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ORIGINAL PAPER

Identification of the flavin-dependent monooxygenase-encoding *YUCCA* gene family in *Populus trichocarpa* and their expression in vegetative tissues and in response to hormone and environmental stresses

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Abstract Auxin is a plant hormone that regulates many processes of plant growth such as apical dominance, cell growth, adventitious rooting, and fruit and seed development. Expression of a family of *YUCCA* genes important for auxin biosynthesis has been shown to be spatially and temporally regulated in *Arabidopsis*. In this study, we report on the identification of 12 *YUCCA* genes from the completely sequenced *Populus trichocarpa* genome database and characterized them by comparing them with *Arabidopsis YUCCA* genes. The *Populus YUCCA* genes are distributed on eight chromosomes. All *Populus* and *Arabidopsis YUCCA* genes can be divided into two phylogenetic groups, one of which can be divided into two subgroups. *Populus YUCCA* genes are expressed in the shoot tip, immature and mature leaf, young root, stem, and bark tissues in an tissue-specific manner. Transcript accumulation of the *Populus YUCCA* genes is reduced by exogenous applications of various plant growth regulators except auxin. Steady-state mRNA levels of different *Populus YUCCA* genes vary considerably depending on the experimental treatments. These results suggest that, similar to the *Arabidopsis* and rice *YUCCA* genes, the *Populus YUCCA* genes may also have diversified roles in *Populus* growth and development, and their expression may be regulated in a complex manner by developmental and environmental cues.

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Abbreviations

ABA	Abscisic acid
BA	6-Benzylaminopurine
BR	Brassinosteroids
GA	Gibberellic acid
JA	Jasmonic acid
IAA	Indole-3-acetic acid
PEG	Polyethylene glycol
SA	Salicylic acid
SF	Subfamily

Introduction

The plant hormone auxin, with indole-3-acetic acid as the major endogenous auxin, plays a crucial role in plant growth and development (Davies 2004). A large volume of physiological studies have demonstrated that auxin promotes apical dominance, adventitious root formation and development, gravi- and photo-tropic responses, vascular differentiation, seed development, leaf expansion, shoot elongation, and organogenesis (Davies 2004). Recent genetic and biochemical studies have revealed pathways for auxin biosynthesis, both tryptophan-dependent and tryptophan-independent pathways (Normanly et al. 2004). Genomics and functional genomics analyses with forward and reverse genetics have identified *YUCCA* genes, a part of the flavin monooxygenase gene family, as the rate-limiting genes in IAA biosynthesis (Zhao et al. 2001). Further analyses of *YUCCA* gene family members in *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) suggest that auxin is locally synthesized in many tissues and organs and expression of the *YUCCA* genes

is regulated spatially and temporally (Kramer 2004; Cheng et al. 2006, 2007a, b; Kim et al. 2007; Yamamoto et al. 2007). *YUCCA*1, 2, 4, and 6 of *Arabidopsis* are expressed in the inflorescence apex and flowers, and have been shown to be important in flower development and plant stature based on multi-mutant analysis (Cheng et al. 2006). These four *YUCCA* genes were found to be necessary for vascular development in leaves (Cheng et al. 2006). One of the petunia *YUCCA* genes, *FZY*, encoding an enzyme with homology to flavin monooxygenases, is expressed in young leaves, bracts and in developing flowers, and is required for the specification of leaf and flower architectures (Sauer et al. 2006). The involvement of *YUCCA* genes in auxin biosynthesis is also supported by the fact that over-expression of *YUCCA* genes in *Arabidopsis* induces the expression of auxin regulated marker genes and leads to typical auxin-overproducing phenotypes (Cheng et al. 2006; Sauer et al. 2006; Kim et al. 2007).

Since auxin regulates many central processes in plant growth and development, deficiency of auxin production could be lethal. Although several auxin-overproducing *Arabidopsis* mutants have been identified and characterized (Zhao et al. 2001; Cheng et al. 2006), auxin-deficient mutants have not been isolated. To safeguard auxin biosynthesis, plants have evolved a family of *YUCCA* genes (Cheng et al. 2006; Yamamoto et al. 2007). There are eleven *YUCCA* members in *Arabidopsis* (Cheng et al. 2006) and nine members in rice (Yamamoto et al. 2007). These genes have been shown to have partially duplicated functions, and mutation in one gene does not completely abolish the particular function (Cheng et al. 2006). To understand the roles of *YUCCA* genes in *Populus,* we identified 12 *Populus YUCCA* genes from the completely sequenced genome of *Populus trichocarpa*, genotype 'Nisqually-1' (Tuskan et al. 2006), and characterized them in terms of gene structure, motif structure, phylogenetic relationship with *Arabidopsis YUCCA* genes, as well as their expression patterns in young *Populus* plants. We also examined their expression in response to plant growth regulators and environmental stresses, and analyzed the *cis*-elements in their promoter sequences. The data from these experiments provide a foundation for future in-depth characterization of the roles of these genes in various growth and developmental processes in *Populus,* a model plant for woody plants.

Materials and methods

Identification and sequence analysis of *YUCCA* genes in *Populus*

PtYUCCA genes were identified by searching the *Populus* genome database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) for genes homologous to all eleven *Arabidopsis YUCCA* protein sequences (Cheng et al. 2006) with *e* values less than 1E-05 by the Blast algorithm. To analyze *cis*-elements in the promoters of the *Populus YUCCA* genes, 1,000 bp upstream regions of the start codon (ATG) were identified and searched for cis-elements using PLACE-Signal Scan Search program (http://www.dna.affrc.go.jp/PLACE/index.html; Higo et al. 1999).

Phylogenetic tree construction

Multiple sequence alignments were performed using the MUSCLE sequence alignment program (Edgar 2004; http://www.phylogeny.fr/phylo_cgi/muscle.cgi). The phylogenetic tree was constructed using PHYML with the protein model WAG by maximum likelihood analysis (Dereeper et al. 2008). Bootstrap analysis of 100 replicates was then performed, and the consensus tree was displayed with bootstrap values.

Gene structure and motif identification

Intron/exon structures were downloaded from the *Populus* genome database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). The chromosome locations of *PtYUCCA* genes were drawn using the Map Chart software (Voorrips 2002). Conserved protein motifs of the *PtYUCCA* genes were determined using MEME-MAST programs (http://meme.nbcr.net/meme/meme.html) with the motif length set as 6–200, and the *e* value to $<1 \times 10^{-10}$ (Bailey et al. 2006).

