

Evolution and divergence in the coding and promoter regions of the *Populus* gene family encoding xyloglucan endotransglycosylase/hydrolases

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Abstract Xyloglucan endotransglycosylase/hydrolases (XTHs) are believed to modify the cell wall structure by cleaving a xyloglucan polymer and transferring the newly generated, potentially reducing, terminal to another xyloglucan. We report here the detailed analysis of 37 *Populus trichocarpa* XTH genes/proteins in their divergence in both the coding and 5' promoter regions. Our results show that the *Populus* XTH genes have experienced whole-genome

and local duplications and pre- and post-speciation divergence. Genome-wide and segmental duplications seem to be dominant in subfamily I and III, while tandem duplication seems to be the major mechanism for the subfamily II expansion, which also has higher average ratios of K_a/K_s compared to those in subfamily I and III. There was a general lack of organ-specific gene expression. In contrast, the expression patterns in subfamily II varied in response to various hormone treatments, with II-A being up-regulated and II-B down-regulated after 2 h of hormone treatment. Expression for this subfamily was verified using the 1.5-kb PtXTH22 promoter that was fused with the GUS reporter gene and transformed into *Arabidopsis*. The PtXTH22 promoter contains auxin response element, ethylene insensitive 3-like factors, and brassinosteroid response *cis*-elements. Histochemical GUS staining of transgenic *Arabidopsis* seedlings confirmed that the PtXTH22 promoter was up-regulated by several hormones.

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Abbreviations

XET Xyloglucan endotransglycosylase
XEH Xyloglucan hydrolase
XTH Xyloglucan endotransglycosylase/hydrolases
BA 6-Benzylaminopurine
IAA Indole-3-acetic acid
SA Salicylic acid
GA Gibberellic acid
BR Brassinosteroid
JA Jasmonic acid
ABA Abscisic acid
GUS β -Glucuronidase

Introduction

Throughout their life cycle, plants constantly alter cell shapes, sizes, and functions partly through cell wall reconstruction response to developmental changes, such as seed germination, vegetative growth, flower and fruit formation, organ abscission, and vascular differentiation (Campbell and Braam 1999; Cosgrove et al. 2002). The changes in cell wall composition and structure are realized via wall-modifying enzymes, many of which are encoded by complex multi-member gene families (Carpita and McCann 2000; Cosgrove 2005; Geisler et al. 2006). One of the key gene families that modify cell walls encodes xyloglucan endotransglycosylases and hydrolases (XTHs), which belong to glycoside hydrolase family GH16 (Campbell and Braam 1999; Rose et al. 2002; Cosgrove 2005; Nishikubo et al. 2010). XTH genes have evolved through gene duplication and divergence. There are 33 XTH members in *Arabidopsis* (Yokoyama and Nishitani 2001), 29 in rice (Yokoyama and Nishitani 2004; Yokoyama et al. 2004) and 41 in *Populus* (Geisler et al. 2006). The *Arabidopsis* XTH genes are divided into three (Yokoyama and Nishitani 2001) or four (Saladie et al. 2006) major molecular phylogenetic subfamilies, and 29 rice XTH genes are divided into two major subfamilies (I/II and III) (Yokoyama and Nishitani 2004). Baumann et al. (2007) separated a large family of *Populus* XTHs into I/II, III-A, and III-B.

Biochemically, XTH enzymes are believed to perform two different enzymatic actions: xyloglucan endotransglycosylase (XET) activity or xyloglucan hydrolase (XEH) activity (Rose et al. 2002; Saladie et al. 2006). In subfamilies I and II, XTH enzymes appeared to be more associated with XET activity by cleaving a cross-linking xyloglucan polymer (donor) backbone and then transferring the newly generated end to another xyloglucan (acceptor) polymer (Campbell and Braam 1999; Rose et al. 2002; Cosgrove 2005), while the subfamily III members are less consistent. Phylogenetic analysis indicated that III-As are more associated with XEH activity and III-B with XET activity (Baumann et al. 2007). In addition, some XTHs in subfamily III-A, such as *TM-NXG1*, act predominantly as XEH, while in vitro *TM-NXG1* can also deliver XET activity at elevated concentrations of acceptor substrates (Baumann et al. 2007).

The physiological functions of XTH genes have been demonstrated in many tissues (Tabuchi et al. 1997; Tabuchi et al. 2001; Vissenberg et al. 2005; Wu et al. 2005). XTHs play a role during (1) the early phases of secondary cell wall deposition, possibly reinforcing the connections between the primary and secondary wall layers (Bourquin et al. 2002; Mellerowicz et al. 2008; Baba et al. 2009), (2) cell wall modification during the development of tracheary

elements (Matsui et al. 2005), and (3) somatic embryogenesis (Malinowski et al. 2004). At the gene family level, the individual XTH genes have been shown to be expressed in a tissue-specific manner (Aspeborg et al. 2005; Geisler et al. 2006; Nishikubo et al. 2007; Georgios et al. 2009). The expression of some XTH genes also respond to one or more phytohormone groups (i.e., auxin, gibberellins, brassinosteroids, cytokinin, ethylene) and diverse types of environmental stresses (dark, high and low temperature shocks, wounding, etc.) (Xu et al. 1995; Yokoyama and Nishitani 2001; Cui et al. 2005; Valeria et al. 2008). Transgenic expression of the fused GUS gene with individual *Arabidopsis* XTH gene promoters confirmed that these genes are developmentally regulated (Becnel et al. 2006).

Although XTH expression is responding to various hormones and other environmental cues, the physiological functions associated with this phylogenetic clade (subfamily) and the evolutionary divergence following genome duplication have not been determined. Since the most recent whole-genome duplication event in *Populus* was relatively recent [65 MYA based on fossil record and 10–12 MYA according to the molecular clock (Tuskan et al. 2006)], a detailed analysis of the promoter regions associated with XTH genes may shed light on the relationship between the phylogenetic classification (evo) and the functions (devo) and contribute to our understanding of the evolution and divergence of this gene family. In this study, we analyzed the expression of 37 XTH genes from the genome of *Populus trichocarpa* in various organs and in response to seven hormones. Our results show that the *Populus* XTH genes have experienced whole-genome and local duplications. Expressional divergence suggests that the promoters of PtXTH in subfamily II, which have 17 members, have concurrently evolved with the coding region and are co-regulated among subfamily members to control the spatial-temporal expression of the PtXTH genes. Inducible expression was verified with the fusion of PtXTH22 promoter with a GUS reporter gene and subsequent histochemical assays in transgenic *Arabidopsis*.

Materials and methods

Identification and sequence analysis of XTH genes in *P. trichocarpa*, ‘Nisqually-1’

XTH genes in *Populus* were initially identified from Supplemental Table 2 of Geisler et al. (2006). The amino acid lengths of PtXTH4 and PtXTH9 were 98 and 126, respectively, and were considered truncated and were not included in this study. According to the *Populus* genome assembly v2.0 (<http://www.phytozome.net/poplar>), PtXTH1 no longer exists, and PtXTH33 and PtXTH28 reside on the

same position with PtXTH13 on chromosome 5; therefore, PtXTH33 and XTH28 no longer exist. Gene model (POPTR_0002s06120) has high AA sequence similarity and a conserved sequence tag “HDEIDFEFLG” of XTH to other members, which was not in the list of earlier research (Geisler et al. 2006), and was designated as PtXTH42 in our study. In total, 37 *P. trichocarpa* XTH genes were used in this study (Table 1).

The chromosome locations of XTH genes in *Populus* were drawn using the MapChart software (Voorrips 2002).

To analyze *cis*-elements in the promoter of the *Populus* XTH22 gene, 1,500-bp upstream regions of the start codon (ATG) were extracted and queried for potential transcription factor binding sites using MatInspector 8.0 (Cartharius et al. 2005).

