Isolation and Characterization of Homeodomain-Leucine Zipper III Protein 1 Gene in Poplar

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ABSTRACT Transcription factors are the regulatory proteins which activate or repress their target genes. We isolated homeodomain-leucine zipper III (*PagHD-ZIP III_1*) gene, a plant-specific transcription factor known to play crucial roles in plant development, from poplar (*Populus alba* × *P. glandulosa*). The *PagHD-ZIP III_1* is 2,723 base pairs long and encodes a putative 838 amino acid protein with an expected molecular mass of 92.3 kDa. The *PagHD-ZIP III_1* protein has four conserved regions including HD, bZIP, START and MEKHLA. Southern blot analysis indicated that *PagHD-ZIP III_1* belong to a small gene family in the poplar genome. *PagHD-ZIP III_1* is expressed highly in the stem. Moreover, the expression of *PagHD-ZIP III_1* is induced by salt, cold, wounding and a plant hormone JA. Our results indicate that *PagHD-ZIP III_1* is involved in abiotic stress response and hormonal signaling in poplar.

Keywords Jasmonic acid, *PagHD-ZIP III_1*, *Populus alba* × *P. glandulosa*, Stress, Transcription factor, Wounding

INTRODUCTION

Transcription factors (TFs) in eukaryotes are important regulatory proteins, which activate or repress the expression of their target genes by binding to specific DNA sequences adjacent to the genes (Long and Benfey 2006). TFs play significant roles in the response and adaptation of plant to various types of abiotic stress. Recently, fine-tuning of TF expression has shown to increase plant biomass and abiotic stress tolerance in a number of plant species (Jeong *et al.* 2012; Lindemose *et al.* 2013).

Homeodomain-leucine zipper (HD-ZIP) proteins are a plant specific TF, which plays an important role in the plant development (Elhiti and Stasolla 2009). HD-ZIP proteins contain homeodomain (HD) and leucine zipper (LZ) motif. The HD domain is involved in the specific binding to DNA by its helix III, while the LZ domain has the ability to dimerize, which is necessary for binding to the target sequence (Johannesson *et al.* 2001). The HD-ZIP proteins are divided into four subfamilies (HD-ZIP I, II, III, and IV) based on their sequence conservation, structural features

and functions (Hu *et al.* 2012). HD-ZIP III proteins have been extensively implicated in the regulation of primary and secondary vascular tissue pattern formation as well as lateral organ and cambial polarity in woody plants (Côté *et al.* 2010).

Arabidopsis thaliana contains 48 members of HD-ZIP genes including five HD-ZIP IIIs, which have key roles in the development process (Prigge *et al.* 2005). The functionally well-characterized HD-ZIP class III includes five genes - *PHABULOSA(PHB)/ATHB14*, *PHAVOLUTA(PHV)/ATHB9*, *REVOLUTA (REV)/INTERFASCICULAR FIBERLESS1(IFL1)*, which direct the development of the apical meristem, the vascular bundles, and the adaxial domains of lateral, and *ATHB8*, and *CORONA(CNA)/ATHB15/INCURVATA4 (ICU4)*, which have been suggested to direct vascular development (Emery *et al.* 2003; Green *et al.* 2005). Compared to the largely investigated functions of *Arabidopsis* HD-ZIPs, only two *Populus* HD-ZIP genes (*POPREVOLUTA* and *POPCORONA*) have recently been characterized. These two HD-ZIP III genes are largely involved in regulating cell differentiation during secondary growth (Robischon

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et al. 2011).

In recent years, a number of researches have been conducted to increase lignocellulosic biomass as a promising alternative energy source. Since HD-ZIP III genes have shown to regulate xylem development and cell wall biosynthesis, tree breeding program can use them to identify molecular markers and to improve plant growth traits. In this study, we isolated *PagHD-ZIP III_1* and analyzed its characteristics and expression patterns.

MATERIALS AND METHODS

Plant materials and growth conditions

Leaf suspension cells of poplar (*Populus alba* × *P. glandulosa*) were cultured in the MS medium (Murashige and Skoog 1962) containing 2,4-dichlorophenoxyacetic acid 1.0 mg/L, 1-naphthalene acetic acid 0.1 mg/L, 6-benzylaminopurine 0.01 mg/L, and sucrose 30 g/L. Cells were sub-cultured every three to four weeks when they reached the stationary phase by adding 0.4 g fresh cellular mass to 100 ml of liquid MS medium and maintained at 22±1°C under 20 µmol m⁻² s⁻¹ cool-white fluorescent light with constant agitation at 130 rpm (Lee *et al.* 2005). To examine tissue-specific gene expression patterns, leaf- and stem-tissues were collected from one-year-old poplar trees and flower-tissues were harvested from 25-year-old trees.