Quantitative real-time RT-PCR to determine gene expression in six tissues and in response to hormones and stresses

Tissue-specific expression was analyzed from six tissues of in vitro grown plants: shoot tip (first and second nodes), young (third node) and mature leaves (seventh and eighth nodes showing signs of senescence), bark, stem, and root. *P. trichocarpa* (Nisqually-1) plants were grown on Murashige and Skoog medium (Murashige and Skoog 1962), 3% sucrose, 0.3% activated charcoal (Fisher Scientific, NJ, USA), and 0.3% gelrite (PhytoTechnology Laboratories, USA) at 23–25°C. The photoperiod was 8 h dark and 16 h light with a light intensity of 125 μmol m^{-2} s^{-1} provided by fluorescent illumination.

To analyze gene expression in response to hormones, 4-week-old whole plants were removed from tissue culture and transferred to a solution containing deionized water and 1 μM of one of the following plant hormones: 6-Benzylaminopurine (6-BA), 1 μM indole-3-acetic acid (IAA), 1 μM salicylic acid (SA), 1 μM gibberellic acid (GA_3), 1 μM brassinosteroids (BR), 1 μM jasmonic acid (JA), and

1 μM abscisic acid (ABA) (Fisher Scientific, NJ, USA) for a duration of 8 h, as used by Yokoyama and Nishitani (Yokoyama and Nishitani 2001). The plants placed in deionized water were used as the control. For high salt, drought, and flood treatments, plants were removed from the media and hydroponically grown in a solution supplemented 250 mM NaCl, 15% (w/v) polyethylene glycol (PEG) 8000 (Fisher Scientific, NJ, USA), and deionized water for 16 h, respectively. For high and low temperature treatments, plants in jars were moved to a chamber at 40 and 0°C for 16 h, respectively. The plants without any treatments were used as the controls.

The total RNA was extracted by using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis MO, USA) and treated with AMPD1 DNase I (Sigma-Aldrich, St. Louis, MO, USA) to remove any traces of DNA. The oligonucleotide primers were designed based on the identified 3′ untranslated region (UTR) and the 3′ terminal sequences of the predicted coding region of the *PtYUCCA* genes 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 nucleotide and three other nucleotides were different from the 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 in an ABI 7000 Sequence Detection System (Perkin–Elmer Applied Biosystems, Foster City, CA, USA.) using a Power SYBR Green PCR Master Mix Kit (Applied Biosystems, Warrington WA1 4SR, UK). The primers used in this study are listed in Table 1. To validate the proper concentration of cDNA, four genes, actin2, UBQ, TUA, and 18S, found to be the most stably expressed reference genes out of 10 housekeeping genes in *Populus* (Brunner et al. 2004), were chosen as the reference genes, and geNORM software (Vandesompele et al. 2002) was employed to determine which reference gene(s) is best for normalization. In the preliminary experiment to select reference genes, the Actin2, UBQ and TUA genes were found to be the most stable, and a normalization factor was calculated based on these three reference genes (data not shown). Each experiment was repeated three times.

N_0 (the starting concentration of the target gene) and the efficiency of PCR for each individual sample were derived from the slope of the regression line fitted to a subset of baseline-corrected data points in the log-linear phase using LinRegPCR (Version 11.0, Ruijter et al. 2009, Ramakers et al. 2003). The normalized N_0 was the average N_0 of three replicates per biological sample divided by the normalization factor, and then N_0 of the target gene and N_0 of the control were used to calculate the relative expression ratio (Ruijter et al. 2009). The control for the tissue expression profile was the stem tissue; the controls for the hormonal and stress treatment were non-treated plants.

Table 1 Primer sequences used in real-time PCR

Name of primers	Sequences of primers (5′–3′)
*PtYUCCA*F-1	GGAACAGCCTCTGATGCTGTG
*PtYUCCA*R-1	ATGGGAATTGCAAGATTTATCGT
*PtYUCCA*F-2	GGAGCTTCAATGGATGCTAAAA
*PtYUCCA*R-2	GCCGCTTCTTCATTCCTACA
*PtYUCCA*F-4	CAAGAAGAGGCCTGTTAGGAACT
*PtYUCCA*R-4	TGATAACATGGGAATTACAAGATCTAATAC
*PtYUCCA*F-5	ATCATGGCTCAAGGAGGACA
*PtYUCCA*R-5	AAACCAACACTGTAAAGTCCATTCTT
*PtYUCCA*F-6&2	GTGCCATCTTGGCTAAAGGAAG
*PtYUCCA*R-6&2	CACTCCCCTTTCCATCCATTT
*PtYUCCA*F-7	AGCACTTGACATTGCCAAAAT
*PtYUCCA*R-7	CTGTGACGAGCTGCCACA
*PtYUCCA*F-7&3	TTGACATTGCCAAAATCTGGAA
*PtYUCCA*R-7&3	TCCTGTGACGAGCTGCCAC
*PtYUCCA*F-8	TGATGCCATTAGAATTGCACAA
*PtYUCCA*R-8	CAACGTCTATGGCAAGCTGTT
*PtYUCCA*F-9	TGGCTATAGAAGCACCGTACTGG
*PtYUCCA*R-9	CATTTCCCCCTTTCCAATGA
*PtYUCCA*F-10	GGAAGTG GAATGCCAAAACTCT
*PtYUCCA*R-10	CGTTCACTTCTCAATGCCAAGT
*PtYUCCA*F-11	TGGTCAACAAGTGGAAAGGATC
*PtYUCCA*R-11	AGCAGATTTGACAATTCAGAATCAG
*PtYUCCA*F-12	AAAGCCAGGTTACCCTAACCAT
*PtYUCCA*R-12	AATGAGTGCCTTGATATCGTTGA
Actin F	TTCTACAAGTGCTTTGATGGTGAGTTC
Actin R	CTATTCGATACATAGAAGATCAGAATGTTC
TUA-F	AGGTTCTGGTTTGGGGTCTT
TUA-R	TTGTCCAAAAGCACAGCAAC
UBQ-F	GTTGATTTTTGCTGGGAAGC
UBQ-R	GATCTTGGCCTTCACGTTGT
18S-F	AATTGTTGGTCTTCAACGAGGAA
18S-R	AAAGGGCAGGGACGTAGTCAA

Results

The *Populus YUCCA* gene family and phylogeny of *Populus* and *Arabidopsis YUCCA* genes