Phylogenetic tree construction

Multiple protein sequences were aligned with ClustalW. A phylogenetic tree was created with maximum-parsimony

Table 1 The primer sequences of the XTH genes in *Populus* for real-time PCR

Gene number	Forward primers (5' to 3')	Reverse primers (5' to 3')
PtXTH2	TTGTGATTCAAATAATTTCTCCCCT	CGTTTGGACCCATTGAAGCT
PtXTH3	GAGGGCTGCCCTTCTGA	GCAATCTCGAGCCTAGCAATAT
PtXTH5	CAATTTGGGATGCAAGCTACATT	CAAGAACACTCCACCGCAGTC
PtXTH6	GCTCCTCCAGTAACTCTGGCTA	TTCTTTTGACCCATTTATCTTC
PtXTH7	GACTATAACTACCAACCGTTCATTGG	CAGACGGTGGGCTGCAA
PtXTH8	AATTTGGGATGCAAGCTACATT	GAACACTCCACCGCAGTCC
PtXTH10	GATGTGCAAGGTAGAAAATTGCTC	GGCTAAACCTGCGTCTTCGA
PtXTH11	GGTCTAATGGAGTCTCTTCTTGCAAT	CCGCACCCATTCAGCTG
PtXTH12	GCCAATGCTTGTATTTGGTCACTAC	TGCCAGGCATTGTCTTGCA
PtXTH13	GACAGTACCTGGCAAGTTCAAGC	ACTTCTGCTGCACCCATCG
PtXTH14	TTCCAAGCCTTCCAGCG	GGAGCATCAAGCTCATTGGTTT
PtXTH15	TGGTGAATACCCGAAAAGTACTG	CATAAGTCAAATCTTCTTCCTGATATCTCA
PtXTH16	CTCTCAGATGAACAGAAAATGGACTA	TGTCTTTGCAATAATCATAGATCACA
PtXTH17	CCACTTCTCCTCCCGCCT	CTCTCCTGCCTTGTTGAATCAA
PtXTH18	CCACTTCTCCTCCCGCCC	CCTCTCCTGCCTTGTTGAATCA
PtXTH19	CTTGTAATTTGGTCTTCTGGCAG	CCCACCTGAGCCTTTGGAA
PtXTH20	GGTCTTACGGAGCATCTTCTGTAAA	CCCAGATCCATGAGTTGCTGA
PtXTH21	GGTCCTATGGCACTTCTCTCT	TTGGTATGCCATGCACGA
PtXTH22	AATATCCAGTTTTGTGCTGTATGGA	GTGGCATTACCACAGTTTCA
PtXTH23	AAGTGAATGGACCAGTTAGCATA	GGATGTCCACCAGTTCGCT
PtXTH24	TGCTTATGGTCTTCAGGATCG	TCTGCCAAGCATTGTTTGTG
PtXTH25	CCTGAATGCGTATCAACTACTACTAAGA	TTTGTGATCTGAGAGATGCCAT
PtXTH26	GGTTGTGAGGGCGTCGGTA	CTGATCCCACCAACGCTTAC
PtXTH27	TCTGTGCCACACAGGGTACC	CCTGTATTGCAAGGCATCAAGA
PtXTH29	AAGATCGATGCTTGTCCCTTG	TATCTTTCCTTGTTCCACCAA
PtXTH30	TTCGTCCCTCCTACAAAG	AGCGTTATCATCTGCTGTATC
PtXTH31	TGTTGCTAGTACACCAATTTCAA	CGGAGAGGGCGGACACA
PtXTH32	GCTAGCTACACCAATTTCAAAGCA	GACCGGGCGGCACCAT
PtXTH34	TAGATGGGTGCGAGGCT	CTTCTGGTCCCACCATCTAGC
PtXTH35	TTGATGCAATGTGAGTGTCCAGT	CCACCAGTACCTTTTCTACCA
PtXTH36	AGGACCAGCAACCTGTGCTT	CGTTGAGGGCTTGTAAGTAGC
PtXTH37	CAATGGTGCATCTTCTGTGGTA	CAAGCTCTTCTGAAAGCCAAGC
PtXTH38	TTGCATTTCAACCAAAGTGC	GTCCGTCAACAGGGTCCA
PtXTH39	TGGAGGCAAATACAGAGTTAATTACAA	TCAATTGGATCGACAGCACAA
PtXTH40	CCCCATCAGAGTGTGTGATC	GGCCTCCGCCAAAAGTAAC
PtXTH41	CCCCATCGAGGAATTTCTCT	CATAGCTGACCGTTGTCTCTCT
PtXTH42	GATTGGACACAAGCTCCTTTTCAT	TGTCTTGACAGAAATGGGAGA

analysis using PAUP software (version 4.0; Sinauer Associates, Inc., Sunderland, MA, USA). The phylogenetic tree selected for this study represents the most parsimonious of 9,698 rearrangements that were generated. The numbers of bootstrap replicates was set for “100.”

Quantitative real-time RT-PCR

Five organs from 4-week-old in vitro subcultured explants were analyzed for organ-specific expression following procedures described by Ye et al. (2009a, b). Shoot tip (including first and second expanding leaves), young leaf (third expanding leaf), mature leaf (seventh and eighth fully expanded leaves showing an initial sign of yellowing), root, and bark (including phloem and cambium) tissues were sampled, with the whole plant as the control (CK). The organs were harvested from *P. trichocarpa* (‘Nisqually-1’) plants grown in vitro on medium containing Murashige and Skoog salts (Murashige and Skoog 1962), 2% sucrose, 0.3% activated charcoal (Fisher Scientific, NJ, USA), and 0.3% gelrite (PhytoTechnology Laboratories) at 23–25°C under a 16-h photoperiod with a fluorescent illumination of 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

To analyze hormone response, 4-week-old whole plants were removed from the culture jars and transferred to a solution containing deionized water and one of the following plant hormones (each at 1 μM): 6-benzylaminopurine (6-BA), indole-3-acetic acid (IAA), salicylic acid (SA), gibberellic acid (GA_3), brassinosteroids (BR), jasmonic acid (JA), and abscisic acid (ABA) (Fisher Scientific, NJ, USA) for 2 and 8 h. The treated plants were then frozen in liquid nitrogen. The plants receiving no hormone treatments were used as the control and were handled in an identical manner.

The total RNA was extracted by using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and treated with AMPD1 DNase I (Sigma-Aldrich, St. Louis, MO, USA). The oligonucleotide primers (Table 1) were designed based on the identified 3'-untranslated region and the 3' terminal sequences of the predicted coding region using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The stringency of the primers for each gene was set so that at least one 3'-end and three other nucleotides were unique compared to sequences of other genes in the gene family. The size and homogeneity of the PCR products were examined according to the procedure described previously (Udvardi et al. 2008).

Real-time PCR was conducted using a Power SYBR Green PCR Master Mix Kit (Applied Biosystems, Warrington, UK) in an ABI 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Actin2, UBQ, TUA, and 18S, found to be the most stably expressed reference genes out of ten housekeeping genes in *Populus*

(Brunner et al. 2004), were used as references. To validate the proper dosage of cDNA, the above four reference genes were selected as reference genes in our experiment and geNORM software (Vandesompele et al. 2002) was employed to determine which reference genes were best for normalization. Actin2 was found to be stable and was used as an internal control. All of the above experiments consisted of three biological replicates, and the experiment was repeated at least three times.

Real-time PCR data were analyzed as previously described by Yuan et al. (2006) (Ct data for each reaction are shown in “Electronic supplementary material 1”). Expression ratio was represented relative to the control value observed for the gene and fold change was calculated by the formula $2^{-\Delta\Delta\text{Ct}}$ (the efficiency of amplification for each pair primer was shown in “Electronic supplementary material 2”), where $\Delta\Delta\text{Ct} = \left(\text{Ct}_{\text{target gene}} - \text{Ct}_{\text{actin}} \right)_{\text{treatment}} - \left(\text{Ct}_{\text{target gene}} - \text{Ct}_{\text{actin}} \right)_{\text{ck}}$. The error bars represented the standard error of the mean. In the formula, the “treatment” was the Ct value for specific tissue or different time point, the “ck” was represented by the whole plants, “actin” was the value for the internal control gene, and the “target gene” was the value for the individual XTH gene. The dissociation curve was examined to ensure data quality based on Yuan et al. (2006) and Ye et al. (2009a).

K_a/K_s test

The number of nonsynonymous substitutions per nonsynonymous site (K_a), the number of synonymous substitutions per synonymous site (K_s), and K_a/K_s value were calculated using K-estimator (Comeron 1995, 1999). The K_a/K_s ratio from paralogous XTH genes (shown in Table 2) in subfamily I, II, and III were individually estimated.

Comparative analysis of paralogous promoter sequences

Comparative analysis of the 1,000-bp region upstream (“Electronic supplementary material 3”) of the translation start codon (ATG) was performed using the GATA program (Nix and Eisen 2005), with window size of 7 and lower cutoff score of 12 bit.

Cloning and functional analysis of the XTH22 gene promoter in *Populus*

To verify the expressional data, we cloned the 1,500-bp promoter of XTH22 gene from the genome of *P. trichocarpa* ‘Nisqually-1’ by the primer pair: 5' AACAACCACA CAATGTT'3 (forward), 5' AACCGTAAACAAGAAATAT TAAACAAC'3 (reverse). The fragment was fused in frame to a GUS reporter gene and cloned into the pMDC 162

Table 2 The synonymous and nonsynonymous substitution rates among paralogous XTH genes in *Populus* from different subfamilies

Subfamily	Paralogous genes	K_a	K_s	K_a/K_s	Average
SFII	18 versus 17	0.00808	0.01091	0.7406	0.2886
	12 versus 42	0.04188	0.11422	0.3667	
	2 versus 19	0.1576	1.3461	0.1171	
	14 versus 21	0.03664	0.40178	0.0912	
	5 versus 23	0.03734	0.25349	0.1473	
	10 versus 24	0.24175	0.89902	0.2689	
SFI	27 versus 26	0.10756	1.31301	0.0819	0.1245
	27 versus 34	0.04098	0.22979	0.1783	
	26 versus 34	0.12442	1.16311	0.1070	
	30 versus 35	0.04437	0.30208	0.1469	
	16 versus 25	0.03632	0.33438	0.1086	
SFIII	31 versus 32	0.02948	0.36796	0.0801	0.1497
	31 versus 7	0.17898	1.16332	0.1539	
	32 versus 7	0.17647	1.01467	0.1739	
	39 versus 40	0.04908	0.28030	0.1751	
	41 versus 3	0.0519	0.3135	0.1654	

The promoters of each paralogous in italics were compared in Fig. 6

vector (Curtis and Grossniklaus 2003) using the Gateway method (Invitrogen). Then, the vector was transformed into *Arabidopsis* using the flower dipping protocol (Clough and Andrew 1998).