Isolation and characterization of HD-ZIP III gene

A poplar cDNA library was constructed as described previously in Lee *et al.* (2005). For expressed sequence tag analysis, the cDNA library was randomly excised *in vivo* using the ExAssist helper phage (Stratagene, CA, USA). The plasmid DNAs were isolated and 5'-single pass sequences were determined. Searches of the public databases were made with BLASTX to select clones homologous to known plant HD-ZIP III genes. The selected cDNA clone was sequenced and then analyzed its amino acid sequence and molecular weight by Vector NTI advance 10.0 (Invitrogen, CA, USA). Alignment and phylogenetic trees were generated by the ClustalW, based on cDNA sequence

or deduced amino acid sequences of HD-ZIP III gene. Finally, the analysis for conserved domain was conducted by Batch Web CD-Search Tool (<http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>).

Genomic DNA isolation and Southern blot analysis

Genomic DNA was extracted from leaves using Mega-Extractor plant genome kit (Toyobo, Japan) and 10 µg of DNA was digested overnight with the restriction enzymes *EcoRI*, *HindIII* or *XbaI*. The DNA was then separated on an 1% agarose gel and transferred to a Hybond-XL nylon membrane (Amersham-Pharmacia, NJ, USA) using the capillary transfer method (Southern 1975). The membrane was prehybridized at 68°C for 30 min in 1× PerfectHYB plus hybridization buffer (Sigma- Aldrich, USA) and 0.1 mg/mL sheared, single-stranded herring sperm DNA. Subsequently, ³²P-dCTP labelled full-length *PagHD-ZIP III_1* probe was added and hybridized at 68°C for 12 h. Following hybridization, the membrane was washed three times in 0.2× SSC and 0.1% SDS at 65°C for 10 min. Detection was done by exposure to an X-ray film at -70°C.

Abiotic stress and plant hormone treatment

To induce gene expression in response to osmotic stress, 4-day-old suspension cultures were treated with 250 mM mannitol or 150 mM NaCl. The suspensions were maintained at 2°C for cold treatment, and were treated with 20 µM ABA, 20 µM gibberellic acid (GA₃), 10 µM jasmonic acid (JA), and 20 µM salicylic acid (SA) for plant hormone responses. For wounding treatment, the leaves of one-year-old plants were collected after being trimmed along their edge with sterile scissors. All suspensions and leaves were immediately frozen in liquid nitrogen and stored at -70°C for further gene expression analysis.

RNA isolation and real-time quantitative PCR

Total RNA was extracted using TRI reagent (Molecular Research Center, OH, USA) and RT-qPCR primers were designed by using Primer3 program (<http://fokker.wi.mit.edu>). As a quantitative control, poplar actin gene was used (Kim *et al.* 2010). *PagHD-ZIP III_1* was amplified with a forward primer (5'-TCCCTCCTGCAATCCTTCTGA

-3') and a reverse primer (5'-CCTTCCAGTTTATCACCTCCAGG-3'), while actin was amplified with a forward primer (5'-GCCATCTCTCATCGGAATGG-3') and a reverse primer (5'-AGGGCAGTGATTTCCTTGCT-3'). Gene expression levels were determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

First-strand cDNA synthesis was performed using a PrimeScript RT Reagent Kit (Takara, Japan) and RT-qPCR was conducted using SYBRGreen PCR Master Mix (BioRad laboratories, USA), according to the manufacturer's instructions. RT-qPCR was carried out using the DNA Engine Opticon™ Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA). All PCR mixtures contained: 1 µL of cDNA, 10 µL of 2× SYBRGreen PCR Master Mix, 1 µL of each PCR primer (10 µM), and 7 µL of nuclease-free water in a 20 µL of final volume reaction mix. The cycling conditions for amplification included 10 min at 95°C for polymerase activation, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec.

RESULTS AND DISCUSSION

Isolation and structural characterization of HD-ZIP III₁ gene

We selected a poplar cDNA clone from the cDNA library that had been prepared from poplar suspension cells (Lee *et al.* 2005) and compared it with previously identified plant HD-ZIP III genes using a BLAST search. DNA sequence of the full cDNA clone was 98% identical to HD-ZIP III (AY919621.1) of *Populus trichocarpa*, 87% to HD-ZIP ATHB-15 like isoform 1 (XM_002283967.1) of *Vitis vinifera*, and 80% to ATHB-15 (EU550335.1) of *Arabidopsis thaliana*. Therefore, we named this cDNA clone as *PagHD-ZIP III₁* (*Populus alba* × *P. glandulosa* HD-ZIP III 1).