From the genome database, 12 open reading frames (ORFs) encoding putative *YUCCA* proteins were identified and all were included in this study (Table 2). Ten out of twelve are located in eight chromosomes and two others (*YUCCA*10 and 11) are in two scaffolds that have not been assigned to

Table 2 The list of 12 *YUCCA* genes in poplar *(Populus trichocarpa)* from the poplar genome database

Gene #	Chromosome localization linkage group: nucleotide position	Predicted size amino acid	Gene model
*PtYUCCA*1	Poptr1_1/LG_VI:16815965–16817800	411	eugene3.00061793
*PtYUCCA*2	Poptr1_1/LG_VI:16444857–16447520	436	eugene3.00061754
*PtYUCCA*3	Poptr1_1/LG_VIII:11784884–11786818	421	fgenesh4_pg.C_LG_VIII001540
*PtYUCCA*4	Poptr1_1/LG_XVIII:5094221–5096064	416	eugene3.00180377
*PtYUCCA*5	Poptr1_1/LG_VII:10556589–10558442	401	gw1.VII.3430.1
*PtYUCCA*6	Poptr1_1/LG_XVIII:4718788–4721576	404	gw1.XVIII.1357.1
*PtYUCCA*7	Poptr1_1/LG_X:5654664–5656576	422	eugene3.00100414
*PtYUCCA*8	Poptr1_1/LG_II:23490607–23492221	422	fgenesh4_pg.C_LG_II002544
*PtYUCCA*9	Poptr1_1/LG_V:12464501–12466435	381	fgenesh4_pg.C_LG_V001046
*PtYUCCA*10	Poptr1_1/scaffold_57:557671–561321	389	fgenesh4_pg.C_scaffold_57000067
*PtYUCCA*11	Poptr1_1/scaffold_137:150066–151492	383	fgenesh4_pg.C_scaffold_137000015
*PtYUCCA*12	Poptr1_1/LG_XIV:14587923–14589958	377	fgenesh4_pm.C_LG_XIV000597

http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

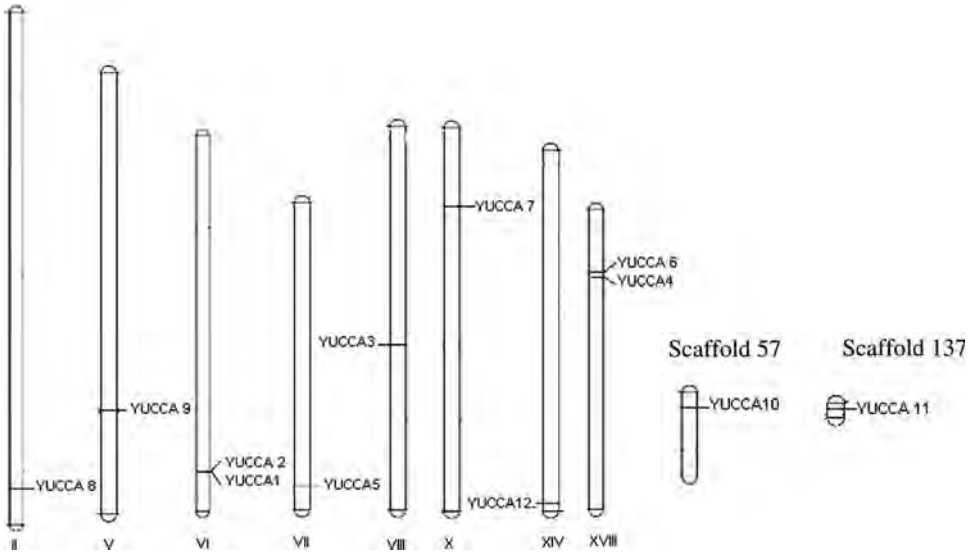

Fig. 1 Locations of 12 *Populus YUCCA* genes in the chromosomes of *P. trichocarpa*. The *number* under each *vertical bar* indicates the chromosome number

specific chromosomes (Fig. 1). Pairwise comparisons showed that the overall *YUCCA* protein sequence of *Arabidopsis* and *Populus* range in their similarities from 37 to 90%. The high homology matches suggest that all sequences identified belong to the *YUCCA* gene family (Fig. 2).

Based on the phylogenetic tree, the *YUCCA* genes in *Arabidopsis* and *Populus*, both being core eudicot species, can be divided into two large groups, one of which can be divided into two subgroups as indicated by circles (Fig. 3a). Based on their locations in the *Populus* genome, it is clear that the *YUCCA* gene family in *Populus* has expanded after genome-wide duplications (Tuskan et al. 2006). As in *Arabidopsis*, *PtYUCCA*1 and 4 are paralogs and 2 and 6 are paralogs. Since *PtYUCCA*1 and 2 are in chromosome 6 and *PtYUCCA*4 and 6 are in chromosome 18, it is likely that these paralogs were produced by whole-genome duplication (Tuskan et al. 2006).

The gene and conservative motif structures of *Populus* and *Arabidopsis YUCCA* proteins

Populus and *Arabidopsis YUCCA* genes share relatively few common features in gene structures in terms of exon numbers, locations, and intron lengths (Fig. 3b). Each of the three pairs of *PtYUCCA* paralogous genes (*PtYUCCA*1/4, 2/6 and 3/7) shared similar gene structures, while those pairs in *Arabidopsis* had respectively very different gene structures (Fig. 3b). Since both *Populus* and *Arabidopsis* are core eudicots, the substantial divergence of gene structures of these gene pairs between *Populus* and

Fig. 2 Predicted protein sequence alignment of 12 *Populus YUCCA* proteins using MUSCLE (3.6) multiple sequence alignment program

Arabidopsis might have originated before divergence of eurosid I and eurosid II. Interestingly, the *AtYUCCA*5 and 8 had no introns (Fig. 3b).

In contrast to the diverse gene structures, all *Populus* and *Arabidopsis YUCCA* proteins contained motifs 1–10 (Fig. 4). Furthermore, the subfamilies and paralogs shared similar motif structures (Fig. 4). *PtYUCCA*1 and 4, and *PtYUCCA*2 and 6 contained almost the same motifs with some variations at the carboxyl ends.