Tissue specificity and induced expression of XTH22 promoter in *Arabidopsis* was examined by histochemical staining for GUS activity using X-gluc (Sigma-Aldrich, St. Louis, MO, USA). The transformed *Arabidopsis* plants with three biological replicates, which were collected at 6, 12, 18, 26, 32, and 60 days after sowing, were stained. To determine whether the XTH22 promoter responded to hormone treatments, the 8-week-old transformed *Arabidopsis* plants were treated with 1 μ M of either 6-BA, IAA, SA, GA₃, BR, JA, or ABA for 8 and 24 h, as described above, and were then assayed by staining for GUS activity.

Results

Chromosomal distribution and phylogenetic tree of XTH genes in *Populus*

Forty-one open reading frames potentially encoding XTH proteins were identified from earlier research (Geisler et al. 2006). PtXTH4 (294 bp) and PtXTH9 (378 bp) with short sequences, which were considered pseudogenes or incomplete sequences, were not included in this study. Gene model (POPTR_0002s06120), which was not listed by earlier research (Geisler et al. 2006), was designated as PtXTH42 in our study (“Electronic supplementary material 4”). According to the *Populus* genome assembly v2.0 (<http://www.phytozome.net/poplar>), PtXTH1, PtXTH33, and PtXTH28 no longer exist. PtXTH2 and PtXTH41

now reside on chromosome 5 (LG5_427531–428762) and chromosome 1 (LG1_1357663–1360135), respectively. PtXTH5, PtXTH20, and PtXTH32 were assigned to chromosome 6. Thus, 37 XTH genes were identified in the *Populus* genome on 16 chromosomes (Fig. 1). There are 10, 17, 10 and 1 member in subfamily I, II, III, and IV, respectively, in *Populus*, compared with 9, 13, 7, and 4 members in *Arabidopsis*, respectively. The phylogenetic tree between *Arabidopsis* and *Populus* XTH genes (Fig. 2) and the locations of the XTH genes in the *Populus* genome demonstrated that further expansion has occurred in *Populus* XTH gene family after the divergence of the *Arabidopsis* and *Populus* lineages, especially in subfamily II. This subfamily II-specific expansion seems to be caused by tandem duplications (PtXTH17 and 18, 12 and 42, 24 and 10) (Fig. 1). The three sister locus pairs from subfamily II were inferred to be paralogs in phylogenetic tree (Fig. 2). None of XTH genes in subfamily I, III, and IV were found in tandem clusters.

Expression patterns of the XTH genes in five organs in *Populus*

Four genes were expressed at levels four times higher in the shoot tips compared with that in whole-plant reference/controls (Fig. 3a). In young leaf tissues, of the 37 XTH genes in *Populus*, 12 genes were expressed lower than in the controls and the rest had higher expressions than the control, indicating no clear association with the subfamily structure (Fig. 3b). Similarly, in mature leaves, approximately half of the genes were expressed higher and another half lower than in controls, showing no clear association with the subfamily structure (Fig. 3c). In root tissue,

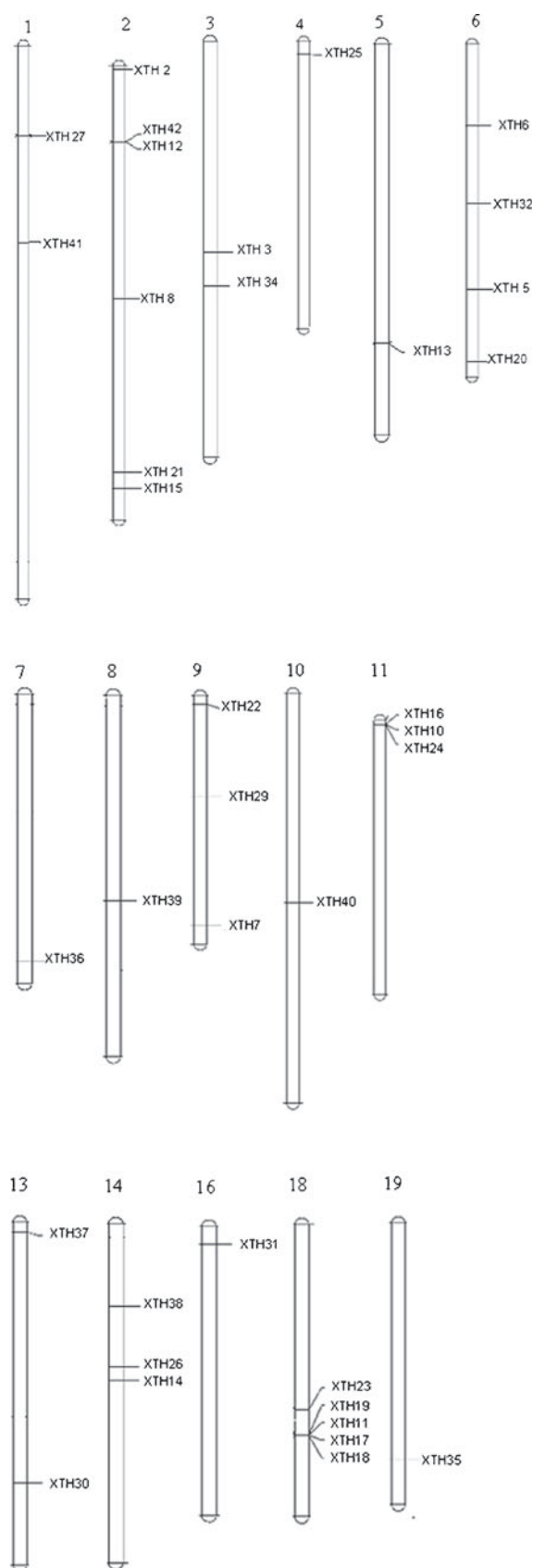


Fig. 1 The chromosome locations of the XTH genes in the sequenced *Populus* genome

substantially more PtXTH genes showed higher expression across all subfamilies and all but two subfamily III genes were expressed higher than in the controls (Fig. 3d). In the bark tissues, expression of all PtXTH genes except PtXTH36 (in subfamily I), PtXTH 38, and PtXTH 39 (in subfamily III) were higher than that in controls and expression levels were considerably higher than those in other tissues (Fig. 3e).

Although there is no clear association of subfamily structure with tissue-specific gene expression, nearly all genes were preferentially expressed in the bark tissues; several individual genes showed clear trends in tissue-preferential expression. For example, PtXTH20 and PtXTH24 were expressed at a low level in shoot tips, young and mature leaves, and roots, but at a high level in the bark, while PtXTH36 show the reverse trend (Fig. 3).

XTH gene expressions in response to hormone treatments

In drastic contrast to the lack of tissue-specific expression patterns in subfamilies, expression of the XTH genes in *Populus* showed distinct patterns in response to various hormone treatments, particularly within the subfamily II. After 2 h of treatment, the genes in subfamily II clearly separated into two general groups in response to all hormones (Fig. 4). One group (II-A, blue circles) showed an overall up-regulation and another group (II-B, red circles) showed a general down-regulation, particularly in response to 6-benzylaminopurine (BA), indole-3-acetic acid (IAA), salicylic acid (SA), gibberellic acid (GA), and brassinosteroids (BR) (Fig. 4a–e). Interestingly, PtXTH24 and PtXTH10, a pair of paralogs, fell into the two alternate subgroups. PtXTH24 was generally up-regulated, while PtXTH10 was down-regulated. The subfamily I genes showed less consistent expression among the members in response to various hormones. Overall, they showed much lower expression than the II-A members and mostly fourfold down-regulated in response to BA, IAA, SA, GA, and BR (Fig. 4a–e), but a 16-fold increase in response to jasmonic acid (JA) and abscisic acid (ABA) (Fig. 4f, g). The subfamily III XTH genes seemed to have no clear expression patterns in responses to BA, IAA, GA, and SA (Fig. 4a–g), with the exception of PtXTH22 which was consistently up-regulated across all hormone treatments. Interestingly, PtXTH22 contains no introns compared with other PtXTH genes with two or three introns (“Electronic supplementary material 5”). The sole subfamily IV member PtXTH15 showed consistent down-regulation, ranging from four- to 64-folds, in response to all hormones tested (Fig. 4a–g).