An open reading frame of *PagHD-ZIP III₁* was 2,514 bp long (Fig. 1). The estimated molecular mass of the putative protein, which consisted of 838 amino acids (aa) residues, was 92.3 kDa and its isoelectric point was 6.09. The major component of deduced amino acids consisted of 10.5% of leucine, 8.7% of serine, and 8.2% of alanine. The

identified HD-ZIP genes in *Populus* encode proteins ranging from 70 to 855 amino acids in length with an average of 465 aa (Hu *et al.* 2012).

HD-ZIP III proteins contain four conserved regions (Hu *et al.* 2012). When the aa sequence of *PagHD-ZIP III₁* was compared with other plant HD-ZIP III proteins, all four regions were shown to be well conserved (Fig. 2). Unique features of all HD-ZIP proteins are the presence of the HD domain, which binds to the specific DNA sequence, and ZIP domain, which dimerizes proteins to bind to the target sequence (Johannesson *et al.* 2001). It is also known that HD-ZIP III and IV contain a steroidogenic acute regulatory protein related lipid-transfer (START) domain (Schrack *et al.* 2004). One of the defining features of HD-ZIP III proteins is the presence of a MEKHLA domain at the C terminus, which is involved in light, oxygen, and redox potential sensation (Ohashi-Ito and Fukuda 2003). MEKHLA domain shares significant similarity with the PAS domain, reported to dimerize with the AP2 domain of the transcription factor DRN/ESR-1 involved in embryo patterning and auxin transport (Mukherjee and Bürglin 2006).

The amino acid sequence of *PagHD-ZIP III* protein was at maximum 99% identical to *PtrHox16* (Poptri.003G050100) in *P. trichocarpa*, 91% to MdHB15 (AC113685.1) in apple (*Malus × domestica*), and 87% to ATHB-15 (NP_175627.1) in *A. thaliana* (Fig. 2). Consequently, we concluded that *PagHD-ZIP III₁* isolated in this study is a member of plant HD-ZIP III.

Southern blot hybridization

In order to investigate how many copies of *PagHD-ZIP III₁* genes are present in poplar (*Populus alba* × *P. glandulosa*), Southern blot analysis was performed. A full-length of *PagHD-ZIP III₁* cDNA probe was hybridized with genomic DNA, which was digested with *EcoRI*, *HindIII*, and *XbaI*, respectively. In a result, all lanes showed five to seven bands (Fig. 3), suggesting that the *PagHD-ZIP III₁* gene belonged to a multi-copy gene family in *Populus alba* × *P. glandulosa*. Previous reports were also shown that the number of HD-ZIP III genes were different between plant species. For example, *Arabidopsis* HD-ZIP III subfamily comprises of only five genes (Prigge *et al.* 2005), while *P. trichocarpa* has 8 genes (Hu *et al.* 2012),

which possesses a number of introns (as many as 17) (Hu *et al.* 2012). Côté *et al.* (2010) also demonstrated that transcripts of HD-ZIP III genes differed in abundance depending on organs and tissues, with a strong indication of functional diversification between the gene families. Therefore we assumed that the *PagHD-ZIP III_1* gene might also play different roles in the organ- or tissue-specific manner.

Tissue-dependent expression pattern of *PagHD-ZIP III_1*

Comparative expression analysis of *PagHD-ZIP III_1* in leaf, stem, flower, and suspension cells, revealed that the transcript was most abundant in stem tissues (Fig. 4). *P. trichocarpa* has eight members of HD-ZIP III genes, which shows tissue-specific expression pattern (Hu *et al.* 2012). For example, *PtrHox5*, 7, and 16 were highly expressed in shoot tip, cambium layer, and root, while higher expression

