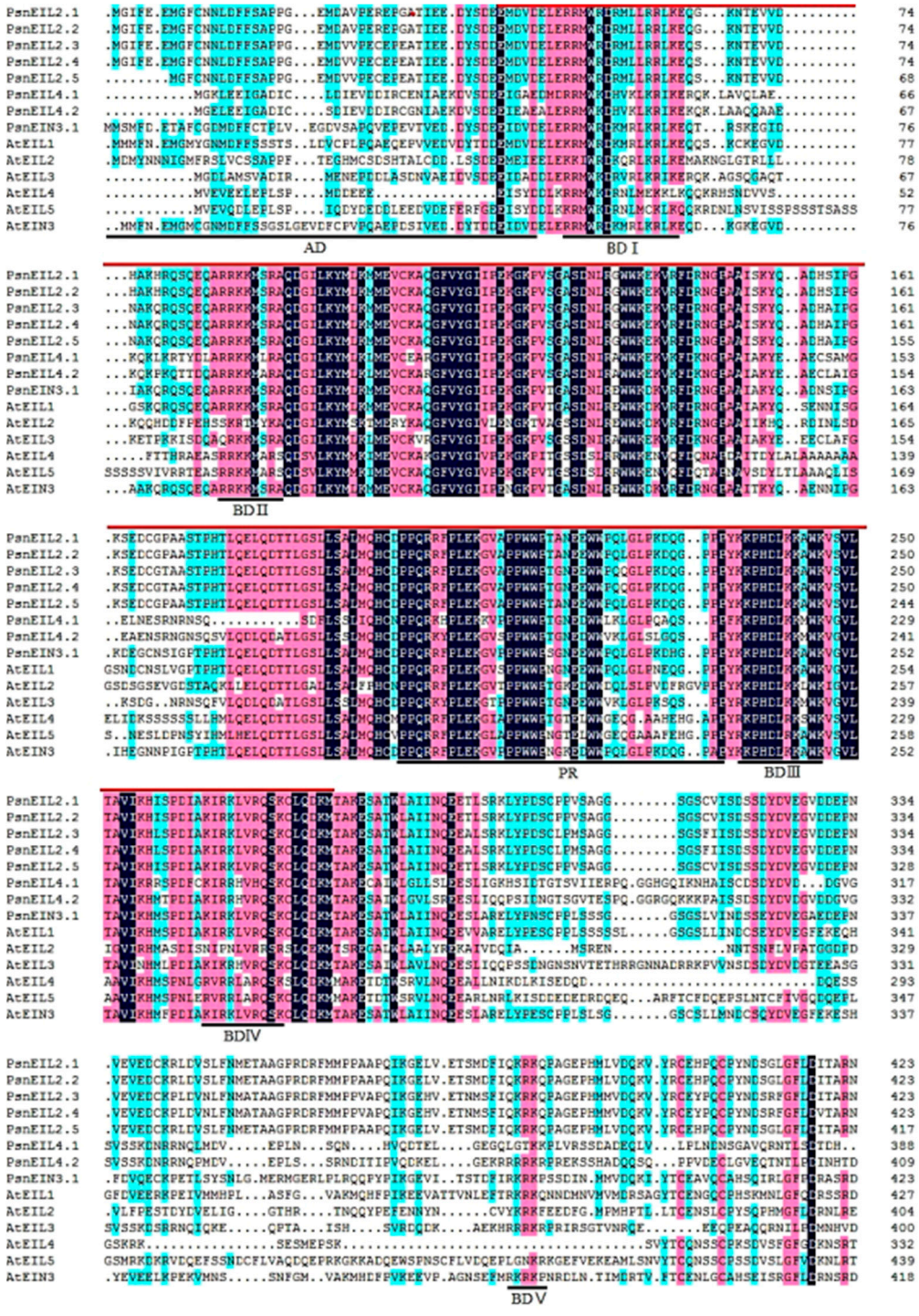


Figure 2. Sequence alignment of all identified EIN3/EIL genes in *Populus* × *xiaohei* and *A. thaliana*. Sequence comparisons were made via alignment using ClustaW, and identical or similar residues are shaded in different background colors. The areas with 100% similarity are displayed in black, pink indicates similarity of 75%, and blue indicates similarity of 50%. The approximate position of the EIN3 structural domain is shown as a red line above the matched sequences. Amino acid enrichment regions are indicated by black lines below the matched sequences: AD: acidic amino acid enrichment region; BDI–V: basic amino acid enrichment regionI–V; PR: proline enrichment region.

3.2.3. Phylogenetic Analysis of PsnEIN3/EIL Genes in *Populus* × *xiaohei*

In order to reveal the evolutionary relationship of EIN3/EIL genes between *Populus* × *xiaohei* and other species, a total of 30 EIN3/EIL protein sequences were analyzed

to construct a phylogenetic tree, including sequences from *A. thaliana* (At: 6), *Theobroma cacao* (Tc: 3), *O. sativa* (Os: 6), *Actinidia chinensis* (Ac: 4), *Z. Mays* (Zm: 2), and *Cucumis melo* (Cm: 1), and *Populus* × *xiaohei* (Psn: 8). The phylogenetic tree was constructed by NJ using MEGA6.0 software (Figure 3).

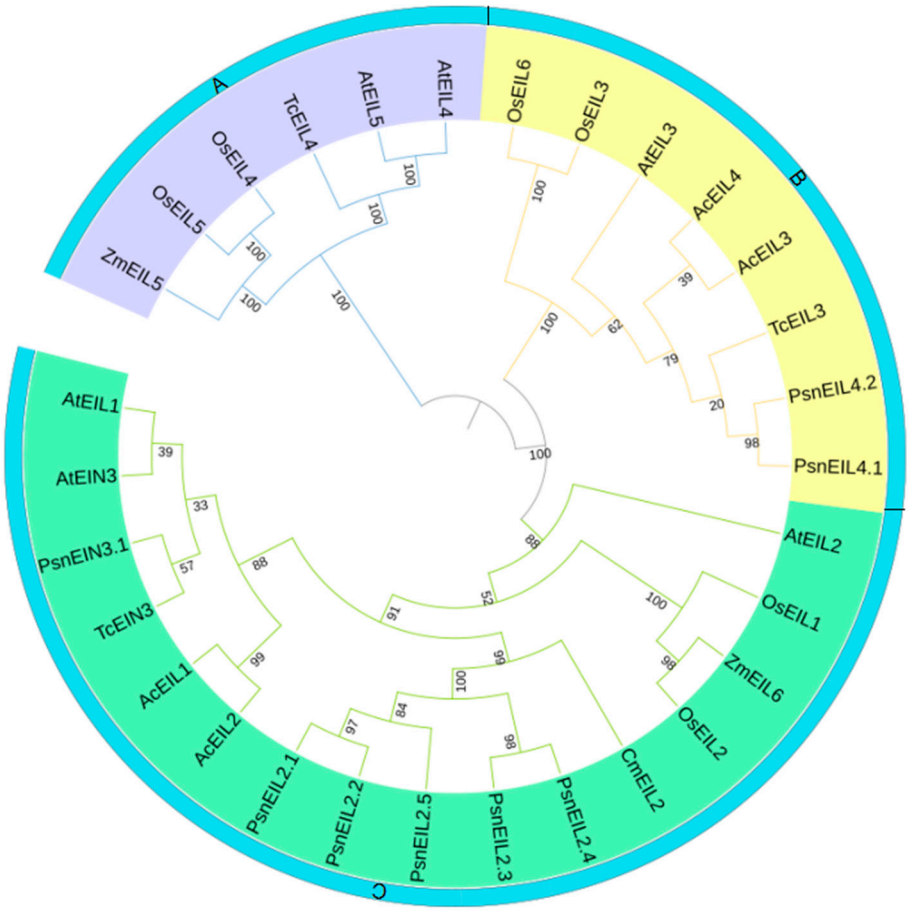


Figure 3. Phylogenetic relationships of EIN3/EIL genes of *Populus* × *xiaohei* and other species. The phylogenetic tree was generated from the alignment of 30 EIN3/EIL protein homeodomain sequences with 1000 bootstrap replicates. EIN3/EIL members are distributed in three main clades, namely clades A, B, and C marked in green, blue, and yellow, respectively. Bootstrap values are shown near the nodes. (At: *Arabidopsis thaliana* [*AtEIN3* (NP_188713.1), *AtEIL1* (NP_180273.1), *AtEIL2* (NP_001332194.1), *AtEIL3* (NP_177514.1), *AtEIL4* (NP_20131.1), *AtEIL5* (NP_196574)]; Tc: *Theobroma Cacao* [*TcEIN3* (EOY34301.1), *TcEIL3* (XP_017982625.1), *TcEIL4* (EOX92159.1)]; Os: *Oryza sativa* [*OsEIL1* (XP_015629857.1), *OsEIL2* (XP_015646574.1), *OsEIL3* (XP_015648717.1), *OsEIL4* (XP_015625091.1), *OsEIL5* (CAD40887.2), *OsEIL6* (XP_015612094.1)]; Ac: *Actinidia chinensis* [*AcEIL1* (PSS14564.1), *AcEIL2* (PSS14565.1), *AcEIL3* (ACJ70676.1), *AcEIL4* (PSR86855.1), *AcEIL5* (PSS21513.1)]; Zm: *Zea Mays* [*ZmEIL5* (KJ726967.1), *ZmEIL6* (KJ728014.1)]; Cm: *Cmaris Melo* [*CmEIL2* (NW_007546307.1)]).