When the *Populus* plants were treated with the same hormones for 8 h, greater differentiation among and within subfamilies was observed (Fig. 5a–g). In the subfamily II,

Fig. 2 The phylogenetic tree of the *Populus* and *Arabidopsis* XTH genes

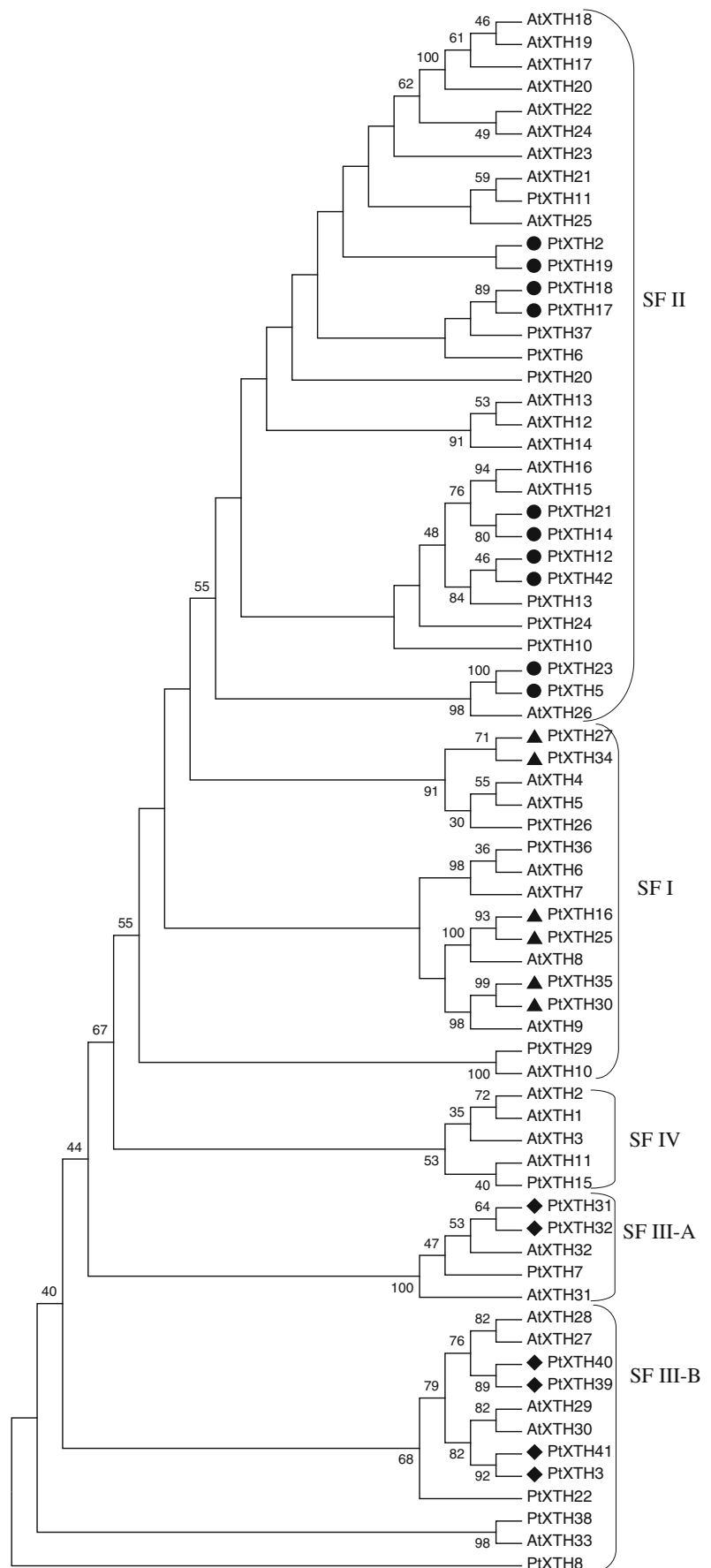


Fig. 3 The tissue-specific expression of the *Populus* XTH genes arranged in the same order as in the phylogenetic tree (see Fig. 2). **a** Shoot tip, **b** young leaves, **c** mature leaves, **d** root, **e** bark. Expression ratio was represented as the value relative to the control value observed for the gene. The fold change was calculated by the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (C_{t_{target\ gene}} - C_{t_{actin}})_{tissue\ x} - (C_{t_{target\ gene}} - C_{t_{actin}})_{CK}$.

The *error bars* represented the standard error of the mean. Tissue X was a specific tissue; CK was represented by the whole plant. Actin was the selected internal control gene, and the target was the XTH gene. The value in Yaxis is $-\Delta\Delta Ct$. The *number* under the subfamily indicates the XTH gene number. The tissues were collected from 4-week subcultured *in vitro* plants

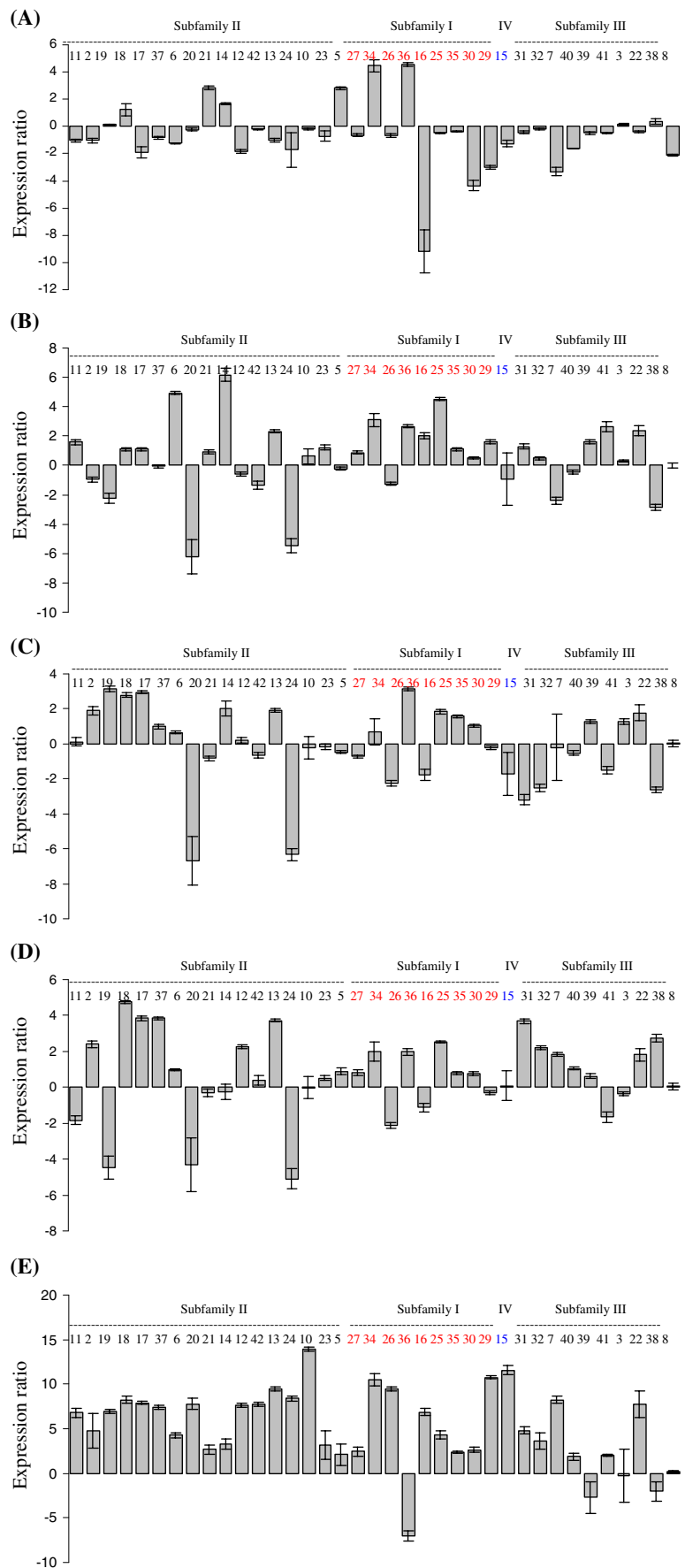


Fig. 4 The expression of the *Populus* XTH genes after induction with seven hormones treated for 2 h. **a** BA, **b** IAA, **c** SA, **d** GA, **e** BR, **f** JA, **g** ABA. The expression ratio was represented as a relative value to the control value observed for the gene. The fold change was calculated by the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{\text{actin}}})_{\text{time 2}} - (C_{t_{\text{target gene}}} - C_{t_{\text{actin}}})_{\text{time 0}}$.