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1  ATGGCAATGTCTGCAAGGATGGTAAGCAGCCTATCATGGCAATGGCAAGTATGTCCGGTACACGCCTGAGCAGGTGCAAGCCCTGGAAGGCTCTAT
   M A M S C K D G K Q P I M D N G K Y V R Y T P E Q V E A L E R L Y
100 CATGATTGTCCAAACCCAGCTCCATTGCGCCGAGCAGCTCATTAGGGAGTGTCCAATTCTCTCAACATTGAGCCCAACAAATCAAAGTTTGGTTT
   H D C P K P S S I R R Q Q L I R E C P I L S N I E P K Q I K V W F
199 CAAAATAGAAGATGTCGAGAGAAGCAGAGGAAAGAGCGTCTCGCCTCCAGGCGGTGAATAGGAAGCTGACTGCAATGAACAAGCTTCTGATGGAAGAG
   Q N R R C R E K Q R K E A S R L Q A V N R K L T A M N K L L M E E
298 AATGATAGTTGTCAGAAGCAGGTGTGCGAGCTGGTGTATGAGAATGGGTACTTTGCGCAACATACCCATAACACACCGCTTGCACCAAGATACAAGC
   N D R L Q K Q V S Q L V Y E N G Y F R Q H T H N T P L A T K D T S
397 TGTGAATCAGTGGTGACAAGCGGTCAACACCACCTGACACCTCAGCATCCGCCAAGGGATGCTAGTCTGCAGGCTTTTGTCCATTGCACAAGAGACT
   C E S V V T S G Q H H L T P Q H P P R D A S P A G L S I A E E T
496 TTAACAGAGTTTCTTTCAAAGGCTACTGGAAGTGTGTAGAGTGGGTCCAAATGCCTGGAATGAAGCCTGGTCCGATTCCAGTGAATCGTTGCTATT
   L T E F L S K A T G T A V E W V Q M P G M K P G P D S S G I V A I
595 TCTCATGGTTGTACTGGCGTGGGAGCAGAGCTTGTGGCCTAGTGGGTCTAGAACCTACAAGGGTTGCTGAAGTCTCAAGGATCGGCCATCATGGTTT
   S H G C T G V G A R A C G L V G L E P T R V A E V L K D R P S W F
694 CGTGATTGCCGAGCTGTGGATGTGCTTAATGTGCTGCCACCGCAATGGTGAACCATGAGCTGCTTTATATGACGCTCTATGCCCACTACGTTG
   R D C R A V D V L N V L P T A N G G T I E L L Y M Q L Y A P T T L
793 GCACCTGGTCTGACTTCTGGTTGTTGCTTATACTTCTGTTTGTAGAGATGGAAGCCTTGTGGTATGTGAGAGTCACTGAAAATACTCAGAAATGGC
   A P G R L R Y T L R Y T L S V L E D G S L V V C E R S L K D R P S W F
892 CCGAGCATGCCACAGTGCAGCATTTTGTGAGAGCAGAAATGCTCCCAAGTGGGTATCTGGTACGGCCTTGTGAAGGTGGTGGTTCAATTATACACATA
   P S M P P V Q H F V R A E M L P S G Y L V R P C E G G G S I I H I
991 GTTGACCATGGATTGGAGCTTGGAGTGTGCTTACTACGGCTGTGATGATGAATCCTCACTGTCTCAAAAGACAAATCGTGGCT
   V D H M D L E P W S V P E V L R P L Y E S S T V L A Q K T T M V A
1090 CTACGCCAGCTGCGGCAGATAGCTCAGGAAGCTTCTCAGTCTAATGTGACCAACTGGGGCAGACGACCTGCAGCTCTACGAGCACTGAGCCAGAGTTG
   L R Q G L R Q I A Q E A S Q S N V T N W G R R P A A L R A L S Q R L
1189 AGCAGGCTTTTAATGAGCTCTCAATGGATTAGTGTGAGGATGTTGGAATGATGCGATGGATGTTACTATCTCTCGTAAGTCA
   S R G F L N E A L N G F S D E G W S M I G N D G M D D V T I L V N S
1288 TCTCCTGACAAGTTGATGGGTTCAAATCTTCTCTCACTAATGGGTTTCCAGCTGTCAGCAGTGTCTGCTGTGTCTAAAGCATCGATGCTTTTACAG