Tissue-specific expression

Since it has been shown that *YUCCA* gene family members in *Arabidopsis* and rice are regulated spatially and

Fig. 2 continued

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PtYUCCA5     EVLGRSTFSLSMWLLNW-FPVKLVDRFLLICSQLILGDTHKMGIRRPKMGPLEQKNSTGK
PtYUCCA8     EIMGKSTFELAVLLMSW-LPLWLVDKLMLIMAWLVLGNTDKYGLKRPSMGPLTLKNTMGK
PtYUCCA3     EILGRSTFELAVTMMKW-LPLWMVDKILLLFAWLILGNLEKYGLTRPCLGPLQLKNTQGK
PtYUCCA7     EVLGRSTFELAVTMMKW-LPLWMVDKVLLLLSRLILGNVEKYGLKRPCLGPLQLKDTQGK
PtYUCCA2     EMLGKSTFGLSMWLLKW-LPMRLVDRFLLIVSRLMLGDTARLGLDRPELGPLELKNLSGK
PtYUCCA6     EMLGQSTFGLSMWLLKW-LPVRLVDRFLLIVSRLMLGDTARFGLERPELGPLELKNLSGK
PtYUCCA1     EMFGMSTFGIAMALLKW-FPLRLVDKFLLLVANLILGNTEQLGLKRPKTGPIELKNVTGK
PtYUCCA4     EMFGMSTFGVAMALLKW-LPLRLVDKLLLLGANLTLGNTDQLGLKRPKTGPIELKNVTGK
PtYUCCA12    EMV-----YLGLNMLKY-FSCGMVDKVMVMLSKLVYGDLSKHGIKRPKEGPFFMKVAYGK
PtYUCCA11    EMV-----SLAMFLLKF-LSVTSVDKILAKLCKLRFDDLSEYGIQRPKEGPFYLKTTQGR
PtYUCCA10    NIV-----YIGMRLLSYGVPCNIVDFIVVLLSKLQHGDISNYGFPRPTRGPFYIKQRVGR
PtYUCCA9     EIV-----FLGMCFLKY-LPCRLVDFIATTLSKIKFGDTSKYGIQRPTEGPFYIKAKTGR
                 ::.      :.: ::.: ..   ** .    ..:  .:    *: **  **:  *   *.

PtYUCCA5     TPVLDVGAFSKIKSGKIKVVCGVQRFTASGAEFVDGHVENFDSVILATGYRSNVTSWLKE
PtYUCCA8     TPVLDIGALEKIRSGDINVVPGIKRFSRGQVELVNGEILDIDSVILATGYRSNVPYWLQE
PtYUCCA3     TPVLDIGALEKIRSGKIKVVPGIKRFSSGKAELVNGEILQIDSVILATGYRSNVPSWLKE
PtYUCCA7     TPVLDIGALEKIRSGKIKVVPGIKRFSSGKVELVNSEILEIDSVILATGYRSNVPSWLKE
PtYUCCA2     TPVLDVGTLAKIKSGDVKVCPGIKKLKRHTVEFLDGKMENFDAIILATGYKSNVPSWLKE
PtYUCCA6     TPVLDVGTLAKIKSGDIKICPEIKKLKRHTVEFIEGKTENFDAIILATGYKSNVPSWLKE
PtYUCCA1     TPVLDVGALSQIKSGKIKVMEGVKEVTKNGVKFMNGQEKKFESIILATGYKSNVPTWLKG
PtYUCCA4     TPVLDVGALSQIKSGKIKVMEGVKEITKNGVKFMDGQEREFESIILATGYKSNVPTWLKG
PtYUCCA12    YPVFDVGTCNKIKSGEIQVLPALESIRGNEVVFENGKSHPFDTIVFCTGFERSTNKWLKG
PtYUCCA11    SPTIDVGCVDRIKQGKIKVFPSMANIEGKKIEFMNGESNQFDVIIFATGYRSTVGRWLKG
PtYUCCA10    TPTIDVGAVEKIRRKEVQVFPSIRGIQGSKIEFANGEVKQFDAIIFATGYKSTVRHWLKG
PtYUCCA9     SPTIDVGAVQKIKTGEIQVFPSIANIEGTEITFENGKSKQYDAIIFATGYRSTVLDWLKD
                  *.:*:*   .*.  .:::   :  .      : :.     : :::.**:  ..  **:

PtYUCCA5     DNFFNEKDGYPRNPFPDNWKGKNGLYSVGFTRRGLLGSSIDAQRVAEDIARQ--------
PtYUCCA8     GEFF-SKNGFPKAPFPNGWKGNAGLYAVGFTRKGLSGASSDAIRIAQDIGKVWKEETKQT
PtYUCCA3     NEFF-SEDGIPKNQFPNGWKGNAGLYAVGFTKRGLSGASLDAISVAFDIAKSWKEETKQK
PtYUCCA7     NEFF-TEDGIPKNPFPNGWKGNAGLYAVGFTRRGLSGASLDAMSVALDIAKIWKEETKQK
PtYUCCA2     GDMF-EKDGFPKRPFPNGWRGECGLYAVGFTKRGILGASMDAKRIAEDIERYCRNEEAAP
PtYUCCA6     GDMFSEKDGFPRRPFPNGWKGECGLYAVGFTKRGILGASVDAIRIAEDIE----------
PtYUCCA1     CDFF-TKDGMPKTPFPNGWKGENGLYTVGFTRRGLLGTASDAVKIAQDIGDQWKTIKGN-
PtYUCCA4     CDFF-TKDGMPKTPFPKGWKGGNGLYTVGFTRRGLLGTASDAVKIAHDIAGEWKAAKDS-
PtYUCCA12    DDYLLNEDGIPKPGYPNHWKGKDGLYCIGLSRRGLYGASADAQNVVNDIKALI-------
PtYUCCA11    GEELFDDNGFPKQDLVNKWKGSNGLYCVGFARNGLLAISSDAKNVSQDISTLILNCQIC-
PtYUCCA10    GQDLFDGSGMPKLCFPNIWKGKNGLYCSGFARRGLFGISVDSQNIAKDIDLALRSERLTL
PtYUCCA9     GKDLFNENGMPKLRFPNHWKGGNGLYCAGFSRSGLMGISLDAQQIATDIGLAWKGASK--
                  . :   .* *.    . *.*  ***  *:. *: . : *:  :  **