The phylogenetic tree clearly divided the EIN3/EIL proteins into three clades, A, B, and C. Clade C had the most members (16) and contained *EIL1*, *EIL2*, and *EIN3* proteins, followed by groups B and A with 8 and 6 proteins, respectively, which consisted of *EIL3*, *EIL4*, and *EIL5* proteins. Each clade contained EIN3/EIL proteins from monocots and dicots, and at least one member of the *AtEIN3/EIL* family was included in each clade in this phylogenetic tree. This indicates that the EIN3/EIL family divergence earlier than the divergence of *Populus* and *A. thaliana*. As can be seen from the figure, this protein family had high bootstrap values at most nodes of the phylogenetic tree, indicating that this family of genes is highly conserved in evolution. *PsnEIN3.1* and *TcEIN3*, *PsnEIL2* and *CmEIL2*, and *PsnEIL4* and *TcEIL3* were clustered in the A2, B2, and C1 sub-clades,

respectively. Meanwhile, the eight *Populus* × *xiaohei* genes were classified into two groups, among which the five homologous genes of *PsnEIL2* were extremely similar. Therefore, we speculated that the five genes of *PsnEIL2* were formed by duplication during the evolutionary process. Moreover, the five *PsnEIL2* and *PsnEIN3.1* genes were clustered together with *AtEIL1* and *AtEIN3*, but not with *AtEIL2*. Studies have shown that when a gene undergoes chromosomal tandem or partial duplication, it may lead to complete functional redundancy among the initially homologous genes [57], and both *AtEIL1* and *AtEIL2* genes can partially complement the phenotype of the *AtEIN3* variant [58]. These results suggest that the functions of *AtEIN3*, *AtEIL1*, and *AtEIL2* are partially similar and that the six *Populus* × *xiaohei* genes are functionally redundant and functionally similar to the *A. thaliana* EIN3/EIL genes.

3.2.4. Analysis of Conserved Motifs Analysis of EIN3/EIL Proteins

In order to further analyze the protein characteristics of the EIN3/EIL gene family, the online analysis software MEME was used to find conserved motifs in the EIN3/EIL proteins of *A. thaliana* and *Populus* × *xiaohei* (Figure 4). As a result, a total of 10 most conserved motifs (motif1–10) were identified in EIN3/EIL proteins, ranging in length from 21 to 50 amino acid residues (Figure 4B). Among these motifs, according to a previous study, motifs 2–5 located at the N-terminal may constitute a conserved domain of EIN3/EILs, further confirming that the N-terminal of EIN3/EIL members is a highly homologous sequence [59]. Moreover, the existence of such conserved motifs associated with the EIN3 domain also suggests the highly conserved structure of *PsnEIN3/EIL*.

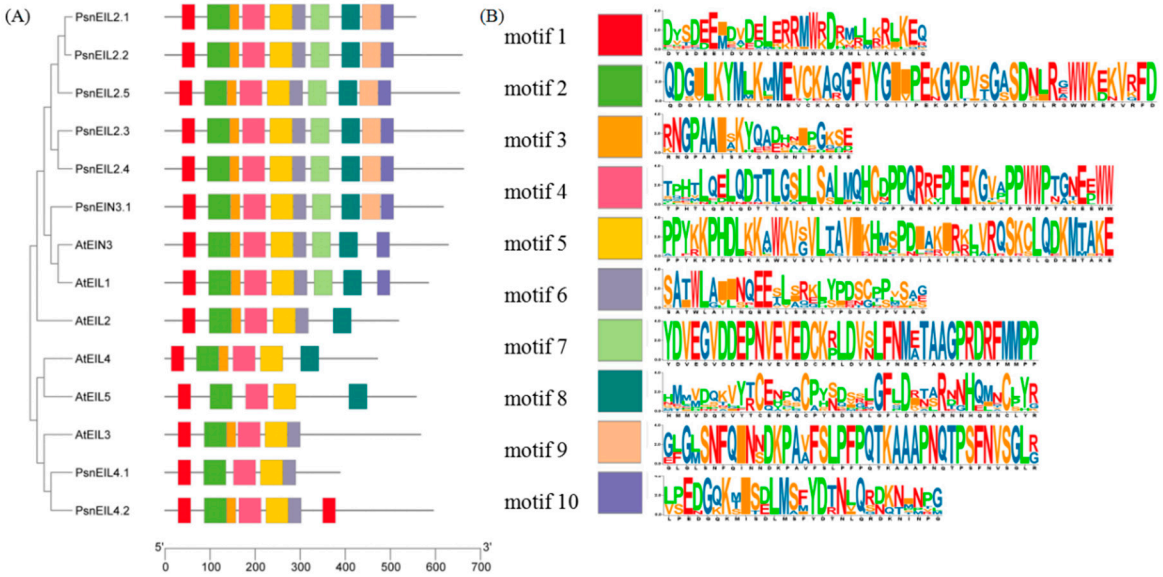


Figure 4. Analysis of the conserved motifs of *EIN3/EIL* genes in *Populus* × *xiaohei* and *A. thaliana*. Structural features of EIN3/EIL transcription factors in *Populus* × *xiaohei* and *A. thaliana*. (**A**) The NJ tree and motif analysis were constructed from a total of 14 EIN3/EIL genes from *Populus* × *xiaohei* and *A. thaliana*. The numbers below the motifs correspond to their approximate position and length in the residue. The EIN3 domain is located in the first half of the protein, between approximately 80 and 280 residues. (**B**) LOGO of 10 conservative motifs was predicted by the MEME tool.

As shown in Figure 4A, there were 10 conserved motifs (motifs 1–10) present in *PsnEIL2* and *PsnEIN3.1* proteins, and five conserved motifs (motifs 1–2 and motifs 4–6) in *PsnEIL4.1*. *PsnEIL4.2* had six conserved motifs (motifs 1–6). This difference in the number of conserved motifs may be connected to the differential expression and related functions of the genes. Consistent with the phylogenetic tree results, most of the closely related EIN3/EILs exhibited highly similar motif compositions and distribution patterns. *PsnEIL2* was significantly different from *PsnEIL4*, which was homologous to *AtEIL3*, indicating the

possibility of different functions. It is noteworthy that the distribution of the *PsnEIN3/EIL* protein motif has typical characteristics: all members of the *PsnEIN3/EIL* contained five classes of identical motifs (motif 1, motif 2, motif 4, 5, and 6); the distribution order of the identified motifs is highly consistent, and the positions are basically the same, indicating that this gene family has been in evolution. Remarkably, *PsnEIL2* and *PsnEIN3.1* proteins contained a unique motif 9, suggesting that this motif may have a special function that distinguishes it from other EIN3/EIL proteins. The high conservation of EIN3/EIL family proteins in the N-terminal region is evidence of their evolutionary conservation and also indicates that they have important functions. We speculated that the conserved N-terminal motif may be closely related to the active center of this family of proteins. Moreover, functional differences of EIN3/EIL members are generally derived from carboxy-terminal sites of the proteins [19,60].

3.2.5. Chromosomal Location, Duplication Analysis, and Ka/Ks Calculation

To better understand duplication events and the distribution of the *PsnEIN3/EIL* genes, the identified *PsnEIN3/EIL* members were mapped to poplar chromosomes. The five genes identified were located on different chromosomes (Figure 5). The reported overlapping functions of the EIN3/EIL genes in the ET signaling pathway suggest synergistic cross-talk between the gene family members.

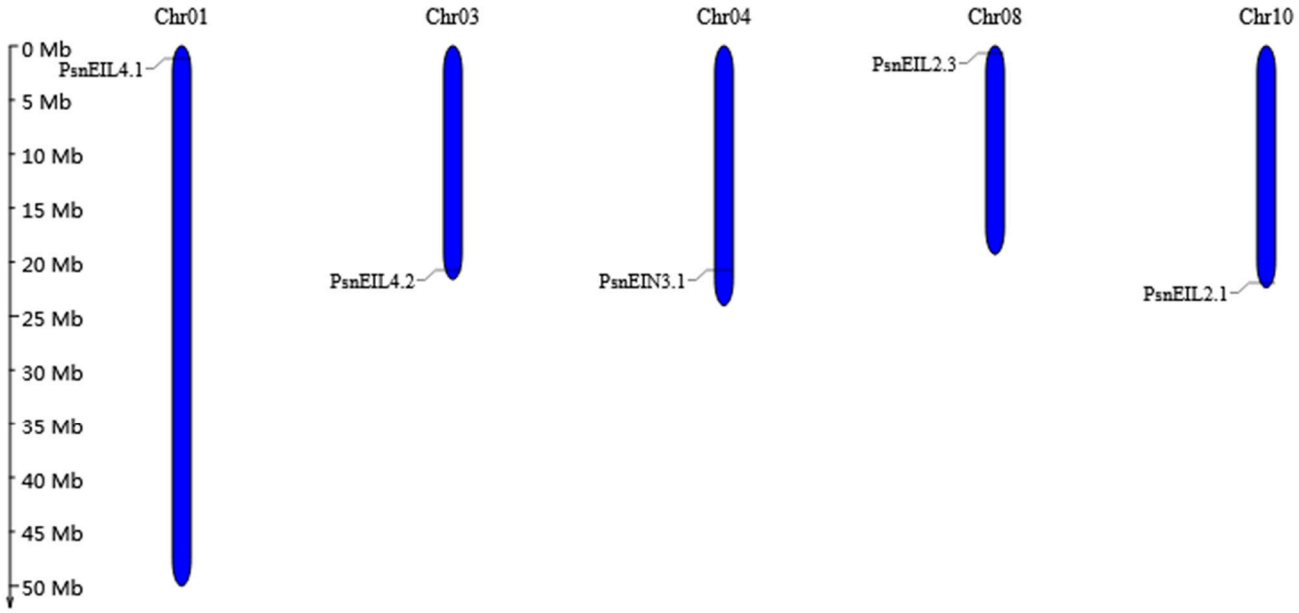


Figure 5. Chromosomal location of *PsnEIN3/EIL* genes. EIN3/EIL genes were mapped to different poplar chromosomes using the MapGene2Chrom tool. Gene positions on chromosomes are as follows: Chr01:1163628-1167197, Chr03:20939782-20943057, Chr04:20998609-21002260, Chr08:626991-631538, and Chr10:22117935e22122336). Chr represents the chromosome. The rule on the left indicates the physical map distance among genes (Mbp).