   S P D K L M G S N L S F T N G G F P A V S S A V L C A K A S M L L Q
1387 AATGCCCTCTGCAATCTCTGAGATTCTTGCAGAGCAGAGTCCAGATGAGGAGTAAACAATATTGATGCTATGACGCTGACGCGGTTAAAGTT
   N V P P A I L L R F L R E H R S E W A D N N I D A Y A A A A V K V
1486 GGTCTTTTAGCTTACAAGTTCTCGAGTTGGAAGTTTCGGGGGTCAAGTTATATTCTCGCTGGCTCACACTATTGAACATGAAGAGTCTCTGGAGTGC
   G P F F S L Q G G S R V G S F G G Q V I L P L A H T I E H E E F L E V
1585 ATAAACCTGGAAGGTGGGCCATTCTCCTGAAGATCCAAATATGCCGAGATGTGTTCTTTTGAACCTCTGCTGTGGAATGGACGAGAAATGCTGTT
   I K L E G V G H S P E D P I M P R D V F L L Q L C C G M D E N A V
1684 GGCGCATGTGCTGAACTTATATTGCTCCCATTTGATGCGACTTTTGTGATGATGACCGCTTTTACCTTCTGGTTTTCGCATCATTTCCCTTGATTCT
   G A A C A E L I F A P I D A T G F A D D A P L L P S G A G F R I I P L D S
1783 GGAAGCAAGCCTTCCAAATCGTACCTTAGATCTTCTGCTGCTCTTGAAGTCCGACGAGCTGGAAACGAGCATCCAGTATCATTTCTGCTAAT
   G K E A S S P N R T L D L A A A L E V G P A G N R A S S D H S A N
1882 TCTGGTTCACAAAGATCTGTAATGACAATCGCATTGTAATTTGCATTGAGAGCCACATGCAAGAACATGTAGCATCAATGACTCGGCAATATATCCGA
   S G C T R S V M T I A F E F A F E S H M Q E H V A S M T R R Q Y I R
1981 AGCATTATATCTTCAGTTTCAGAGGTGGCATTAGCAGTATCTCTCTCATCTTGTGTTTACAGGCTGGTCTTCCGTCACCACTGGGTACTCTGAAGCA
   S I I S S V Q R V A L A L S P S H L G S Q A G L R S P L G T P E A
2080 CAGACACTTGTCTGTTGATCTGCCAGAGCTACAGGAGCTATTTGGGTGTGGAGTACTCAAATCCAAATGGCGAAGGAAGTGAATCTATTCTGAAAACC
   Q T L A R W I C Q S Y R S Y L G V E L L K S N G E G S E S I L K T
2179 TGTGATCATTCAGATGCTATTATGTGCTGCTCATTGAGAGTGTTCGCGTCTTTACTTTTGAACCAAGCTGGAGTGTGATGATGCTGTGAGACACA
   L W H H S D A I M C C S L K V L P V F T F A N Q A G L D M L E T T
2278 TTAGTTGCACTGCAAGACATACTTTGGAAGATATTTGATGATCATGGAAGAAAACTCTCTGCTCAGAAATCCACAGATCATGCAACAGGGTTTT
   L V A L Q D I T L E K I F D D H G R K T L C S E F P Q I M Q Q G F
2377 ACTTGCTCTCAAGCGGTGATTGTTGTCAAGCATGGGAGACAGTTTCAATATGAAAGAGCTGTGGCCTGGAAAGTGTGAATGAAGAAGAAATGCG
   T C L Q G G I C L S S M G R P V S Y E R A V A W K V L N E E E N A
2476 CATTGATCTGCTTTATGTTTATAAAGTGGTCTTTGTCTGAGACTTGAACCAAGATCTAGTGGAGTGGCTGATTAAAGAGTGAGAACTCTATACCTG
   H C I C F M F I N W S F V *
2575 TCTTATTTGCTGGCTGTATTCTTCTATGTATAACATGTTTACAACCGTGGAGCACCTAGTTCATTGCTATGTTATAAATTTGTGTGGGGTTTGTTA
2674 TCGTAGAGATTATGGACGAGACGATCTAGTTGCCGGGGATCATTTTGT