Time 2 was 2-h time point for different treatments; the non-treatment plant represented time 0

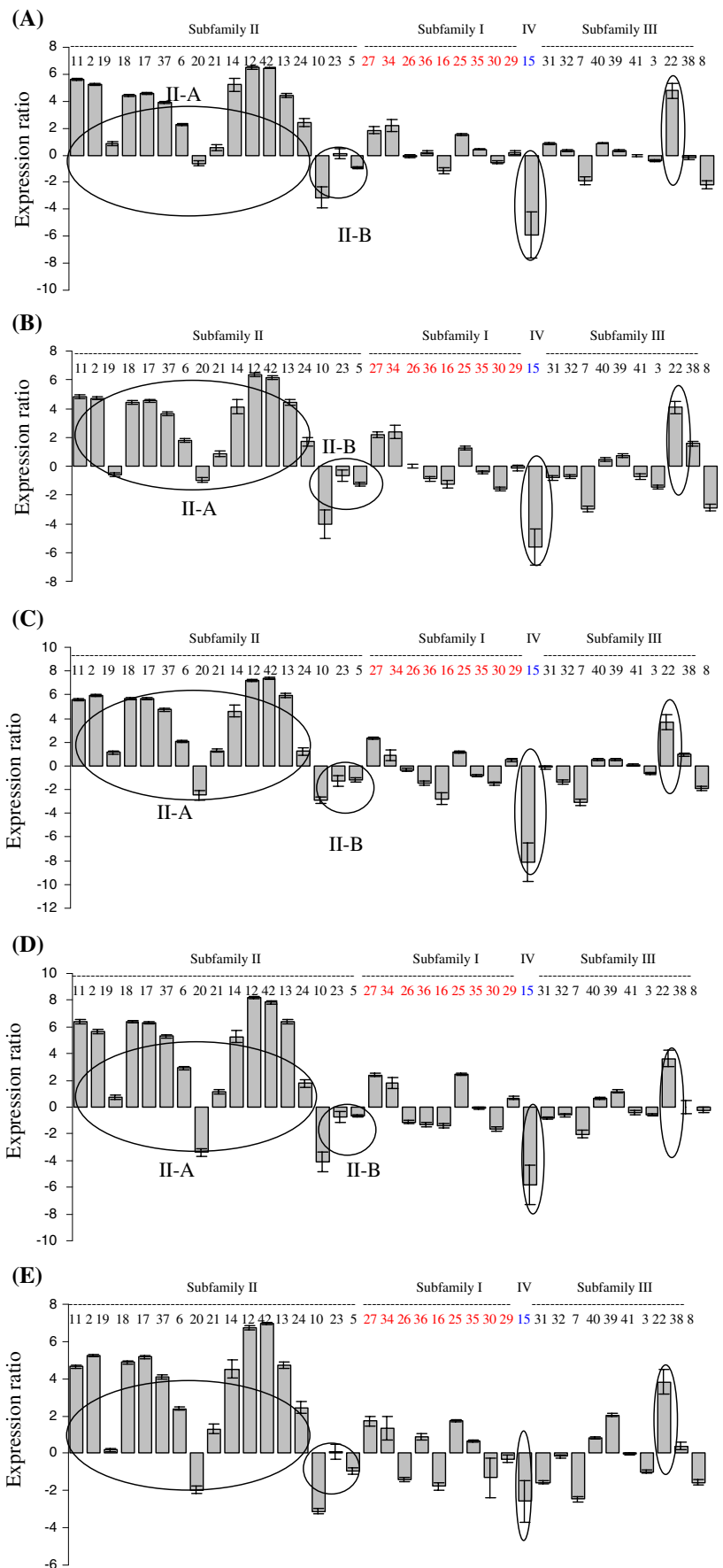
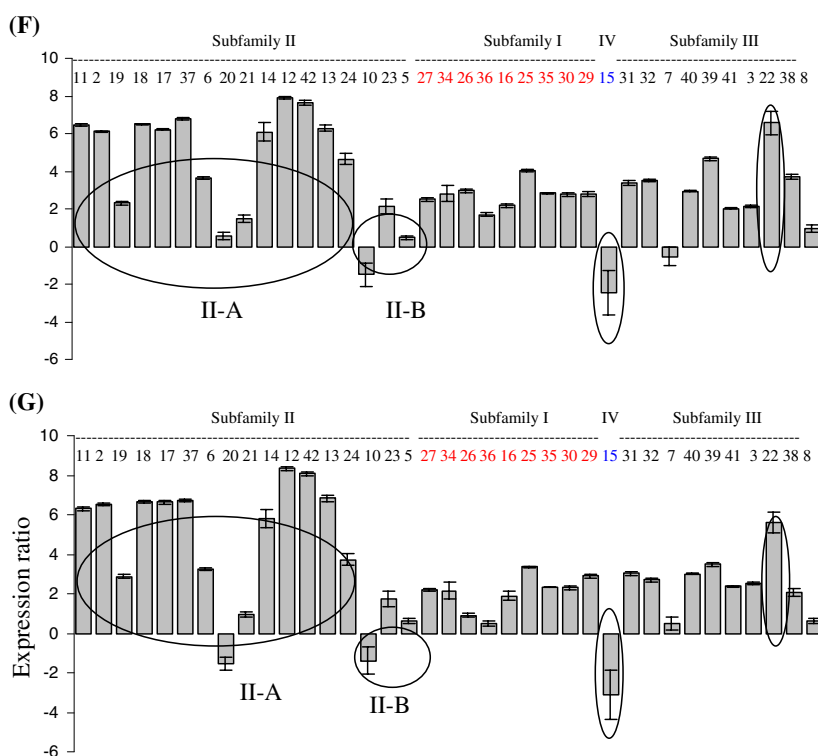


Fig. 4 (continued)



the up-regulating II-A group in Fig. 4 further differentiated into several groups: PtXTH20 and PtXTH19 became mostly down-regulated; the rest remained up-regulated, except PtXTH24 that became down-regulated, joining its paralogous member PtXTH10. The II-B in Fig. 4 remained down-regulated but the overall expression levels were dramatically reduced comparing with those treated for 2 h. In the subfamily I, nearly all genes were down-regulated in response to BA, GA, BR, and ABA, with either moderately up- or down-regulated in response to IAA, SA, and JA. The most substantial, consistent reduction in expression was for PtXTH26 (Fig. 5a–g). In subfamily III, no trend could be drawn comparing 2- and 8-h responses (comparing Fig. 4 with Fig. 5). The PtXTH22 gene, however, remained up-regulated in response to all hormones after 8 h of treatment (Fig. 5a–g).

The K_a/K_s ratio test of coding regions and comparative analysis of promoter regions from paralogous genes

The *Populus* XTH genes in paralogous clusters PtXTH18 and PtXTH17 in subfamily II had higher K_a/K_s ratio, while the other paralogous genes had lower K_a/K_s values (Table 2). The average K_a/K_s ratio was 0.2886, 0.1245, and 0.1497 in subfamily II, I, and III, respectively (Table 2), indicating that members in subfamily II are still in the process of active positive selection.

The 1,000-bp region upstream of the translation start codon (ATG) of the paralogs in *Populus* was compared

using the GATA software. The results indicated that there was divergence in the upstream regions of the paralogs, such as paralogous genes PtXTH17–PtXTH18 in subfamily II, PtXTH16–PtXTH25 in subfamily I and PtXTH41–PtXTH43 in subfamily III. However, PtXTH17–PtXTH18 shared a longer similar upstream region than that in other paralog pairs (Fig. 6). Conserved regions and microsynteny remained in the promoters of paralogous gene pairs (Fig. 6).

Verification of ectopic expression of GUS under control of PtXTH22 promoter

Because the intronless PtXTH22 gene had consistently high expression in response to various hormone treatments, its promoter was analyzed and fused to the GUS reporter gene and then introduced into the *Arabidopsis* genome. The results indicated that the promoter of the PtXTH22 gene included ARE (auxin response element) (from position –1,383 to –1,395 bp), EINL (ethylene insensitive 3-like factors) (from position –1,176 to –1,186 bp), and BR (brassinosteroid response element) (from position –713 to –729 bp) *cis*-elements. The transgenic *Arabidopsis* seedlings carrying the PtXTH22 promoter::GUS fusion showed GUS activity in 6-, 12-, 18-, 26-, 32-, and 60-day-old seedlings (Fig. 7). In 6-day-old transgenic seedlings, GUS activity was detected in the hypocotyl and cotyledons, but no activity was detected in young roots. The GUS gene was highly expressed in the two cotyledons, hypocotyl, and root

Fig. 5 The expression of the *Populus* XTH genes after induction with seven hormones treated for 8 h. **a** BA, **b** IAA, **c** SA, **d** GA, **e** BR, **f** JA, **g** ABA. Expression ratio is represented as the relative value to the control value observed for the gene. The fold change was calculated by the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \frac{(Ct_{target\ gene} - Ct_{actin})_{time\ 8} - (Ct_{target\ gene} - Ct_{actin})_{time\ 0}}{}$. Time 8 was 8-h time point for different treatments; the non-treatment plant represented time 0