Duplicate genes may occur through new functionalization, sub-functionalization, and non-functionalization during genetic evolution [61]. Therefore, it is important to detect duplication events in *PsnEIN3/EIL*.

The synteny relationship of EIN3/EIL genes was analyzed using the genome information from *Populus* × *xiaohei*. After screening the identity and position of sequences, a total of three pairs of EIN3/EIL syntenic paralogs were found, and three fragment duplication events were identified among the six *PsnEIN3/EIL* genes comprising the three pairs of *PsnEIL2.1* and *PsnEIL2.3*, *PsnEIL4.1* with *PsnEIL4.2*, and *PsnEIN3.1* with *EIN3*-like genes. The results suggest that parts of the *PsnEIN3/EIL* genes may have been caused by gene

duplication events, and these duplication events were the main factor for the *PsnEIN3/EIL* gene family amplification (Figure 6).

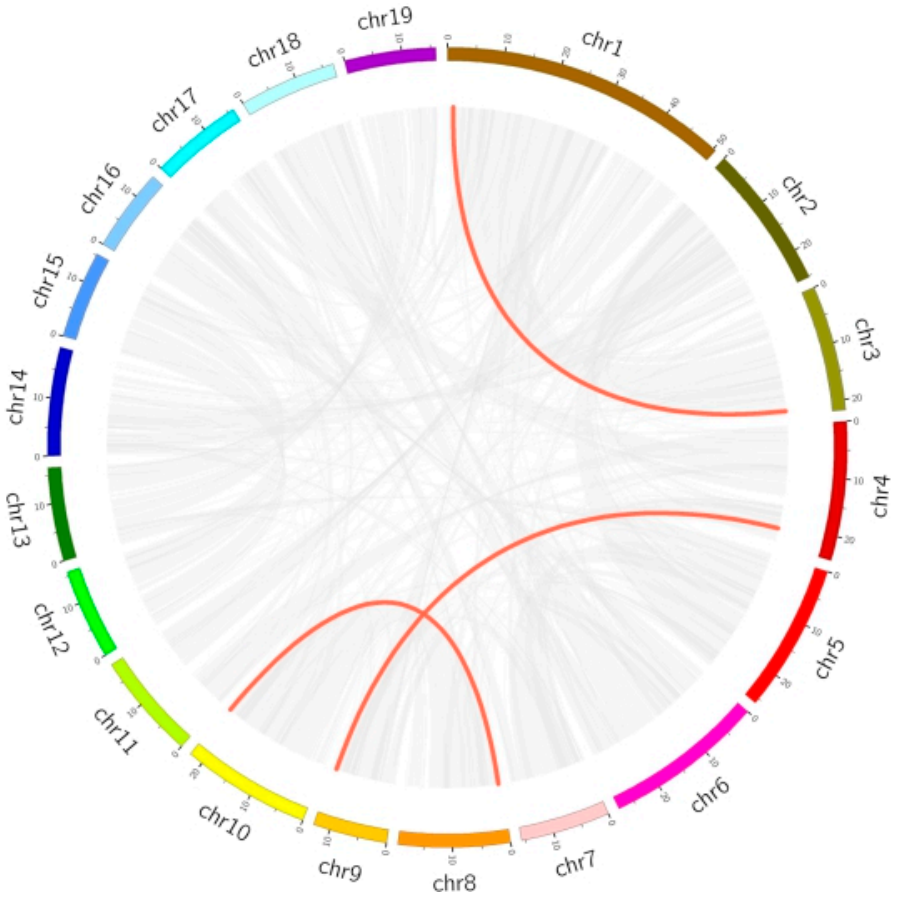


Figure 6. Genome-wide synteny analysis of *PsnEIN3/EIL* genes. chr1–19 represent chromosomes in *Populus* × *xiaohei*. All identified EIN3/EIL genes were mapped onto corresponding chromosomes. The red lines link the syntenic paralogs.

To further understand the gene duplication mechanism of the EIN3/EIL gene family in *Populus* × *xiaohei*, a comparative map with the dicotyledonous plants *A. thaliana*, *Glycine max*, and *M. pumila* was constructed (Figure 7).

As shown in Figure 7, no homologs were found between the *Populus* × *xiaohei* and *A. thaliana*. In contrast, two and four pairs of homologs existed between *Populus* × *xiaohei* and *G. max* and *M. pumila*. The results showed that the *PsnEIN3/EIL* genes have experienced a high degree of divergence during evolution and are homologous within the dicotyledons.

After a brief period of relaxed selection early, most duplicated genes are silenced, with only a few survivors experiencing the strong purifying selection [61]. Selective pressure can provide insights into the duplication of genes the EIN3/EIL gene family. The calculated non-synonymous (Ka) and synonymous substitution (Ks) values are shown in Table S1. The Ka/Ks values of three gene pairs were all less than 1.0. Studies have shown that Ka/Ks = 1.0 means neutral drift; when Ka/Ks > 1.0, the selection is positive, and evolution is accelerated; and Ka/Ks < 1.0 indicates strong purifying selection [45]. The EIN3/EIL gene family in *Populus* × *xiaohei* and its diploid progenitors have undergone purifying selection pressure after the duplication events.

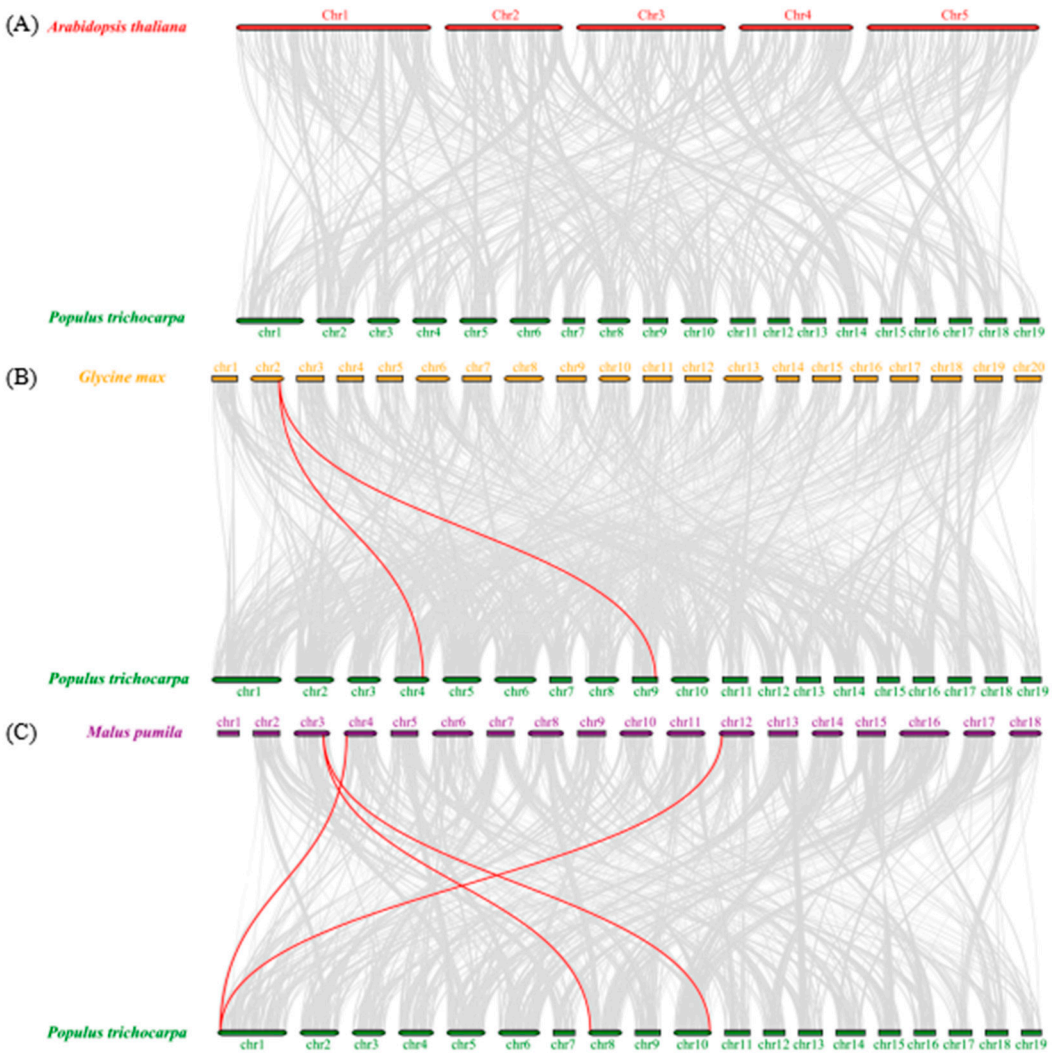


Figure 7. Synteny analysis of EIN3/EIL genes between *Populus* × *xiaohei* and other plants. (**A**) Dicotyledonous plant *A. thaliana*. (**B**) Dicotyledonous plant *G. max*. (**C**) Dicotyledonous plant *M. pumila*. Gray lines in the background indicate collinear blocks within *Populus* × *xiaohei* and other plant genomes; red lines indicate syntenic EIN3/EIL gene pairs.