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Fig. 1. Nucleotide and deduced amino acid sequences of the *PagHD-ZIP III_1*. A stop codon is indicated by an asterisk (*).

levels of *PtrHox44* and *61* were found in shoot tip, cambium layer, root, and phloem tissues. Hu *et al.* (2012) also revealed that *PtrHox31* genes showed the highest

transcript abundance in both differentiating xylems and roots with much lower expressions in cortex and leaf tissues, while *PtrHox23* genes were expressed much higher

HD			
PagHD-ZIPIII	MAMSKDGGKQPMIDNGK	YVRYTPEQVEALERLYHDCPKPSSIRRQQLIRECPILSNIEPKQIKVWFQNRRCREKQKKEASRLQAVNRKLT	90
PtrHox16	MEMSKDGGKQPMIDNGK	YVRYTPEQVEALERLYHDCPKPSSIRRQQLIRECPILSNIEPKQIKVWFQNRRCREKQKKEASRLQAVNRKLS	90
MdHB15	MAMSKDGNKHALDNGK	YVRYTPEQVEALERLYHDCPKPSSIRRQQLIRECPILSNIEPKQIKVWFQNRRCREKQKKEASRLQAVNRKLS	90
ATHB-15	MAMSKDGGKGLDNGK	YVRYTPEQVEALERLYHDCPKPSSIRRQQLIRECPILSNIEPKQIKVWFQNRRCREKQKKEASRLQAVNRKLT	90
bZIP			
PagHD-ZIPIII	AMNKLMEENDRLQKQVSQLVYENGYFRQHTHNTPLATKDTSCSVVTSQGHHLPQHPPRDAS	PAGLLSIAEETLTFELSKATGTAVEW	180
PtrHox16	AMNKLMEENDRLQKQVSQLVYENGYFRQHTHNTPLATKDTSCSVVTSQGHHLPQHPPRDAS	PAGLLSIAEETLTFELSKATGTAVEW	180
MdHB15	AMNKLMEENDRLQKQVSHLVYENGYFRQHTQGTTLATKDTSCSVVTSQGHHLPQHPPRDAS	PAGLLSIAEETLAEFLSKATGTAVEW	180
ATHB-15	AMNKLMEENDRLQKQVSQLVHENSYFRQHTPNPSLPAKDTSCSVVTSQGHLASQNPQRDAS	PAGLLSIAEETLAEFLSKATGTAVEW	180
START			
PagHD-ZIPIII	VQMPGMKPGFDSSGIVAISHGCTGVGARACGLVGLPEPTRVAEVLKDRPSWFRDCRAVDVNLNVLPTANGGTIELLYMQLYAPTTLAPGRDF		240
PtrHox16	VQMPGMKPGFDSSGIVAISHGCTGVGARACGLVGLPEPTRVAEILKDRPSWFRDCRAVDVNLNVLPTANGGTIELLYMQLYAPTTLAPGRDF		240
MdHB15	VQMPGMKPGFDSIGIVAISHGCTGVAARACGLVGLPEPTRVAEILKDLPSWLRDCRAVDVNLNVLPTANGGTIELLYMQLYAPTTLAPACDF		240
ATHB-15	VQMPGMKPGFDSIGIIAISHGCTGVAARACGLVGLPEPTRVAEIVKDRPSWFRDCRAVEVMNVLPTANGGTVELLYMQLYAPTTLAPPRDF		240
PagHD-ZIPIII	WLLRYTSVLEDGSLVVCERSLKNTQNGPSMPFVQHFVRAEMLPSGYLVRPCEGGGSIIHIVDHMDLEPWSVPEVLRPLYESSTVLAQKTT		360
PtrHox16	WLLRYTSVLEDGSLVVCERSLKNTQNGPSMPFVQHFVRAEMLPSGYLVRPCEGGGSIIHIVDHMDLEPWSVPEVLRPLYESSTVLAQKTT		360
MdHB15	WLLRYTSVLEDGSLVVCERSLKNTQNGPTMPFVQHFVRAEMLPSGYLVRPCEGGGSIIHIVDHMDLEPWSVPEVLRPLYESSAVLAQKMT		360
ATHB-15	WLLRYTSVLEDGSLVVCERSLKSTQNGPSMPLVQNFVRAEMLSSGYLIRPCDGGGSIIHIVDHMDLEACSVPEVLRPLYESPKVLAQKTT		360