PtYUCCA5     ----------------------------
PtYUCCA8     KRATAC-HRRCISQF-----------
PtYUCCA3     KITVAARHRRCISHF-----------
PtYUCCA7     KKTVAARHRRCISHF-----------
PtYUCCA2     YDH----HHRSVLLLKSSSSSSSPSP
PtYUCCA6     ----------------------------
PtYUCCA1     -------DKSCNSHVIIL----GKPW
PtYUCCA4     -------IRSCNSHVIIHLDHDGAK-
PtYUCCA12    ----------------------------
PtYUCCA11    ----------------------------
PtYUCCA10    YKIF----------------------
PtYUCCA9     ----------------------------
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temporally, we also examined the *PtYUCCA* expression in six different vegetative tissues. Because the primers for *YUCCA*6 failed to distinguish *YUCCA*2 and 6, and the primers for *YUCCA*3 failed to distinguish *YUCCA*3 and 7, the expression data for *YUCCA*6 was the sum of *YUCCA*2 and 6 and that for *YUCCA*3 was a sum of 3 and 7. In six tissues, there was no overall association between tissue-specific expression and phylogenetic subfamilies (Fig. 5). Compared with the expression in the stem (artificially serving as the control), *YUCCA*1 was slightly up-regulated in the shoot tip and bark, but the expression in young leaves, mature leaves, and root was more than two times of

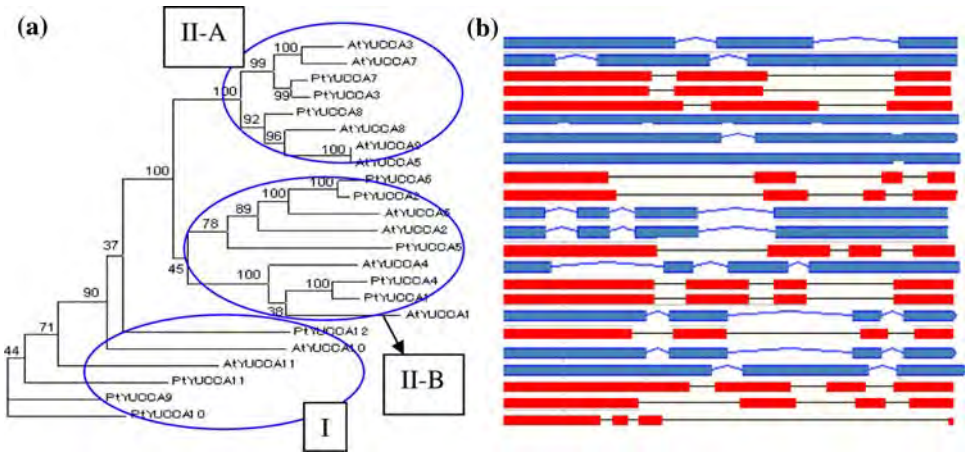

Fig. 3 Phylogenetic tree and gene structures of 12 *Populus* (Pt) and 11 *Arabidopsis* (At) *YUCCA* genes. **a** The phylogenetic tree of *PtYUCCA* and *AtYUCCA*. **b** Gene structures of *PtYUCCA* and *AtYUCCA*. Exons are shown as *open boxes* and introns as *lines*

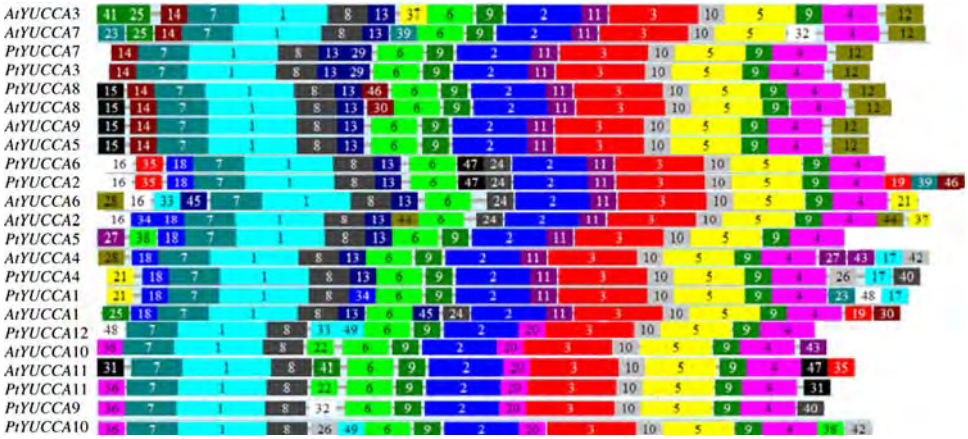

Fig. 4 Motif structures of the 12 *Populus* (Pt) and 11 *Arabidopsis* (At) *YUCCA* proteins. *Boxes* labeled with numbers are protein motifs