3.3. Gene Expression Pattern Analysis of EIN3/EIL Genes

3.3.1. Characterization of Tissue Expression of PsnEIN3/EIL in *Populus* × *xiaohei*

To further investigate the expression of all identified *PsnEIN3/EIL* genes and their potential biological functions, the expression of eight *PsnEIN3/EIL* genes in major tissues (roots, xylem, phloem, third leaves, and terminal buds) was analyzed (Figure 8). As shown in Figure 5, the overall expression levels of eight *PsnEIN3/EIL* genes were not specific to these five tissues, indicating that they may play roles in all examined tissues. There were significant differences in the expression of *PsnEIN3/EIL* genes in different tissues. *PsnEIL2.3* and *PsnEIL4.1* showed markedly high expression in roots. *PsnEIL2.2* was highly expressed in phloem. Other genes were expressed in most tissues. Furthermore, *PsnEIL2.1* and *PsnEIL3.1* had the similar expression patterns, and were expressed in all tissues, except leaves, and, especially, in roots. The expression level of *PsnEIL4.2* was the lowest in xylem and higher in the other four plant parts. These results suggest that the *PsnEIN3/EIL* genes may play different roles in the growth and development of *Populus* × *xiaohei*.

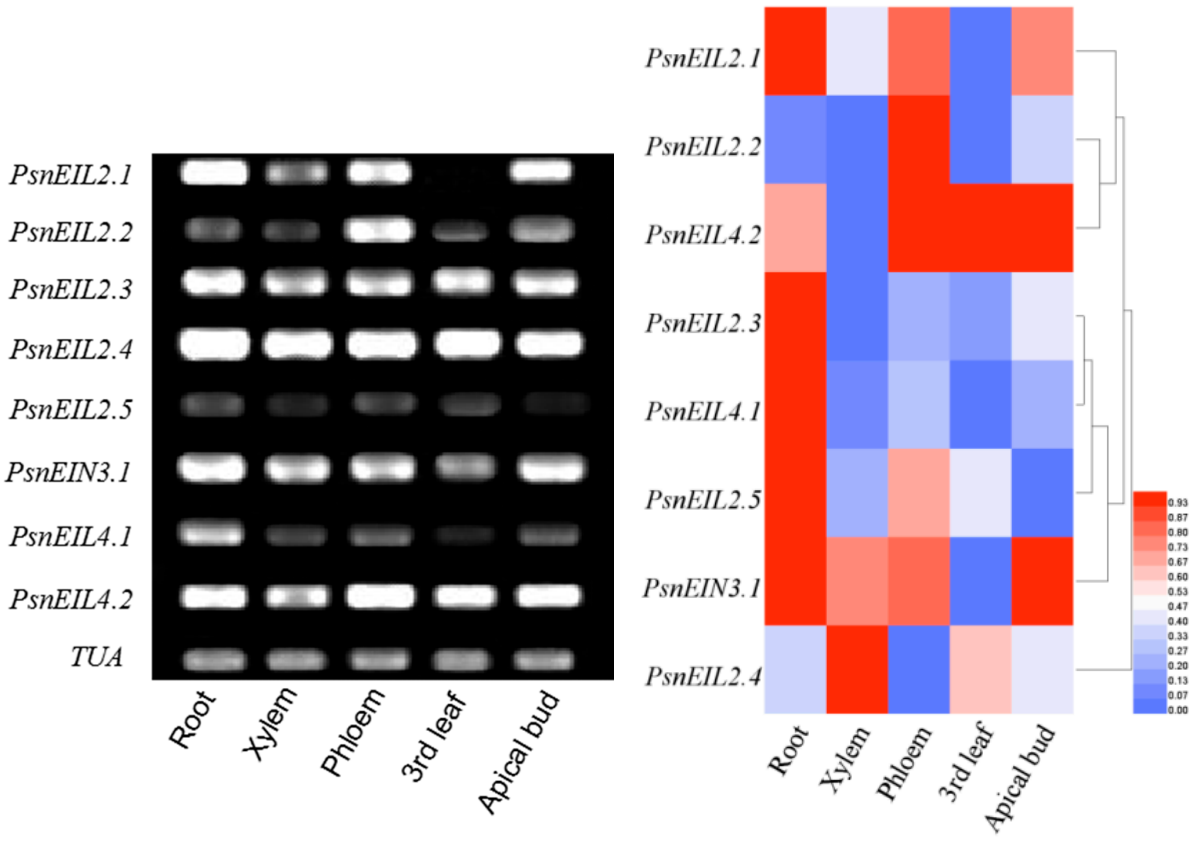


Figure 8. Tissue-specific expression pattern of *PsnEIN3/EIL* genes. The heat map shows the relative expression levels of EIN3/EIL genes in different plant parts (roots, xylem, phloem, third leaves, and terminal buds) of *Populus* × *xiaohei*.

3.3.2. PsnEIN3/EIL Genes Showed Different Expression Patterns during the Development of Stems of *Populus* × *xiaohei*

The tissue expression analysis indicated that all six genes were expressed in both the xylem and the phloem of mature stems, except for *PsnEIL2.3* and *PsnEIL4.1*, which were expressed at relatively lower levels in stems. To further reveal the role of EIN3/EIL genes during stem development, the expression of each gene from the first to the eleventh stem segment was analyzed (Figure 9).

The results showed that the expression patterns of *PsnEIL2.1* and *PsnEIL2.5* showed a general trend of increasing expression with stem development, while the expression patterns of *PsnEIL2.2*, *PsnEIL4.1*, and *PsnEIL4.2* were opposite, with high expression at the early stage of stem development; *PsnEIL2.3*, *PsnEIL2.4*, and *PsnEIL3.1* had the higher expression levels in young stems, but low expression levels in most other stem segments (Figure 6). These results indicated that the roles of EIN3/EIL genes in the development of stem nodes of *Populus* × *xiaohei* were divergent.

3.3.3. Most PsnEIN3/EIL Genes Are Involved in the Maturation and Senescence Processes of Leaves

Analysis of gene expression in five tissues showed that the expression levels of *PsnEIN3/EIL* in the third leaves were different. To further analyze the role of each gene in leaf development, the first young leaves at the top and the senescent leaves at the bottom were collected.

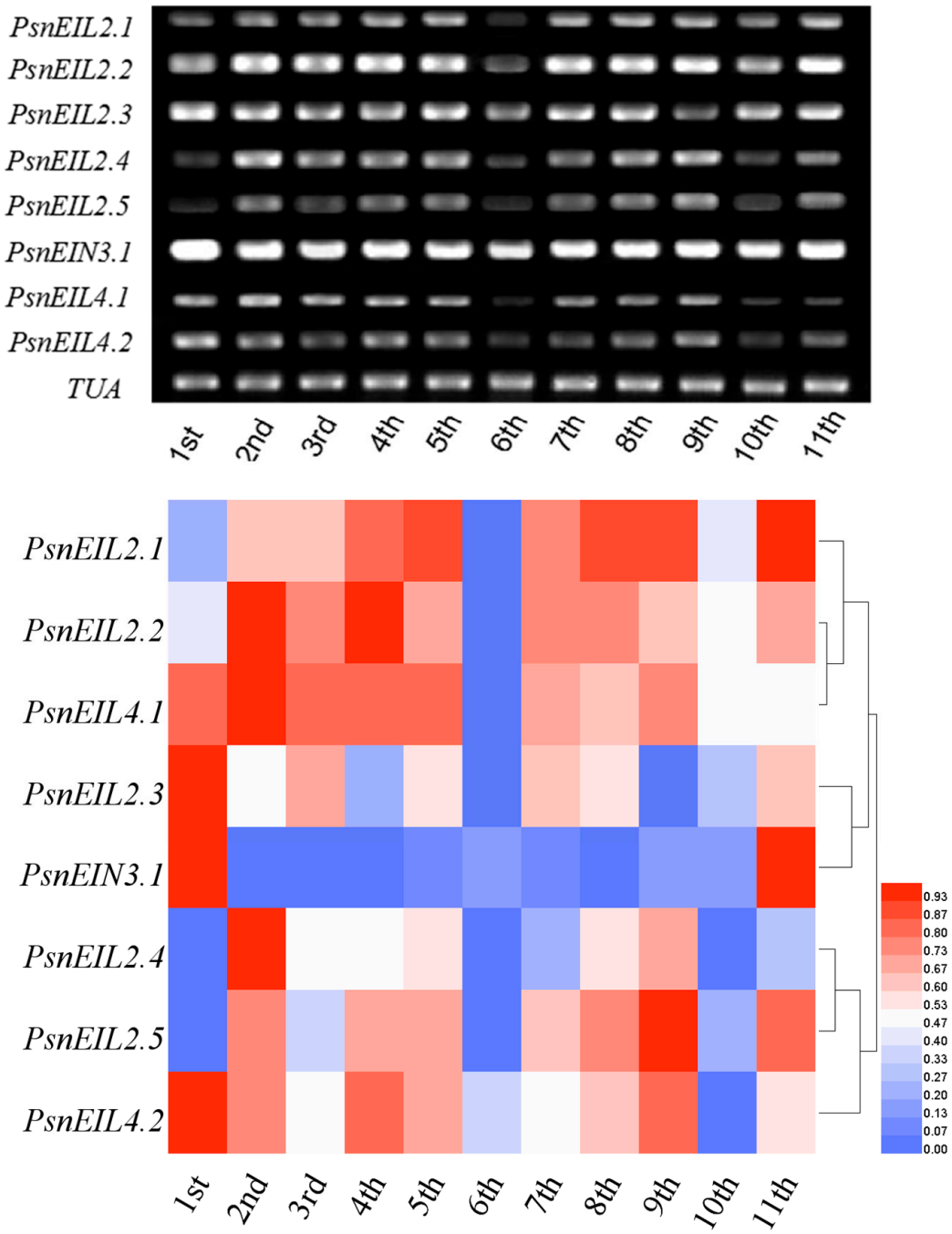


Figure 9. *PsnEIN3/EIL* gene expression patterns in different stem segments of *Populus* × *xiaohei*. Heat map showing the expression levels of the *PsnEIN3/EIL* genes in different stem segments. The first stem segment (1st) to the eleventh stem segment (11th) are presented in abbreviated form.