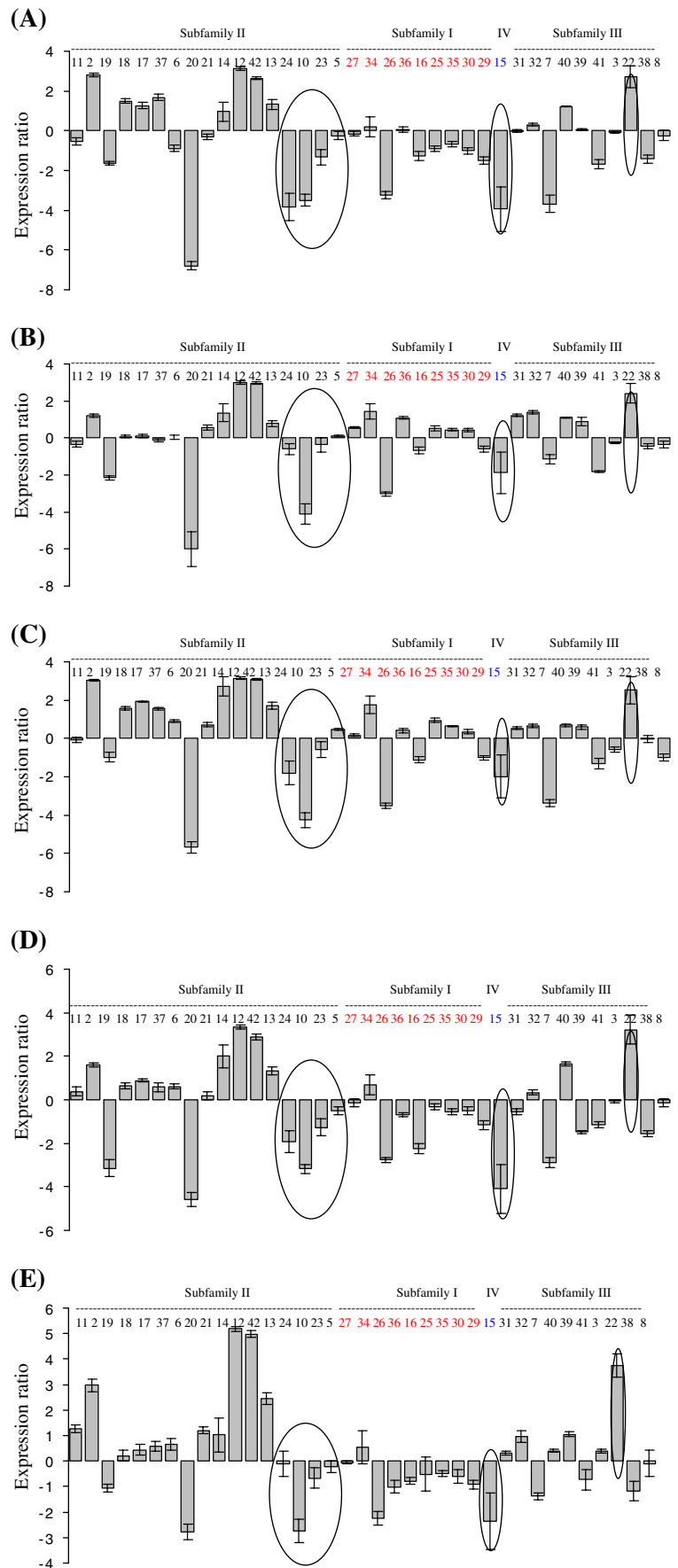
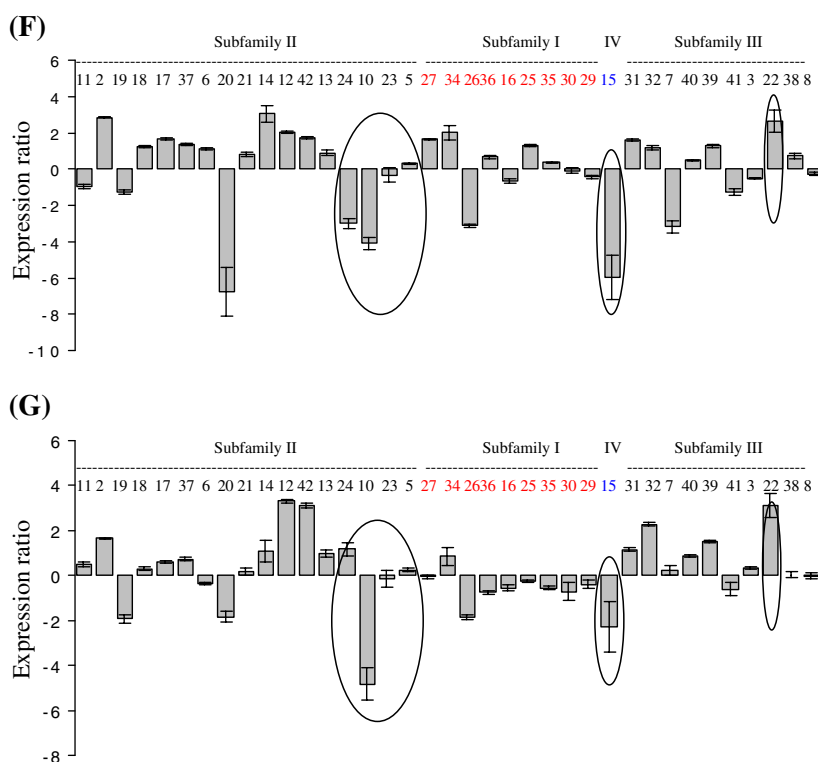


Fig. 5 (continued)



of 12-day-old transgenic seedlings. The 18-day-old seedlings showed high GUS activity in the four younger rosette leaves and root; however, the GUS gene was then expressed at moderate to low levels in the cotyledons and hypocotyl, especially in the middle of hypocotyl. The GUS gene was expressed most strongly in six older rosette leaves and roots in 26-day-old seedlings, but no GUS activity was detected in the rest of the rosette leaves, stem, hypocotyl, and first flower buds. The GUS gene was expressed in cauline leaves, root, and the border of two cauline leaves in 32-day-old seedlings. In 8-week-old plants, a high activity of GUS gene was detected mainly in basal rosettes and there was low activity in cauline leaves; no activity was detected in stems, siliques, and flowers.

GUS gene expression with the PtXTH22 promoter in response to various hormone treatments

Based on the GUS staining patterns of 8-week-old seedlings (Fig. 8), the GUS gene remained up-regulated in response to BA, IAA, SA, GA, BR, JA, and ABA hormones after 8 and 24 h of treatments when compared to levels in response to the no-hormone control treatment. In transgenic control plants, the GUS gene was expressed only in the basal rosettes and main root, with no GUS activity detected in the stem, flowers, and siliques. In contrast, hormone-treated plants stained deep blue for GUS

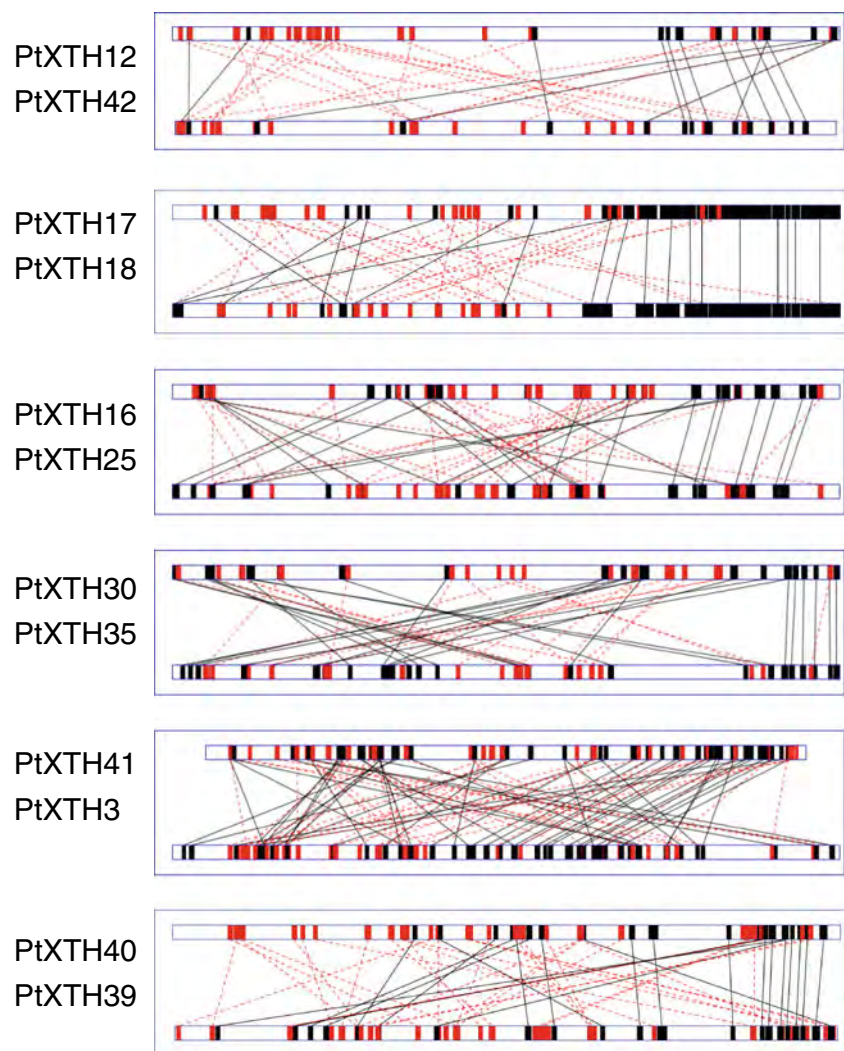
activity in basal rosettes, the cauline leaves, and main root, and GUS activity was intensified in stem, flowers, young siliques, and lateral roots (Fig. 8). There were no obvious differences in GUS gene expression between the 8- and 24-h treatments with SA, GA, BR, JA, and ABA treatments, and the expression level of the GUS gene was slightly lower in the 8-h treatment than that in 24-h treatments with BA and IAA.