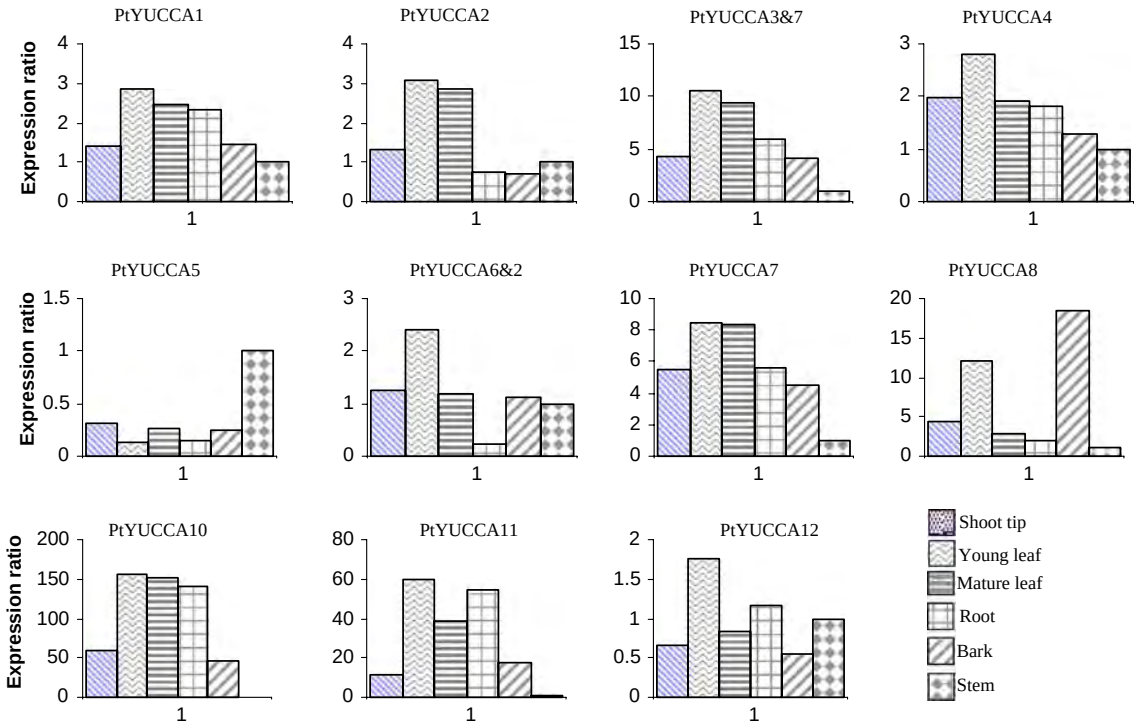

Fig. 5 Tissue-specific expression of 12 *Populus* YUCCA genes. The expression ratio is the starting concentration (N_0) of the individual gene in the respective organ to that of the stem

that in the stem. *YUCCA*2 was up-regulated in the young leaves and mature leaves, and expression in the shoot tip, root and bark tissues was similar with that in stem tissues. *YUCCA*7 and *YUCCA*3/7 were up-regulated in the shoot tip, young leaf, and bark tissues. *YUCCA*4 was up-regulated, while *YUCCA*5 was down-regulated in the other five vegetative tissues at various levels when compared with that in stem tissues (Fig. 5). *YUCCA*6 and/or 2 were expressed at a low level in roots, while higher in young leaves. *YUCCA*8 showed up-regulation in the shoot tip, young leaf, mature leaf, and root tissues, especially in bark tissues (more than 15 times than that in stem tissues; Fig. 5). The *PtYUCCA*9 mRNA was too low to be detected by the real-time PCR method. *YUCCA*10 and *YUCCA*11 were greatly up-regulated in the shoot tip, young and mature leaf, root, and bark tissues (Fig. 5). *YUCCA*12 was slightly up-regulated in the young leaf and root tissues, but down-regulated in the shoot tip, mature leaf, and bark tissues (Fig. 5).

YUCCA gene expression in response to hormonal and stress treatments

It is important to understand what controls the specific expression patterns of the *YUCCA* genes and how *YUCCA* genes are regulated by environmental and developmental signals (Zhao 2008). We examined expression patterns of *PtYUCCA* genes in response to various plant growth regulators. Figure 6 shows considerable variation in expression levels among the genes in response to the same hormone and among the same gene in response to different hormones. *PtYUCCA* genes were down-regulated by applications of all plant growth regulators except that expression of the *YUCCA*2, 7, 8, and 12 genes were induced by exogenous IAA. *YUCCA*12 expression was induced by GA, and *YUCCA*11 expression was induced by BR and BA, in comparison with the controls. Expression of *PtYUCCA*10 was drastically reduced with all hormonal treatments so that expression levels could not be detected using real-time RT-PCR. *Populus YUCCA* genes responded differentially to various stresses (Fig. 7). While most of these stresses repressed expression of *YUCCA* genes, there are several exceptions. *PtYUCCA*2 and *PtYUCCA*8 expression was induced by low temperature and salt treatments, *PtYUCCA*10 and 12 expression was elevated in the presence of salt, and *PtYUCCA*11 expression was induced by high temperature and salt treatments. Expression of *PtYUCCA*9 in response to hormone treatments was too low to detect by real-time PCR, while expression of *PtYUCCA*9 in response to stress treatments can be observed at low levels (data not shown).

Hormone- and stress-response factors in *PtYUCCA* promoters

To determine the possible cis-elements that are responsible for hormonal and environmental regulations of *PtYUCCA*

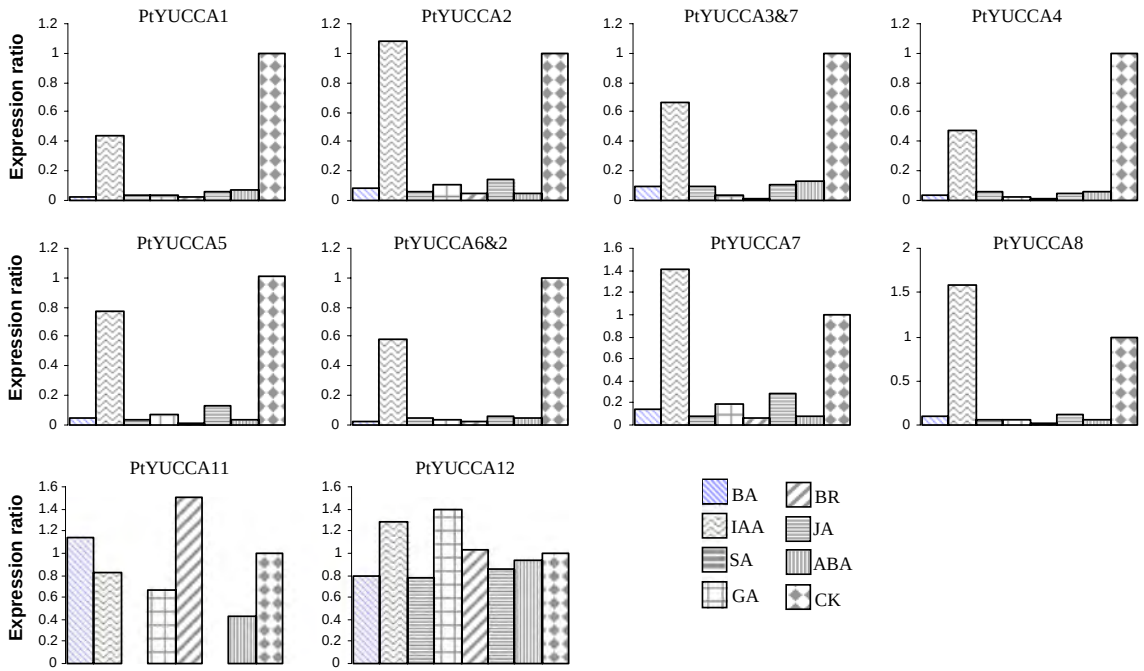

Fig. 6 *Populus YUCCA* gene expression in response to hormonal treatments. The expression ratio is the starting concentration (N_0) of the individual gene in the respective treatment to that of the control (no treatment)