The results showed that except for *PsnEIL4.1* and *PsnEIL4.2*, the expression levels of the other six genes in this gene family were upregulated with the increase in leaf age (Figure 10). At the early stages of leaf development (1st–5th leaves), the genes were expressed at lower levels. The relative expression was gradually increased in the middle and late stages of leaf development (6th–13th leaves). Moreover, by the 14th leaf, the gene expression level had decreased significantly. This indicates that *PsnEIN3.1* and *PsnEIL2* performed semblable functions during leaf development. The *PsnEIN3/EIL* gene family may be involved in the maturation and senescence process of leaves, and the *PsnEIL4* gene and the other six genes possibly have different functions in leaves.

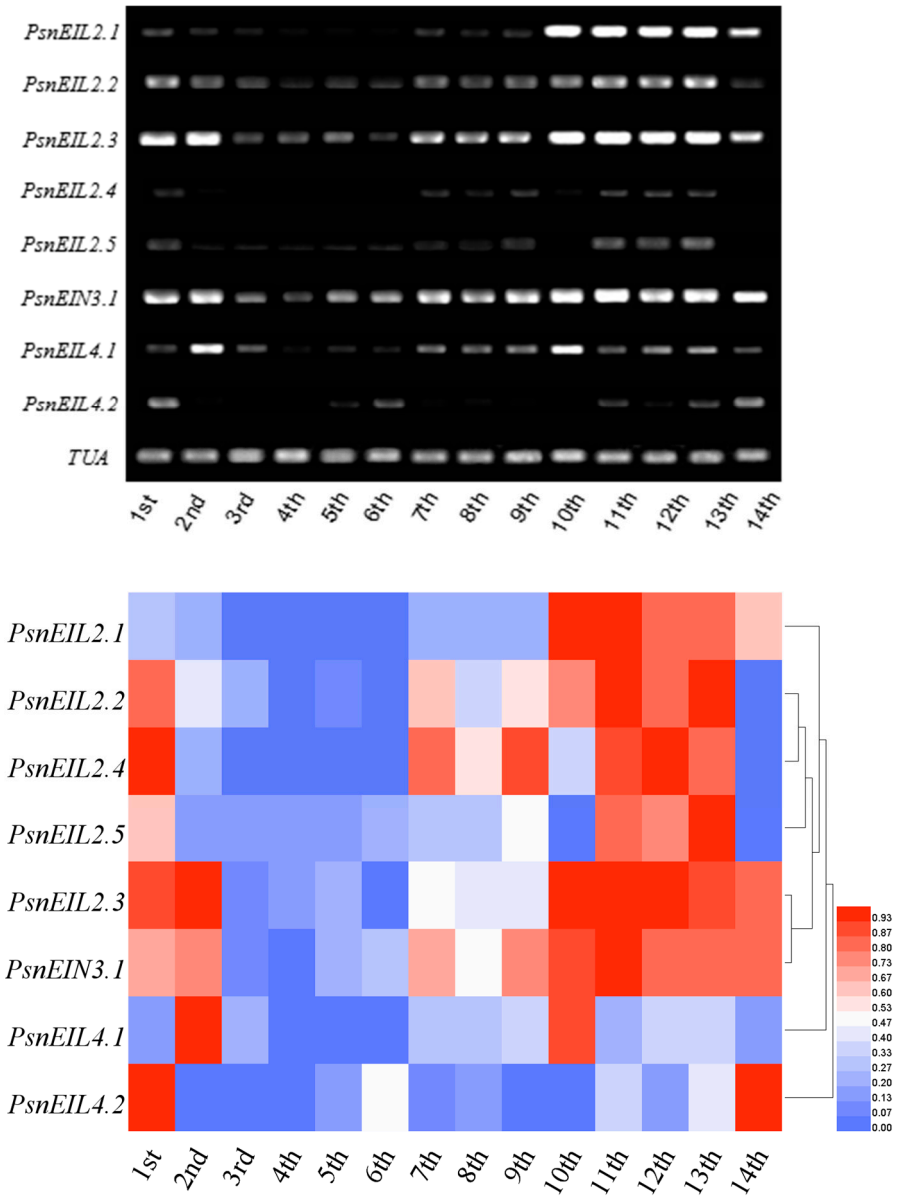


Figure 10. Relative *PsnEIN3/EIL* gene expression patterns in different leaves of *Populus* × *xiaohei*. Heat map showing the expression levels of *PsnEIN3/EIL* genes in different leaf developmental stages. The first leaf (1st) to the fourteenth leaf (14th) are presented in abbreviated form.

3.3.4. The PsnEIN3/EIL Gene of *Populus* × *xiaohei* Could Be Induced by High Salt Concentrations

Salt stress is one of the most serious abiotic factors that limit plant growth and development. *AtEIN3* and *EIL1* have been found to be involved in salt stress response as ET activated transcription factors [62]. The *PtEIN3/EIL* promoter of *P. trichocarpa* was found to contain multiple *cis*-acting elements induced by high salt concentrations (Figure S2, Table S2). In order to explore the effect of abiotic stress on the expression of the *EIN3/EIL* genes, the expression of *PsnEIN3/EIL* in roots and leaves under NaCl treatment was analyzed.

The results showed that *PsnEIL4.2* had similar downregulated expression trends in roots and leaves under NaCl treatment, while other *PsnEIN3/EIL* genes showed different responses to NaCl stress in roots and leaves (Figure 11). In the roots, except for *PsnEIL2.1* and *PsnEIL2.2*, which showed overall upregulation trends in expression, all the other five genes showed a transient decrease in expression at the beginning of treatment, followed by a significant increase, and then a rapid decrease as the treatment time was extended.

In the leaves, NaCl treatment inhibited the expression of *PsnEIL4.2*, and the expression level showed a downregulation trend with the extension of treatment time. The expression patterns of other genes showed increased expression levels with the extension of stress time. *PsnEIL2.1*, *PsnEIL2.5*, and *PsnEIL4.1* did not change significantly during the pre-treatment period, and their expression levels rapidly increased during the first 48 h and then decreased. However, *PsnEIL2.2*, *PsnEIL2.3*, and *PsnEIN3.1* were upregulated in the early stage of treatment, followed by leveling off. Except for *PsnEIL4.2*, the expression of most genes was the highest at 48 h in leaves.

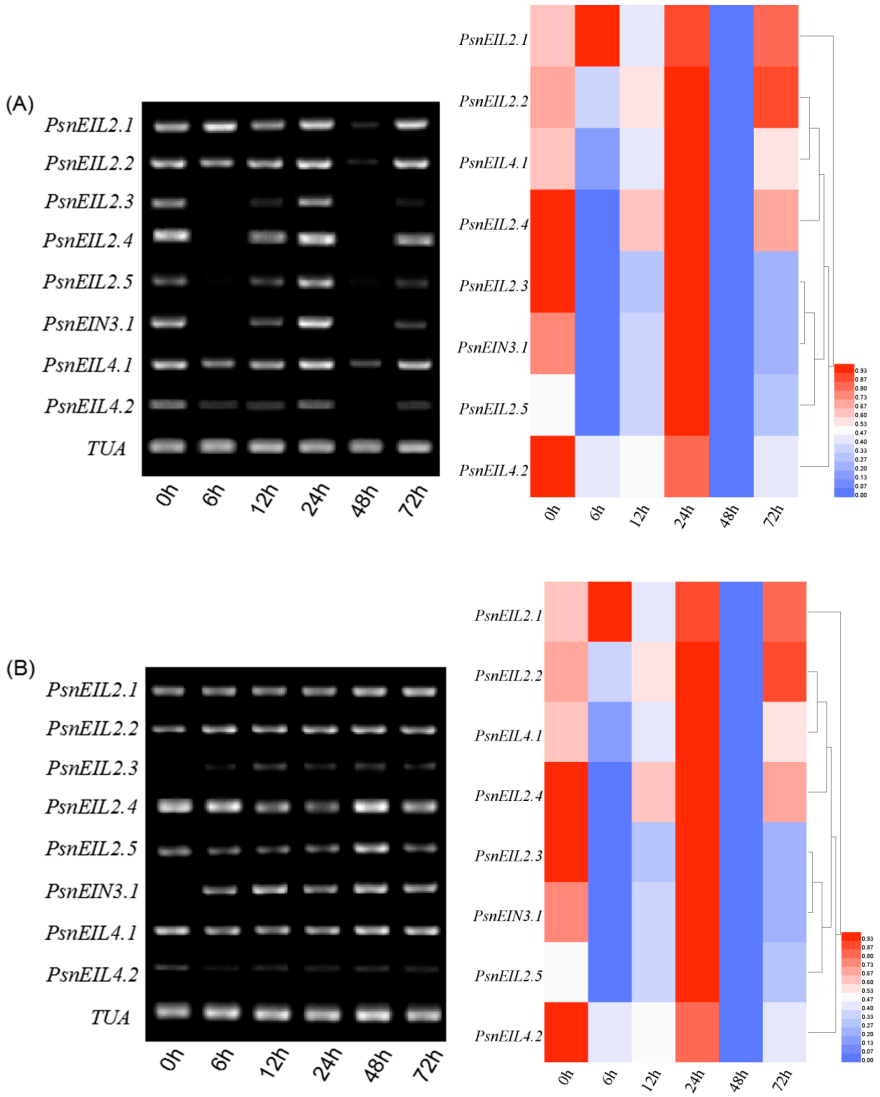


Figure 11. *EIN3/EIL* gene expression patterns of *Populus* × *xiaohei* under Nacl stress. Heat map showing the relative expression levels of *PsnEIN3/EIL* genes during salt damage treatment. sqRT-PCR was used to measure the transcription levels in root and leaf samples under stress treatment, and the data were normalized. (**A**) Roots. (**B**) Leaf. The color scale on the right of the heat map indicates the expression level: red indicates upregulation, and blue indicates downregulation.