PagHD-ZIPIII	MVALRQLRQIAQEASQSN--VTNWGRPAALRALSRQLSRGFNEALNGFSDEGWSMIGNDGMDVITLVNSSPDKLMGNSLFTNGFFAV		448
PtrHox16	MVALRQLRQIAQEASQSN--VTNWGRPAALRALSRQLSRGFNEALNGFSDEGWSMIGNDGMDVITLVNSSPDKLMGNSLFTNGFFAV		448
MdHB15	MAALRQLRQIAHEVSQSN--VTGWGRPAALRALSRQLSRGFNDALNGFTDEGWSMMGNDGMDVITLVNSSPDKLMGLNLSFGNGFFAV		448
ATHB-15	MAALRQLKQIAQEVTTQNTSSVNGWRPAALRALSRQLSRGFNEAVNGFTDEGWSVIG--DSMDDVITLVNSSPDKLMGLNLTFANGFAPV		450
PagHD-ZIPIII	SSAVLCAKASMLLQNVPPAILLRFLREHRSEWADNNIDAYAAAQVKGPFSLQGSRVGSFGGQVILPLAHTIEHEEFLEVIKLEGVGHSP		538
PtrHox16	SSAVLCAKASMLLQNVPPAILLRFLREHRSEWADNNIDAYAAAQVKGPFSLQGSRVGSFGGQVILPLAHTIEHEEFLEVIKLEGVGHSP		538
MdHB15	SNSVLCAKASMLLQNVPPAILLRFLREHRSEWADNNIDAYAAAQVKGPCSLAGSRVGSFGGQVILPLAHTIEHEEFLEVIKLEGVGHSP		538
ATHB-15	SNVVLCAKASMLLQNVPPAILLRFLREHRSEWADNNIDAYLAAQVKGPCSR---VGGFGGQVILPLAHTIEHEEFMEVIKLEGLGHSP		536
PagHD-ZIPIII	EDPIMPRDVFLLQLCCGMDENAVGCAELIFAPIDATFADDAPLLPSGFRIIPLDSGKEASS-PNRTLDAALAEVGPAGNRASSDHSAN		627
PtrHox16	EDPIMPRDVFLLQLCCGMDENAVGTCAELIFAPIDATFADDAPLLPSGFRIIPLDSGKEASS-PNRTLDAALAEVGPAGNRASSDHSAN		627
MdHB15	EDAMPREMFLQLCCGMDENAVGSCAELIFAPIDASFADDAPLLPSGFRIIPLDYGKEASS-PNRTLDALEAGTGTNGKGSSEYSAS		627
ATHB-15	EDAIVPRDIFLLQLCCGMDENAVGTCAELIFAPIDASFADDAPLLPSGFRIIPLDSAKQEVSSPNRTLDALEAGTGTNGKASTDQSGN		630
PagHD-ZIPIII	SGCTRSVMTIAFEFESHMQEHVASMTRQYIRSISSVQRVALALSPSHLGSQAGLRSP	LGTPEAQTARWICQSYRSYLGVELLSNG	717
PtrHox16	SGCTRSVMTIAFEFESHMQEHVASMTRQYIRSISSVQRVALALSP-HLGSQAGLRSP	LGTPEAQTARWICQSYRSYLGVELLSNG	716
MdHB15	AGCVRVMTIAFEFACETHMQEHVASMARQYVRSIISVQRVALALSPSNLSSQAGLRSP	LGTPEAQTARWICNSYRCYLGVELLSNG	717
ATHB-15	STCARVMTIAFEFGIESHMQEHVASMARQYVIRSISSVQRVALALSPSHISSQVGLRTP	LGTPEAQTARWICQSYRGYMGVELLSNS	720
MEKHLA			
PagHD-ZIPIII	EGSESILKTLWHHSDAIMCCSLKVLVFTFANQAGLDMLETTLVALQDITLEKIFDDHGRKTLCEFFQIMQQGFTCLQGGICLSSMGRP		807
PtrHox16	EGSESILKTLWHHSDAIMCCSLKALPIFTFANQAGLDMLETTLVALQDITLEKIFDDHGRKTLCEFFSQIMQQGFTCLQGGICLSSMGRP		806
MdHB15	EGSESILKSLWHHSDAIMCCSLKALPVFTFANQAGLDMLETTLVALQDITLEKIFDDHGRKTLCEFFQIMQQGFTCLQGGICLSSMGRP		807
ATHB-15	DGNESILKNLWHHTDAIICCSMKALPVFTFANQAGLDMLETTLVALQDISLEKIFDDHGRKTLCEFFQIMQQGFACLQGGICLSSMGRP		806
PagHD-ZIPIII	VSYERAVAWKVLNEEENAHICFCFMFINWSFV		838
PtrHox16	VSYERAVAWKVLNEEENAHICFCFMFINWSFV		837
MdHB15	VSYERAVAWKVLNEEETAHCMCFVFNWSFV		838
ATHB-15	VSYERAVAWKVLNEEENAHICFCFVFINWSFV		837