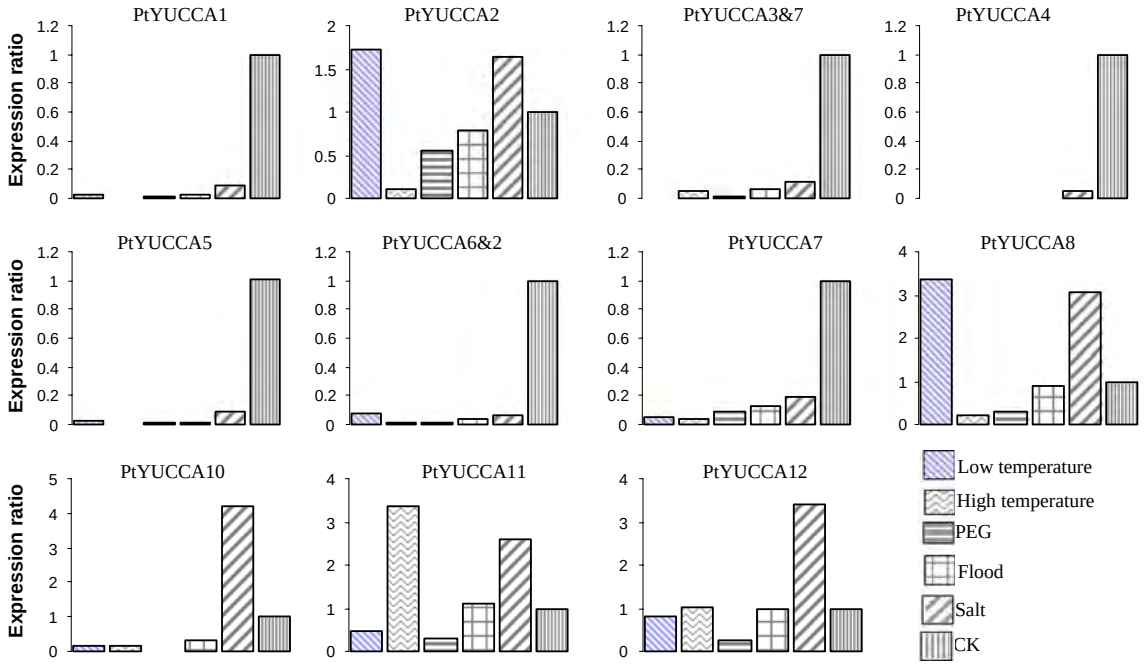

Fig. 7 *Populus* YUCCA gene expression in response to stress treatments. The expression ratio is the starting concentration (N_0) of the individual gene in the respective treatment to that of the control (no treatment)

gene expression, we analyzed the 1,000 bp upstream of the initiation codons. Table 3 shows the presence of various hormonal response elements in each member of *PtYUCCA*, arranged in the order of phylogenetic SFs. Many hormone-responsive elements were identified in the promoter regions of some of the *YUCCA* genes. For example an ABA responsive element, MYCCONSENSUSAT, ranged from 2 to 14 copies, a GA element, WRKY71OS, ranged from 2 to 7 copies, and an SA element, WBOXATNPR1, had up to 4 copies (Table 3). There was no correlation between number of hormone-response elements and level of expression, or between genes having an equal number of hormone-response elements and level of expression (Fig. 6; Table 3). For example, *YUCCA*2, 7, 8, and 12 were up-regulated in response to IAA treatments, while an auxin responsive element, CATATGGMSAUR, was found only in the *YUCCA*8 promoter sequence (Fig. 6; Table 3). There is also no particular association between the subfamilies and the presence of certain elements, except that four and three members of subfamily I contain the ABA-responsive element MYCATRD22 and the JA-responsive element T/GBOX ATPIN2 (Table 3), respectively. No specific element could be identified for *PtYUCCA*3 and 7, which were the only genes up-regulated in response to SA (Fig. 6; Table 3).

In the promoter sequences of the *PtYUCCA* genes, less profuse elements were identified from those responding to stresses than to hormones (Table 4). The most abundant element was salt-responding GT1GMSCAM4, ranging from 0 to 7 copies (Table 4). DREB (dehydration responsive element) element CBFHV was found only in members of subfamily I; and the LTRE (low-temperature response element) element LTRE1HVBLT49 was found only in members of subfamily II. As with hormone-induced expression and elements in the promoter regions, there is no obvious association or correlation of expression levels with the element abundance and the element types (Fig. 7; Table 4). For example, *PtYUCCA*2 and 8 were up-regulated in response to low temperature and salt (Fig. 7), but the elements in their promoter sequences were different (Table 4). The same was true for *PtYUCCA*11 and 12 in response to high temperature and salt (Fig. 7; Table 4).

Discussion

YUCCA genes in *Populus*

Auxin is one of the most important hormones involved in regulation of critical processes of plant growth and development (Davies 2004). Indole-3-acetic acid (IAA), the primary auxin in plants, can be synthesized from either tryptophan-dependent or tryptophan-independent pathways (Woodward and Bartel 2005; Cheng et al. 2006; Zhao 2008). Several tryptophan-dependent auxin biosynthetic enzymes have been discovered in *Arabidopsis*, including SUR1 (Boerjan et al. 1995), SUR2 (Delarue et al. 1998), cytochrome P450 CYP79B2/B3 (Hull et al. 2000; Zhao

Table 3 Hormone-responding elements in promoters of the *PtYUCCA* genes

SF	Gene #	ABA				GA					SA	IAA	JA
		MYCA-TRD22	PROXB BNNAPA	ABREAT-CONSENSUS	MYCCON-SENSUSAT	GARE2-OSREP1	CARE OSREP1	WRKY-71OS	GAREAT	MYBGAHV	WBOXA-TNPR1	CATAT GGMSAUR	T/GBOX ATPIN2
I	1				2			3			1		
	2				4			5			2		1
	3	1			10			4			1		
	4	1	1		6		1	3			2		
	5				10			5	2	1	1	4	1
	6				2			6			2		1
	7	1			14			5	1				
	8	1			6		3	6			4	4	
II	9				6			7			3	2	
	10			1	4	1		2					
	11				4			6	1	1	4		
	12				6	1		2					