3.3.5. Involvement of PsnEIN3/EIL in the Response to Heavy Metal Stress in *Populus* × *xiaohei*

Heavy metal cadmium ($CdCl_2$) stress not only seriously harms the normal growth and development of plants but also affects the yield and quality of crops. It has been found that ET can function under $CdCl_2$ stress through *EIN3*-mediated transcriptional regulation [36]. As a key transcription factor, it is very important to study its expression pattern.

In roots, *PsnEIN3/EIL* genes showed a negative regulation pattern during the entire course of $CdCl_2$ treatment, as most genes continued to be downregulated throughout the treatment process. *PsnEIL2.1*, *PsnEIL2.2*, and *PsnEIL4.2* showed a downregulated trends in the early stage of stress, were temporarily increased at 12 h until 24 h, and then decreased sharply and remained relatively stable (Figure 12). In leaves, the expression levels of *PsnEIL2.3*, *PsnEIL2.4*, and *PsnEIN3.1* were gradually upregulated with the extension of stress time. Gene expression was consistently elevated during the stress treatment. In contrast, the expression trends of *PsnEIL2.1*, *PsnEIL2.5*, and *PsnEIL4.2* in leaves were similar to those in roots, showing an overall downregulation trend. However, the expression levels of *PsnEIL2.2* and *PsnEIL4.1* increased initially and decreased significantly during 48 h of treatment. The results suggest that $CdCl_2$ may participate in the expression of *PsnEIN3/EIL* through the corresponding signal transduction pathway and that there are functional differences between *PsnEIN3/EIL* genes involved in heavy metal stress response of the roots and leaves of *Populus* × *xiaohei*.

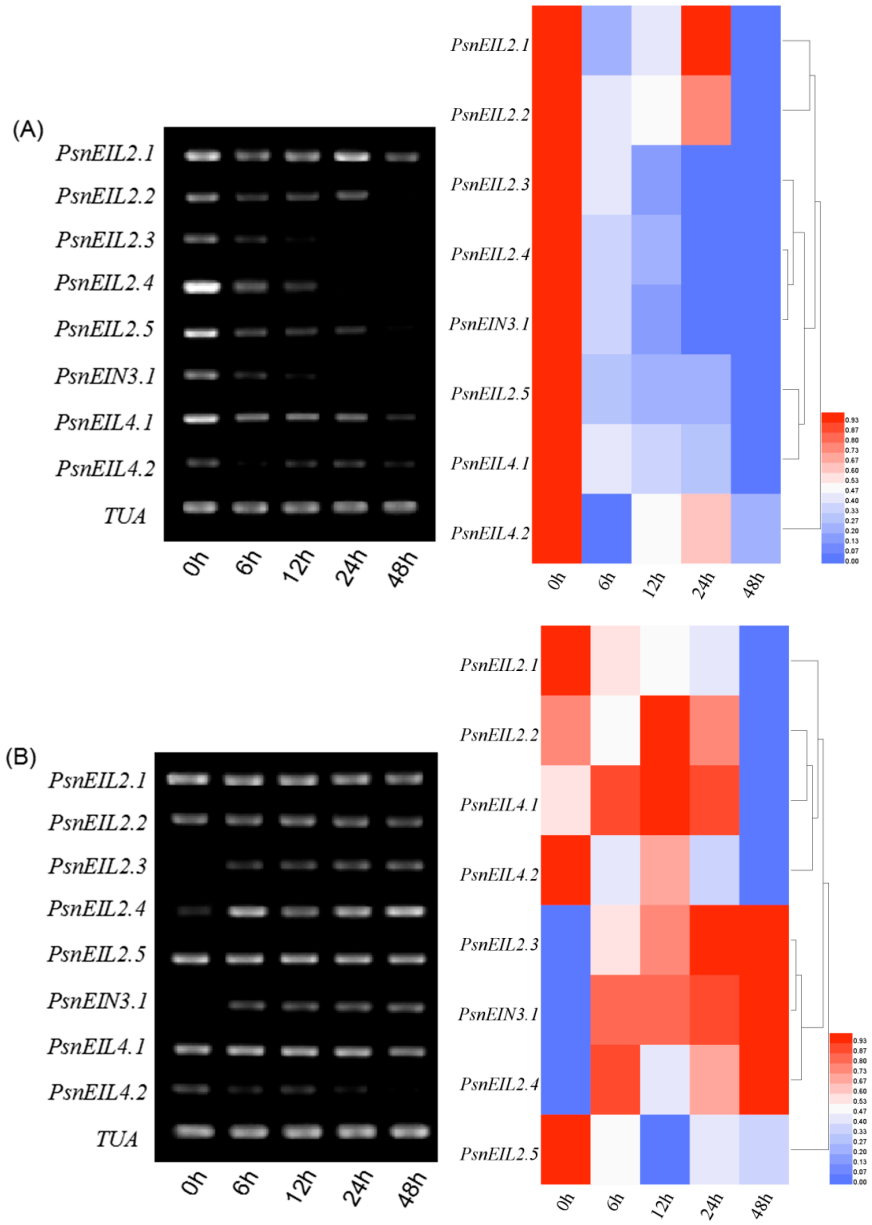


Figure 12. *EIN3/EIL* gene expression patterns of *Populus* × *xiaohei* under $CdCl_2$ stress. (**A**) Root. (**B**) Leaf.

3.3.6. Response of PsnEIN3/EIL Genes to $NaHCO_3$ in *Populus* × *xiaohei*

Land salinization has become one of the most important environmental problems worldwide. Salinization stress not only affects plant morphogenesis but also endangers the plant growth process. In this study, the expression patterns of the EIN3/EIL gene in *Populus* × *xiaohei* were studied by simulating alkali stress with $NaHCO_3$ (Figure 13). The results showed that the *PsnEIL4.2* gene was upregulated in both roots and leaves, and its expression was the highest at 48 h, indicating that this gene had similar functions in roots and leaves. In roots, except for *PsnEIL4.2*, the changes in the expression of *PsnEIN3/EIL* genes during alkaline stress treatment were similar to those during $CdCl_2$ treatment, with a general decreasing trend in expression. The relative gene expression levels of the seven downregulated genes rebounded slightly at 12 h but were still significantly lower than the initial levels. The relative expression levels of *PsnEIN2.3*, *PsnEIL2.4*, and *PsnEIN3.1* in the leaves showed an overall upward trend. The expression levels of *PsnEIL2.1*, *PsnEIL2.2*, *PsnEIL2.5*, and *PsnEIL4.2* were very similar, and they all rapidly increased to the maximum at 48 h of treatment and then decreased. In roots, $NaHCO_3$ stress inhibited the expression of *PsnEIN3/EIL* but had a positive effect on the expression of *PsnEIN3/EIL* in leaves.

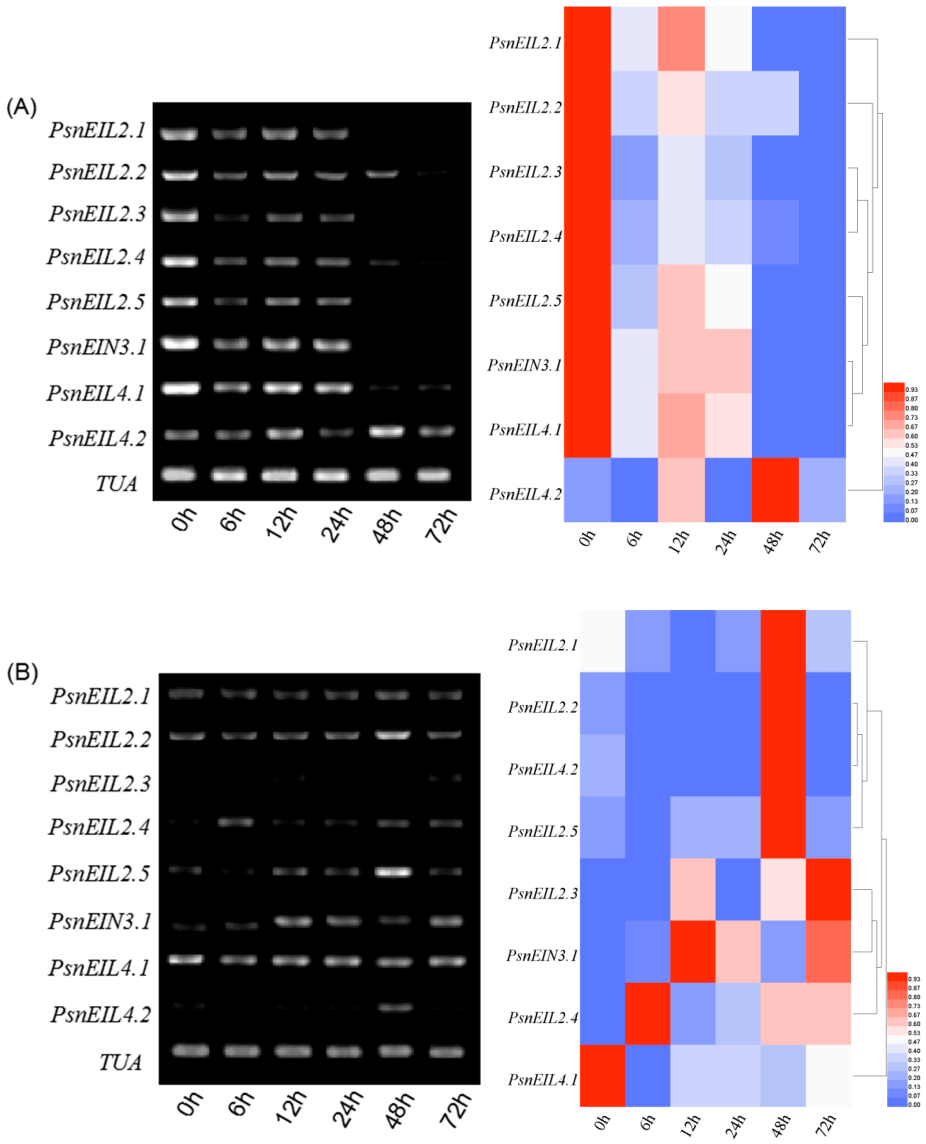


Figure 13. *EIN3/EIL* gene expression patterns of *Populus* × *xiaohei* under $NaHCO_3$ stress. (**A**) Root. (**B**) Leaf.