Discussion

The local duplication contributing to expansions of XTH genes in the subfamily II

The *Populus* genome contains 39 XTH genes, about 15% and 26% more than those found in *Arabidopsis* (Yokoyama and Nishitani 2001) and rice (Yokoyama et al. 2004), respectively. Similar to *Arabidopsis* and rice XTHs, the phylogenetic tree and the locations of the XTH genes in the *Populus* genome also suggest that the PtXTH gene family underwent expansions from segmental and genome-wide duplications before and after speciation, and local duplications resulted in several paralogous sets of genes in the subfamily II, as evidenced by PtXTH17, PtXTH18, PtXTH11, and PtXTH19 clustered in a single chromosome location (Figs. 1 and 2). Other two pairs of

Fig. 6 Comparative analysis of the 1,000-bp region upstream of the translation start codon (ATG) of the paralogs (the 5' terminus on the left and the 3' terminus on the right). Solid dark lines connect similar regions (black boxes) and red broken lines connect matched regions in reversed orientation (red boxes)



paralogous genes (PtXTH12 and PtXTH42, PtXTH24 and PtXTH10) were located in respective single chromosome location. A total of 6 out of 17 genes in the subfamily II are probably from tandem duplications, implying that local duplications have been a major cause for the expansion of PtXTH genes in the subfamily II. These paralogous genes in the subfamily II appear still in the process of rapid positive selection, as indicated by a greater average value of K_a/K_s than those in the subfamily I and III.

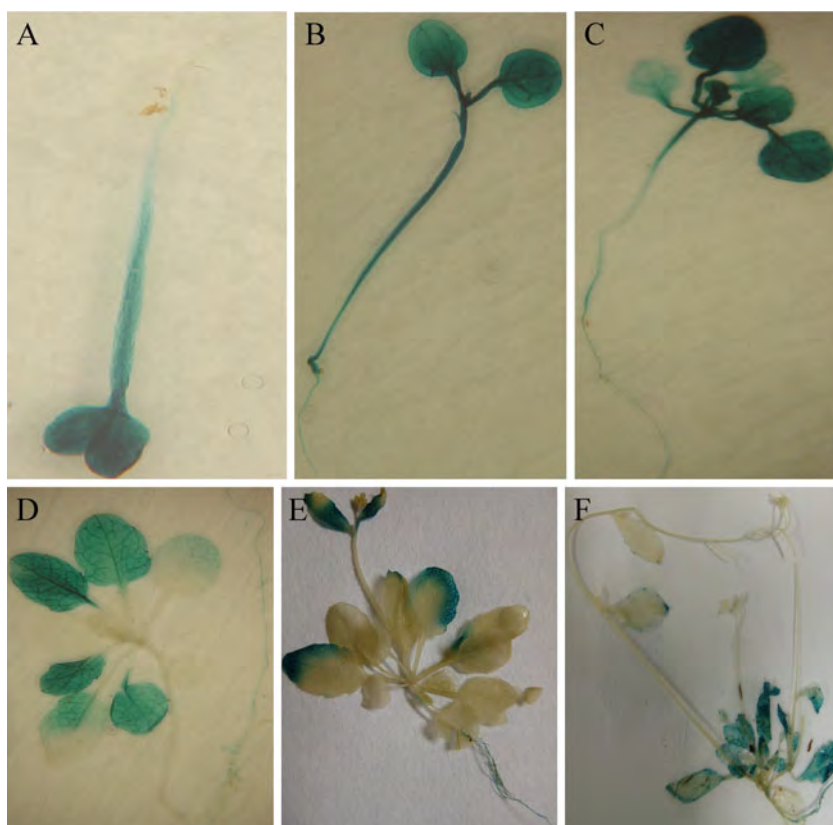
Gene expression patterns suggest divergence and concurrent evolution in the promoters of *Populus* XTH genes

Concurrent to the divergence of the coding region, the individual XTH genes have also diverged extensively in their promoters based on the expression patterns in various

tissues and in response to various hormones. Several paralogous sets of genes, which were considered as recent duplications, were differentially expressed in various organs, suggesting that their divergence occurred after duplication, e.g., PtXTH17 and PtXTH18 in shoot tips, PtXTH10 and PtXTH24 in shoot tips, young leaves, mature leaves, and roots. These results suggest that a single or combinations of the XTH genes could function with spatial and temporal specificity, allowing a plant to alter cell shapes, sizes, and functions during the course of development. This notion has been supported by studies of the entire gene families in *Arabidopsis* (Yokoyama and Nishitani 2001; Becnel et al. 2006) and rice (Yokoyama et al. 2004).

In addition to considerable divergence of expression patterns of the promoter in various tissues, the expression patterns in subfamily II were also consistent in response to various hormones. After 2 and 8 h of hormone treatments, the subfamily II genes clearly showed two general groups,

Fig. 7 The histochemical localization of GUS activity in transgenic *Arabidopsis* seedlings containing the -1500 PtXTH22:GUS fusion gene. **a-f** 6-, 12-, 18-, 26-, 32-, and 60-day-old transgenic seedling, respectively



i.e., the subgroup II-A with up-regulating expression and the subgroup II-B with down-regulating expression. The coding region of these genes, some of which were from tandem duplications, also exhibited high sequence homologies. In addition, the comparative analysis of the 1,000-bp region upstream from paralogous genes suggested that the promoter might have undergone concurrent evolutions with the coding region in the XTH gene family in *Populus*. For example, the paralogs PtXTH17 and PtXTH18 have higher similarity in the coding regions and the promoter regions.

Ongoing individual gene evolution

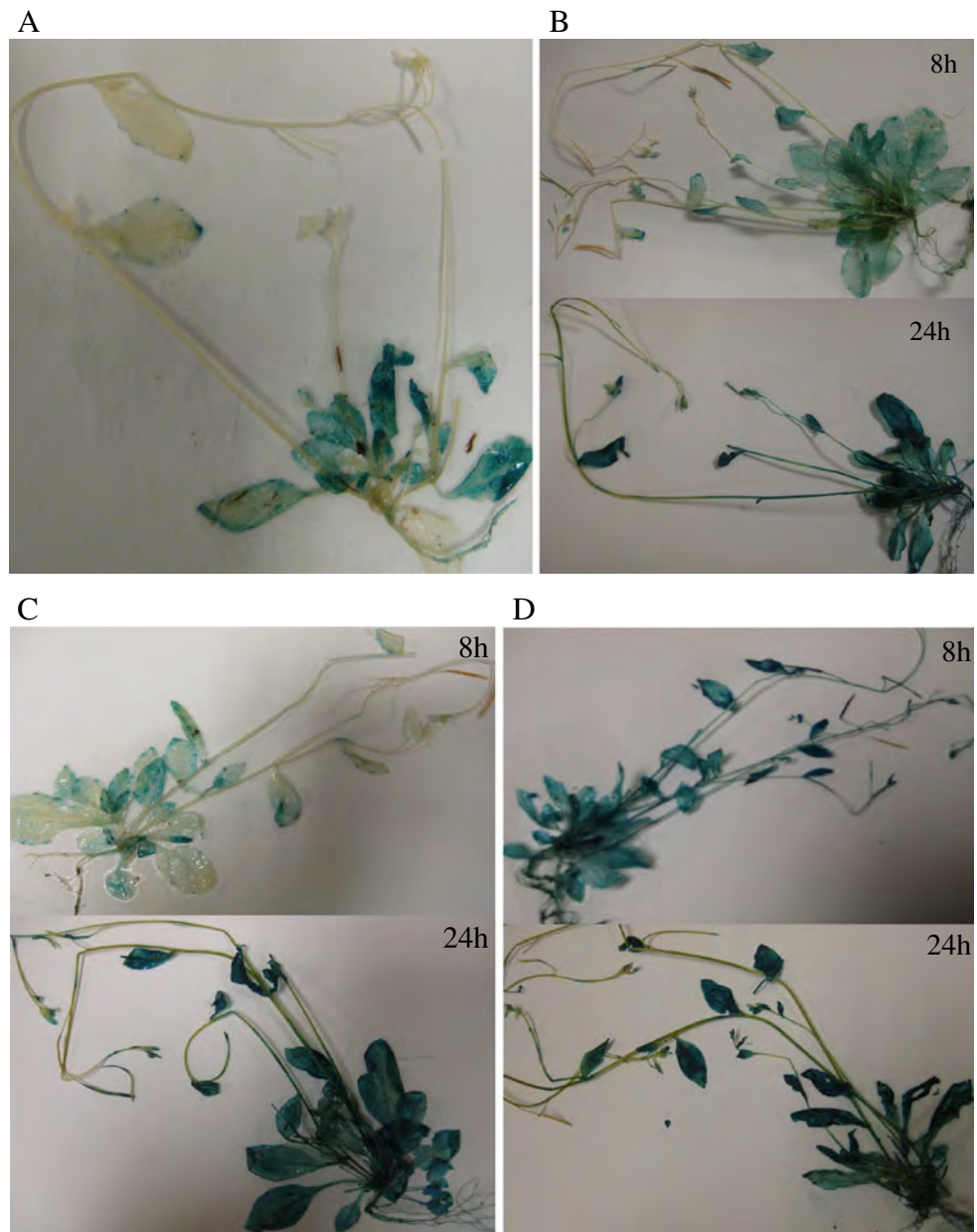
A K_a/K_s ratio greater than 1 indicates an acceleration of protein evolution since the divergence of the two genes, whereas a K_a/K_s ratio less than 1 indicates selective constraint of the two genes. Complete relaxation of selection will result in K_a/K_s of approximately 1 (Hurst 2002; Looman et al. 2002). The K_a/K_s ratios of PtXTH18 to PtXTH17 paralogs are close to 1, which implies that after an early phase of accelerated evolution, these duplicated genes have had neutral evolution to preserve their original functions.