Table 4 Stress-responding elements in promoters of the *PtYUCCA* genes

SF	Gene #	DREB					LTRE			Water		Salt
		CBFHV	DRECRTCOREAT	DRE2COREZMRAB17	ABRE LATERD1	MYB1AT	LTRE1HVBLT49	LTRE ATLTI78	LTRECORE ATCOR15	MYB2AT	MYCATERD1	GT1GM SCAM4
I	1	2				3						7
	2	2			1	4				1		1
	3									3	1	
	4									1	1	7
	5	1	1		1	1			1			2
	6	1	1	1	1	3		1	1	2		1
	7	1				1					1	2
	8	1	1	1		1			1		1	
II	9						1			3		3
	10				1	1	1			1		1
	11		1				1		1	1		4
	12						1			1		3

et al. 2002), and *YUCCA* flavin monooxygenases (Zhao et al. 2001). So far, the roles of CYP79B2/B3 and SUR1/SUR2 in auxin biosynthesis have only been analyzed in *Arabidopsis*. In contrast, the *YUCCA* flavin monooxygenases have been found to exist in all of the plant species whose genome has been sequenced or partially sequenced, including *Arabidopsis* (Zhao et al. 2001; Cheng et al. 2006), rice (Yamamoto et al. 2007), *Populus* (Table. 2), and *Vitis vinifera* (grapevine; our data not shown). These results suggest that the *YUCCA*-dependent auxin biosynthesis pathway is evolutionarily conserved, at least in angiosperms.

To safeguard the production and function of auxin, plants have evolved a family of *YUCCA* genes in their genomes. For example, 11 *YUCCA* genes in *Arabidopsis* (Cheng et al. 2006) and 7 members in rice (Yamamoto et al. 2007) have been identified thus far. We also identified 12 *YUCCA* genes in the recently sequenced *Populus* genome (Tuskan et al. 2006). These *Populus YUCCA* genes share high sequence homology (Fig. 2) and similar motif structures (Fig. 3b), as well as orthologous pairs (Fig. 3a) with the *Arabidopsis YUCCA* genes, probably because both of them are core eudicot species. Variations in gene and motif structures suggest gene divergence after eurosids I and II split. For example, the ancestral orthologous pair of *AtYUCCA*3/7 and *PtYUCCA*3/7 might have duplicated before the split, duplicated again after split, and became extant *AtYUCCA*3 and 7, and *PtYUCCA*3 and 7, respectively. The duplicated genes then diverged after speciation, as witnessed by the different gene structures. Similarly, this is also found with the ancestral paralogous pairs of *AtYUCCA*2/6 and *PtYUCCA*2/6, and of *AtYUCCA*1/4 and *PtYUCCA*1/4. The more similar gene structures of the *Populus* paralogs, *PtYUCCA*2/6, *PtYUCCA*1/4, and *PtYUCCA*3/7, suggest that the duplications in the *Populus* genome may have occurred much more recently or were more conserved.

PtYUCCA genes display distinct expression patterns

One of the important questions regarding auxin-regulated developmental or growth events is how spatio-temporal patterns of auxin biosynthesis are controlled. The recent analyses of *YUCCA* gene expression in *Arabidopsis* (Cheng et al. 2006; Kim et al. 2007; Cheng et al. 2007a, b), rice (Woo et al. 2007; Yamamoto et al. 2007), and petunia (Tobena-Santamaria et al. 2002) clearly demonstrate that each *AtYUCCA* gene is expressed temporally and spatially during plant growth and development, and this expression is important in mediating plant growth and developmental processes (Cheng et al. 2006; Zhao 2008). For example, in *Arabidopsis*, *YUCCA4* is mainly expressed in small regions of the shoot apex and cotyledon tips in the mature embryo, and *YUCCA1* showed strong expression in the floral meristems and at the base of the floral organs (Cheng et al. 2006). *YUCCA1* is also expressed in discrete groups of cells in both stamens and carpels at later stages of flower development, but expression of *YUCCA1* is completely shut down in the fully matured flowers, and was not observed in mature leaves (Cheng et al. 2006). *AtYUCCA2* promoter activity was observed in young leaf primordia and mature leaves. *AtYUCCA6* promoter activity was mainly in stamens and pollen, not in seedlings (Cheng et al. 2006).

Because the original *Populus* genotype for genome sequencing no longer exists and all plant materials currently available are clonally propagated from the original tree and have not reached reproductive stage, we were unable to determine the expression of *PtYUCCA* genes in reproductive tissues of *Populus*. Since much of the expression data in *Arabidopsis* were in reproductive tissues, it is difficult to compare directly between *Populus* and *Arabidopsis* orthologous pairs. Nevertheless, in cultured young *Populus* plants the *PtYUCCA* genes are expressed in a tissue-specific manner in six vegetative tissues (Fig. 5). This suggests that the *YUCCA* genes are also expressed temporally and spatially during *Populus* growth and development, as in *Arabidopsis*. Controlling temporal and spatial auxin biosynthesis by a range of differentially regulated *YUCCA* genes may provide a way to regulate auxin levels in specific tissues and organs throughout the plant life cycle.

PtYUCCA gene expression in response to hormones and environmental cues

Since auxin, GA, BR and ABA are involved in many processes of plant growth and development, much evidence has been accumulated to support that these hormones regulate plant growth and development in a network (Weiss and Ori 2007; McSteen and Zhao 2008). Such crosstalk has been investigated in several cases between two different hormones. For example, the activities of GA and auxin overlap with respect to the regulation of cell expansion and organ differentiation (Weiss and Ori 2007). A connection between auxin and GA metabolism genes and auxin regulation of GA metabolism genes has been shown to be organ specific (Frigerio et al. 2006). BR has also been found to interact with auxin in promoting lateral root development in *Arabidopsis* (Bao et al. 2004). However, it is still unclear how *YUCCA* genes are regulated by environmental and developmental signals (Zhao 2008). Our results show that the *Populus YUCCA* genes are modulated by other plant growth hormones (Fig. 6) and environmental cues (Fig. 7). It is interesting to note that the expression of *Populus YUCCA* genes is down-regulated in most of the hormone or stress treated plant tissues,

suggesting that negative regulatory mechanisms might exist. However, a thorough study is needed to draw any conclusion. Although the hormone and stress response cis elements are present in the *YUCCA* gene promoter sequences, it is difficult to make direct connections between these elements and the expression patterns in response to a specific hormone or environmental cue. Again, further study, such as using expression of *YUCCA* promoter–reporter fusion genes and an auxin marker gene, and the in situ hybridization technique to confirm expression patterns of the *YUCCA* genes in hormone or stress factor-treated *Populus* plants, is needed.

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