3.3.7. Response of PsnEIN3/EIL Genes to PEG in *Populus* × *xiaohei*

Drought as an abiotic stress seriously affects plant growth and development. PEG simulated drought experiments were conducted to analyze the response of *PsnEIN3/EIL* genes to drought stress (Figure 14).

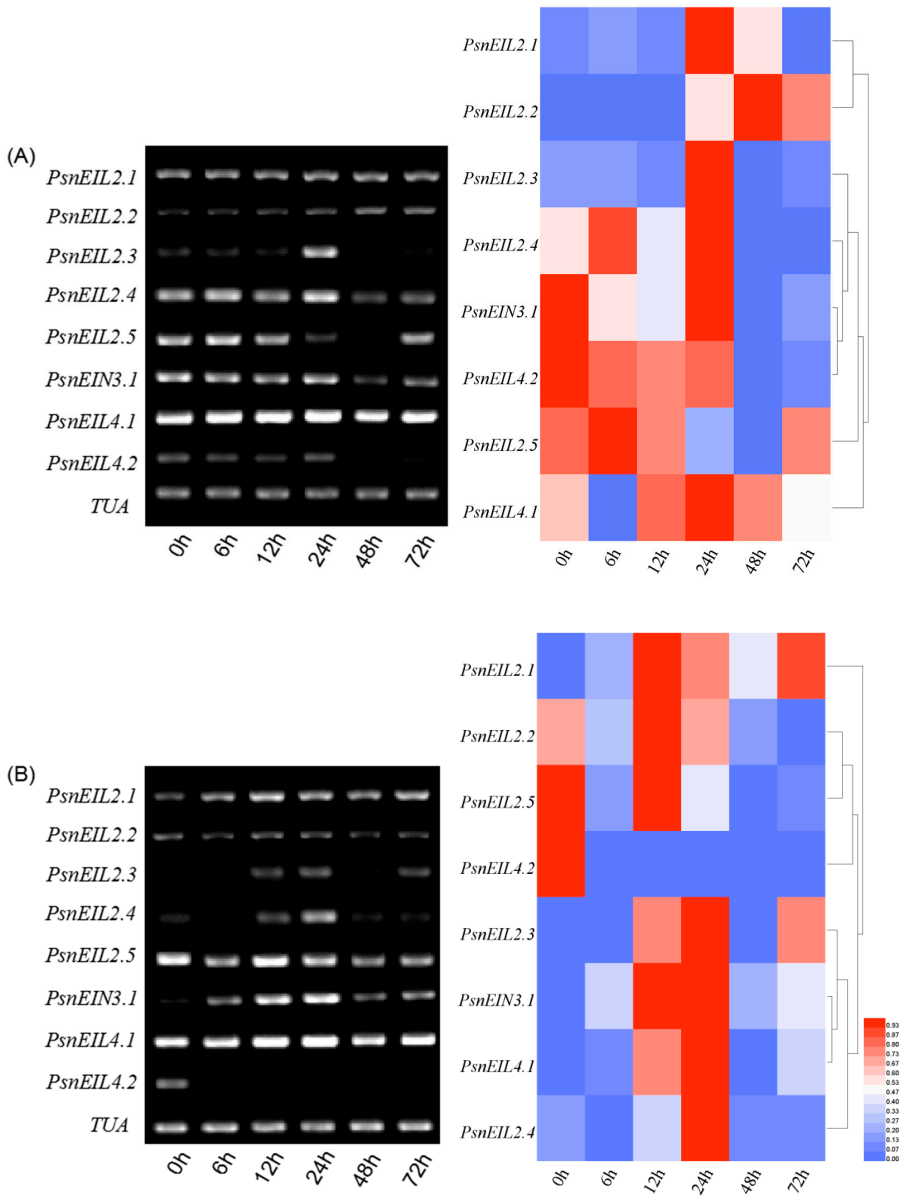


Figure 14. *EIN3/EIL* gene expression patterns of *Populus* × *xiaohei* under PEG stress. (**A**) Root. (**B**) Leaf.

The expression levels of *PsnEIL2.5* and *PsnEIL4.1* in roots were always high during the PEG stress but decreased in the later stages. The expression levels of *PsnEIL2.4*, *PsnEIN3.1*, and *PsnEIL4.2* did not change significantly in the early stages of treatment but decreased significantly after 48 h. In contrast, the expression levels of *PsnEIL2.1*, *PsnEIL2.2*, and *PsnEIL2.3* were upregulated after 48 h, suggesting that *Populus* × *xiaohei* may function in the later stages of drought stress by regulating *PsnEIN3/EIL* gene expression.

PsnEIL4.2 was barely expressed in leaves under PEG drought stress. However, *PsnEIL2.1*, *PsnEIL2.3*, *PsnEIL2.4*, *PsnEIN3.1*, and *PsnEIL4.1* showed a trend of upregulation, in which the relative gene expression levels began to increase at 12 h, and then appeared to decrease at 48 h. The gene expression levels slightly rebounded at 72 h. In con-

trast, the expression levels of *PsnEIL2.2* and *PsnEIL2.5* genes showed an overall downward trend. The results showed that the *PsnEIN3/EIL* genes were involved in the response to drought stress in the roots and leaves of *Populus* × *xiaohei*, and that the *PsnEIL2.2* gene was different from *PsnEIL2.4*, *PsnEIN3.1*, and *PsnEIL4.2* genes in regulating the response to drought stress in the roots.

3.3.8. The PsnEIN3/EIL Genes were Involved in the Abscisic Acid Response of *Populus* × *xiaohei*

ABA, a plant endogenous hormone, not only regulates the physiological roles of bud dormancy, leaf abscission, stomatal closure, and cell growth inhibition but also plays an important role in plant response to stress. Analysis of the *PtEIN3/EIL* gene promoter response elements (Figure S2, Table S2) showed that the *PtEIN3/EIL* gene contained different numbers of ABA response elements. Therefore, the changes in *PsnEIN3/EIL* gene expression level under ABA treatment were analyzed (Figure 15). The results showed that exogenous ABA could induce *PsnEIN3/EIL* gene response, and there were differences in the expression trends. The expression levels of *PsnEIL2*, *PsnEIL4.1*, and *PsnEIN3.1* genes were upregulated with treatment time, except for *PsnEIL4.2*, which was significantly downregulated. The expression level of *PsnEIL2.5* was the highest at 0 h of treatment, then significantly decreased at the initial stage of treatment, and then increased. These results suggest that *PsnEIN3/EIL* may be involved in a hormone response pathway, and *PsnEIL4.2* may play an opposite role in ABA signaling response with other *PsnEIN3/EIL* genes.

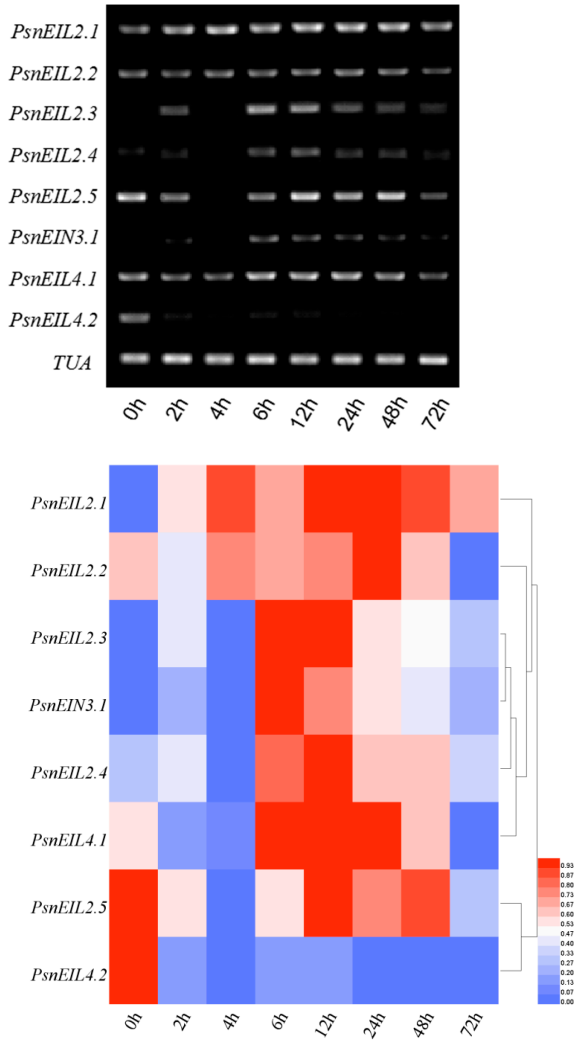


Figure 15. EIN3/EIL gene expression patterns of *Populus* × *xiaohei* under ABA stress.