The observations of K_a/K_s ratios in the XTH gene family in *Populus* confirms the marked differences between tandem and segmental duplications and paralogous and orthologous genes (Nembaware et al. 2002; Yu et al. 2005; Looman et al. 2002). For example, the paralogous genes with tandem duplication have larger K_a/K_s values in subfamily II, and the other paralogs which resulted from the segmental or whole genome duplication tend to have lower K_a/K_s values, which supports the observation that tandem duplicated and paralogous genes have greater K_a/K_s values compared to segmentally duplicated and orthologous genes (Nembaware et al. 2002; Yu et al. 2005; Looman et al. 2002) and that some paralogous genes, such as PtXTH12 and PtXTH42, PtXTH18 and PtXTH17, PtXTH10 and PtXTH24, are from more recent local duplications.

PtXTH22 gene is a unique gene and up-regulated in response to various hormone treatments

To verify the expressional data, we chose PtXTH22 for further verification partially because it is the only XTH gene in *Populus* without an intron and because it showed sensitivity to tissue type and hormone treatment. According

Fig. 8 The histochemical staining for GUS activity in transgenic *Arabidopsis* plants containing the -1500 PtXTH22::GUS fusion gene in response to various hormone treatments for 8 and 24 h, respectively. **a** CK, **b** BA, **c** IAA, **d** SA, **e** GA, **f** BR, **g** JA, **h** ABA

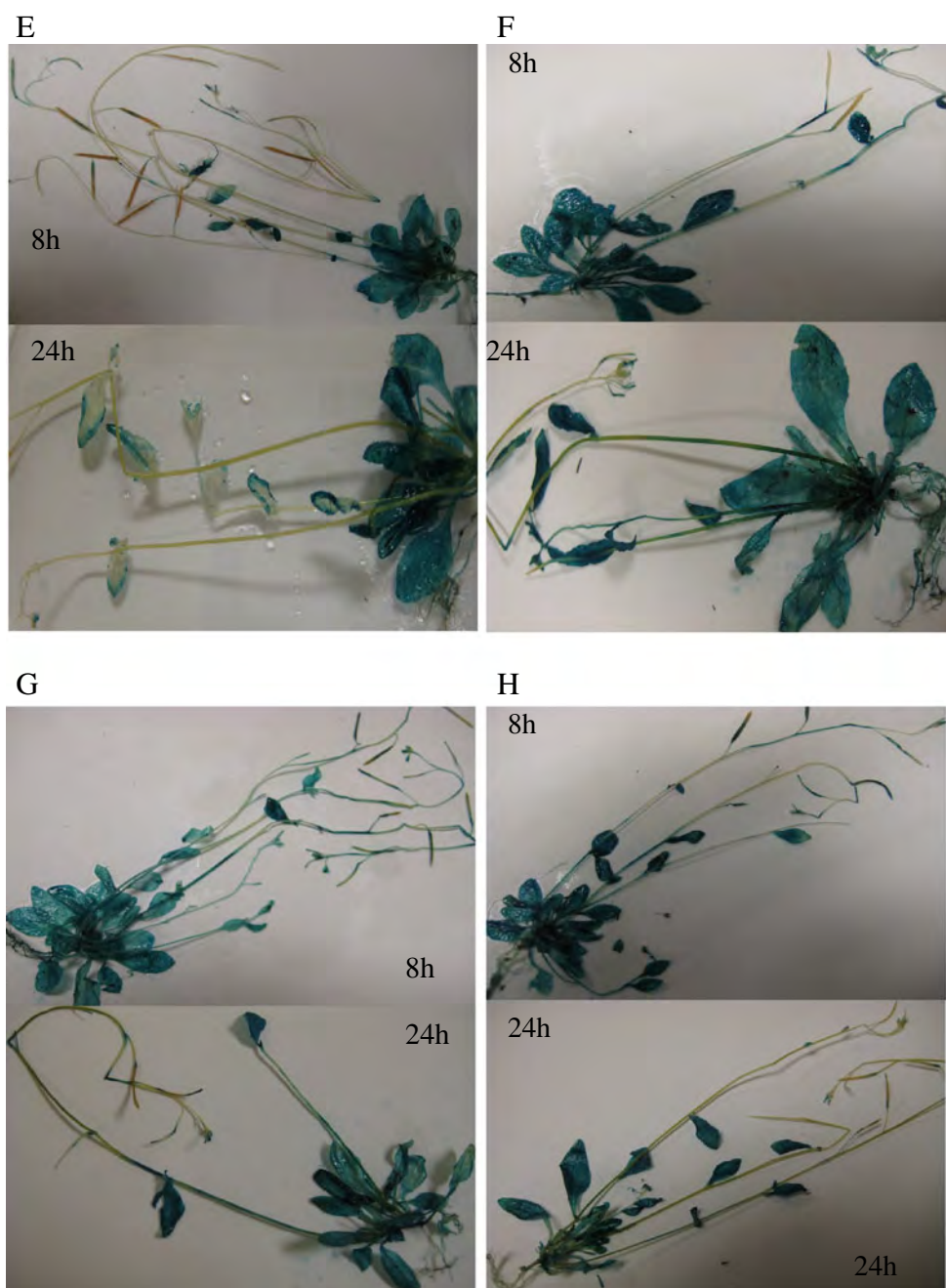


to the hypothesis of Baumann et al. (2007), PtXTH22, which is an XTH subfamily III member, should primarily display the XET activity while *Populus* seedlings undergo wall expansion and reconstruction. The PtXTH22 promoter/GUS construct was preferentially highly expressed in early developmental stages based on GUS histochemical staining, which suggested that it plays an important role during the early phases of secondary cell wall deposition.

Ethylene, SA, JA, and ABA are signals that mediate plant systemic responses (Mauch and Felix 2005), and interactions between these plant hormones may modulate the expression of stress-responsive genes in plants (Narusaka et al. 2003).

Our results demonstrated that expressions of the PtXTH22 gene were strictly up-regulated in response to various hormones through real-time PCR analysis (Figs. 3 and 4), which was further confirmed with fusion gene expression (PtXTH promoter::GUS) in transgenic *Arabidopsis* (Fig. 7). The exact reason that the intronless PtXTH22 gene was consistently up-regulated in response to all hormones is puzzling because these major plant hormones have very different physiological roles in plant growth and development. The consistent up-regulation in response to these hormones may be related to ARE, EINL, and BR *cis*-elements that are present in its 5' promoter region.

Fig. 8 (continued)



Conclusions

It has been widely demonstrated that gene/genome duplications, followed by divergence, have resulted in the current expanded gene families in plants (Yang et al. 2006; Doyle et al. 2008; Comai 2005). The XTH genes in *Populus* clearly have gone through whole-genome and local duplications and post-duplication divergences both in the coding and promoter regions. The divergence through either neofunctionalization or subfunctionalization in the coding region is seemingly correlated with the phylogenetic

subfamilies. The patterns of XTH gene expression in an organ-specific manner and in responses to various hormonal signals in *Populus* may suggest that the promoter region has concurrently diverged to accommodate when and where to exert the XET or XEH activity. Subfamily II showed least variations in responses to hormone treatments, suggesting their origins from recent tandem duplications. This research identified several members of XTH genes in *Populus* that showed consistent induction by hormones, like PtXTH22, PtXTH15, PtXTH12, PtXTH42, PtXTH10, and 20, which merit further characterization.

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