Fig. 2. Multiple alignment of the deduced amino acid sequences of *PagHD-ZIP III_1* with HD-ZIP III from *P. trichocarpa* (PtrHox16), *Malus × domestica* (MdHB15) and *A. thaliana* (ATHB-15). The alignment was produced using ClusterW2. The conserved domains were indicated in the box and bZIP motif was underlined. Domains and motif were marked with an abbreviation such as HD (homeodomain), START (steroidogenic acute regulatory protein related lipid-transfer), MEKHLA and bZIP (basic leucine-zipper motif).

in differentiating xylems and roots than the other tissues. This shows that the abundance of *PagHD-ZIP III_1* transcripts in stem tissue is a unique feature of HD-ZIP III in *Populus alba* × *P. glandulosa*.

Effects of abiotic stress and plant hormone treatments on *PagHD-ZIP III_1* expression

We performed another set of RT-qPCR to gain more

insights into the expression patterns of *PagHD-ZIP III_1* in response to the treatments of various abiotic stresses and plant hormones. The levels of *PagHD-ZIP III_1* transcript in suspension cells were 2.7- and 2.9-fold highly expressed two hours after NaCl and low temperature treatments, respectively (Fig. 5A). HD-ZIP genes in plants were shown to be differentially expressed in response to different environmental cues. For example, *Arabidopsis ATHB7*

Fig. 3. Genomic Southern blot analysis of *PagHD-ZIP III_1* in *Populus alba* × *P. glandulosa*. Genomic DNA digested with *EcoRI* (E), *HindIII* (H) or *XbaI* (X), was fractionated by electrophoresis in an 1.0% agarose gel. The gel was blotted onto nylon membrane, and hybridized with ³²P-labeled full-length *PagHD-ZIP III_1* cDNA.

Fig. 4. Tissue-specific expression of *PagHD-ZIP III_1*. Total RNA was extracted from leaves (L), flowers (F), stems (S) and suspension cultured cells (SC) of *Populus alba* × *P. glandulosa*. Error bars show the standard deviation of expression levels.

Fig. 5. *PagHD-ZIP III_1* expression under various treatment conditions. A. *PagHD-ZIP III_1* expression in response to treatment with mannitol, NaCl and cold for 2 and 10 h. Untreated control cells were incubated for the same durations. *PagHD-ZIP III_1* expression level represented as relative values when compared to those of untreated controls. B. *PagHD-ZIP III_1* expression in response to treatment with plant growth regulators including ABA, JA, SA and GA₃ for 0.5 and 10 h. C. *PagHD-ZIP III_1* expression in response to wounding for 12, 24 and 48 h. Total RNA was extracted from the trimmed leaves at the indicated times. Error bars show the standard deviation of expression levels.

was induced in response to ABA and drought (Olsson *et al.* 2004), while HD-ZIPs in *P. deltoides* showed different expression patterns depending on the plant genotypes or the type of HD-ZIP genes (Hu *et al.* 2012).

The effects of exogenously applied plant hormones at physiological concentrations including JA, SA, GA₃ and ABA on the expression level of *PagHD-ZIP III_1* were also examined. Among the plant hormone treatments, the expression level of *PagHD-ZIP III_1* was highly induced by JA with a 2.8-fold increase (Fig. 5B). Hu *et al.* (2012) reported similar results showing that the treatment of methyl jasmonate increased the *PtrHox18* and *45* but reduced *PtrHox6*, *30*, and *31* in suspension cells of a clone L4 of *P. tremuloides*. The increased expression of *PagHD-ZIP III_1* gene in the JA treatment indicated that this gene might be involved in JA signal transduction pathway. We therefore analyzed the expression of *PagHD-ZIP III_1* gene in response to mechanical damage on leaves of one-year-old plants. The expression levels of *PagHD-ZIP III_1* started to increase 12 hours after wounding and reached to the maximum level (3.1-fold) after 24 hours (Fig. 5C). Thereafter its expression leveled with the pre-treatment level (1.1-fold). The response of HD-ZIP genes to wounding treatment was also reported in *P. tremuloides* (Hu *et al.* 2012). When the mechanical damage was given on young leaves of poplar plants, *PtrHox 17* and *23* were highly expressed but *PtrHox27* and *90* was down-regulated, showing that each HD-ZIP subfamily showed different expression patterns in response to wounding.

CONCLUSION

Although many studies have shown that HD-ZIP genes in plants played important roles in plant development, little is known about how such genes are regulated by various stresses and plant hormones. In this study, we showed that the *PagHD-ZIP III_1* gene was significantly induced in a hybrid poplar (*Populus alba* × *P. glandulosa*) in response to jasmonic acid, NaCl, and low temperature, suggesting that the *PagHD-ZIP III_1* gene might be involved in not only jasmonic acid-mediated developmental process but

also stress response. Since phytohormones play a key role in plant development and response to biotic and abiotic stresses, we expect that this study may enhance our understanding on the functional characterization of *PagHD-ZIP III_1*. Further study such as transgenic modification involving both gain and loss of function may be necessary to reveal the exact function of the protein and to enhance our understanding of its physiological role in poplar.

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