4. Discussion

Poplar EIN3/EIL contains a highly conserved EIN3 domain specific to EIL proteins [59], as well as other important structural features of EIN3/EIL family genes, including an acidic amino acid region, a proline rich region, and five basic amino acid domains (BDI–V). The acidic region and the proline and glutamine rich region are common transcriptional activation regions in plants [17,55,56]. Similar to the EIN3/EIL genes in *Gossypium spp*., *A. thaliana*, *Poplar*, and *C. melon* [63–65], the N-terminal of the *PsnEIN3/EIL* protein is highly conserved, and there are highly conserved protein motifs: GFVYGI, PPQR, PLE, PPWWP, and KPHDL. Compared with the N-terminal sequence, the C-terminal amino acid sequence of *PsnEIN3/EIL* is less conserved [17,21], speculated that the functional difference of EIN3/EIL gene is determined by the C-terminal sequence. The high similarity in the amino acid sequence structure of the *PsnEIN3/EIL* transcription factor in *Populus* × *xiaohei* and other plants such as *A. thaliana* and the highly conserved amino acid sequence of its gene counterpart implies that the gene may have a conserved function associated, suggesting that the gene family may have some similar functions in higher plants. Phylogenetic tree analysis showed that *PsnEIN3.1* clustered with *TcEIN3* and *AtEIN3*, *PsnEIL2* with *CmEIL2*, and *PsnEIL4* with *AcEIL4*. This not only indicated the affinity between *Populus* × *xiaohei* and EIN3/EIL members of other plants but also implies the functional differences among members. The main way of functional differentiation and generation of new genes is gene replication and differentiation [66]. Among the six genes clustered with *AtEIN3*, *AtEIL1*, and *AtEIL2* in the phylogenetic tree, *PsnEIL2* was more likely to have evolved from *AtEIL2*, while *PsnEIL3.1* may be a duplicate gene that emerged during the evolution of *AtEIN3* and *AtEIL1*.

EIN3/EIL genes are expressed in almost all tissues of higher plants, but there are differences in their expression levels and expression patterns. In tobacco, *NtEIN3/EIL* is expressed in roots, stems, leaves, and flowers, with *NtEIL1* and *NtEIL2* expressed at relatively high levels in roots [21]. In contrast, in grape, the expression of EIN3/EIL genes in different tissues is from high to low in stems, leaves and roots. Eight *PsnEIN3/EIL* genes showed different levels of expression in roots, xylem, phloem, terminal buds, and third leaves, and the expression level was higher in roots, followed by phloem. When we analyzed the expression levels of *PsnEIN3/EIL* genes in stem node development, from the 1st node to the 11th node, the expression levels of *PsnEIL2.1* and *PsnEIL2.5* showed an upward trend, and the expression levels of *PsnEIL2.3*, *PsnEIL2.4* and *PsnEIL3.1* were the highest in tender stems, while the expression levels were lower in most other stem nodes. This indicates that the *PsnEIN3/EIL* genes may be involved in the development of roots and stems. *OsEIL1* directly regulates the expression of the downstream target gene *REIN7/YUC8*, regulates the synthesis of auxin in rice primary roots, and participates in the process of ET inhibiting the elongation and growth of primary roots [67]. *A. thaliana EIN3* interacts with *RHD6* (Root Hair Defective 6) to activate the expression of *RSL4* (RHD6-like 4), thereby promoting root hair elongation [68]. In other tissues, different genes showed different expression levels, suggesting that *PsnEIN3/EIL* performed different functions in different tissues, including leaf growth and development as well as in response to stress. In the expression analysis of leaf development, the expression of other genes in the middle and late stages of leaf development (6th–13th leaves) showed that overall upregulation trend, except for *PsnEIL4.1* and *PsnEIL4.2*, indicating that *PsnEIN3/EIL* may be related to accelerated senescence. In *A. thaliana*, *EIN3* accelerates ET- mediated leaf senescence through direct activation of *A. thaliana* chlorophyll catabolism genes [17,27,69]; the *EIN3* gene can cause senescence and the non-sprouting phenotype of transgenic *A. thaliana* leaves [30]. Ectopic expression of *MdEIL1* in *A. thaliana* promotes leaf senescence by directly binding to the promoter of its target gene *miR164* [70]. The above evidence confirms that EIN3/EIL is involved in leaf senescence.

Recent studies have found that in addition to playing an important role in plant development [71,72], EIN3/EIL transcription factors are also involved in the regulation of plant hormones and the response to stresses, such as salinity, heavy metals, and

drought [2,13,32,62]. We analyzed the cis-acting elements of the *PtEIN3/EIL* gene promoter of *P. trichocarpa*, and found that it contained multiple hormones and response elements to stress. After exogenous ABA treatment, *PsnEIN3/EIL* showed an overall upward trend, except for *PsnEIL4*, indicating that *PsnEIN3/EIL* was involved in the response to ABA signaling. During NaCl, $CdCl_2$, $NaHCO_3$ and PEG treatments, *PsnEIN3/EIL* genes were all induced by stress to varying degrees, although showing individual differences. The expression levels of *PsnEIN3/EIL* were changed under salt stress induction; *PsnEIL2.1* and *PsnEIL2.2* were upregulated in both roots and leaves of *Populus* × *xiaohei*, suggesting that *PsnEIL2.1* and *PsnEIL2.2* played key roles in enhancing the salt tolerance of *Populus* × *xiaohei*. Currently, there are few studies on the salt tolerance of *EIL2* genes, but a large number of studies have shown that *EIN3*, *EIL1*, and *EIL2* can partially complement the phenotype of *EIN3* mutants [63], indicating that there is functional redundancy among *EIN3*, *EIL1*, and *EIL2* genes. In *A. thaliana*, under high salt stress, the germination of an *ein3eil1* double mutant strain was significantly inhibited, while the germination of an *EIN3* overexpressed strain was significantly increased, and the resistance at the seedling stage was enhanced, suggesting that the expression of the *EIN3* gene plays an important role in alleviating the inhibition of germination and growth injury induced by high salt stress [73]. The expression of the *EIN3* gene in *A. thaliana* can also prevent the accumulation of reactive oxygen species (ROS) involved in excessive salt stress and improve tolerance to salt stress [62]. *A. thaliana* double mutants *ein4-1*, *ein3-1*, and *eil1-1* showed higher tolerance to cadmium stress [36]. The relative expression levels of eight *PsnEIN3/EIL* genes in the roots of *A. nigra* showed a general downward trend. The expression trends of *PsnEIL2.1*, *PsnEIL2.5*, and *PsnEIL4.2* genes in leaves were similar to the pattern in roots, with downregulated expression. It has been speculated that the EIN3/EIL genes, which were significantly downregulated in *Populus* × *xiaohei* under $CdCl_2$ stress, may be involved in the process of its stress response and play a positive role. The tolerance to drought stress was enhanced by overexpression of the mulberry *MnEIL3* gene in *A. thaliana*; in addition, *MnEIL3* also enhanced the activity of the *MnACS1* promoter in response to drought stress. Therefore, *MnEIL3* may play an important role in plant resistance to abiotic stresses [34]. The expression levels of *PsnEIL2.1* and *PsnEIL2.3* genes were upregulated in both roots and leaves after drought treatment, suggesting that these two genes play positive roles in coping with drought stress in *Populus* × *xiaohei*. Similarly, in *A. thaliana*, *EIN3* was able to respond to PEG-simulated drought stress and positively regulated the morphological growth of *A. thaliana* under drought stress [35]. The above studies suggest that *PsnEIN3/EIL* may be involved in the response to multiple abiotic stresses.

5. Conclusions

In this study, the ORF sequences of eight *PsnEIN3/EIL* genes were successfully cloned, all of which contained a highly conserved EIN3 homeodomain. Bioinformatic analyses showed that the genes belonged to the EIN3/EIL family. Phylogenetic analysis of 30 EIN3/EIL genes from different species showed that the EIN3/EIL genes were divided into three clades: A, B, and C. *EIL3* and *EIL4* belonged to clade A and clade B, while *EIL2* and *EIN3* were mainly belonged to clade C. An sqPCR analysis showed that the expression characteristics of EIN3/EIL family members differed in different tissues, with *PsnEIL2.3* and *PsnEIL4.1* being highly expressed in roots. *PsnEIL2.2* and *PsnEIL2.4* were mainly expressed in phloem and xylem, respectively. The relative expression levels of a total of six homologous genes in the *PsnEIL2* and *PsnEIL3* subfamilies in leaves were generally upregulated with leaf maturation. *PsnEIN3/EIL* genes also showed varying degrees of response to NaCl, $CdCl_2$, $NaHCO_3$, and PEG stresses. The expression of *PsnEIN3/EIL* genes in roots showed an overall downregulation trend under NaCl, $CdCl_2$, and $NaHCO_3$ stresses. However, the relative expression levels of *PsnEIL2.3*, *PsnEIL2.4*, and *PsnEIN3.1* in leaves under three kinds of stress increased with the extension of stress time. Under drought stress, the *PsnEIL2.1* expression pattern was similar in roots and leaves, showing a

trend of upregulation. This study suggests that EIN3/EIL genes of *Populus* × *xiaohei* may be involved in the response to various abiotic stresses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13030382/s1. Table S1: Information on the segmental duplications analysis of the *PsnEIN3/EIL* genes, Table S2: PlantCARE prediction results of the *PtrEIN3/EIL* genes, Figure S1: Phylogenetic tree of *Populus* × *xiaohei* and *A. thaliana*, Figure S2: The EIN3/EIL gene family promoter element prediction of *P. trichocarpa*.

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