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Characterization and expression analysis under bending and other abiotic factors of *PtaZFP2*, a poplar gene encoding a Cys2/His2 zinc finger protein

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Summary In plants, mechanoperception and transduction of mechanical signals have been studied essentially in *Arabidopsis thaliana* L. and *Lycopersicon esculentum* L. plants, i.e., in nonwoody plants. Here, we have described the isolation of both the full-length cDNA and the regulatory region of *PtaZFP2*, encoding a member of Cys2/His2 zinc finger protein (ZFP) family in *Populus tremula* L. × *Populus alba* L. Time course analysis of expression demonstrated that *PtaZFP2* mRNA accumulated as early as 5 min in response to a controlled stem bending and is restricted to the organ where the mechanical stimulus is applied. The real-time quantitative RT-PCR experiments showed that *PtaZFP2* was also rapidly up-regulated in poplar stems in response to gravitropism suggesting that *PtaZFP2* is induced by different mechanical signals. Abundance of *PtaZFP2* transcripts also increased highly in response to wounding and to a weaker extent to salt treatment and cold, which is consistent with the numerous putative cis-elements found in its regulatory region. As in other species, these data suggest that Cys2/His2 ZFPs could function in poplar as key transcriptional regulators in the acclimation response to different environmental factors.

Keywords: C2H2 zinc finger putative transcription factor, environmental stresses, gravitropism, mechanical stress, *Populus*.

Introduction

Plants undergo continuous exposure to biotic and abiotic stresses in their natural environment (Fujita et al. 2006). Many abiotic stresses induce morphogenetic responses that comprise three components: (i) modification of cell

elongation, (ii) localized variation of cell division and (iii) alterations in cell differentiation status. The stress-induced morphogenetic response is postulated to be part of a general acclimation strategy whereby plant growth is redirected to diminish stress exposure (Potters et al. 2007). In the case of external loads such as wind or snow (Telewski 2006), the stress-induced morphogenetic response is called thigmomorphogenesis (Boyer 1967, Jaffe 1973). This includes reductions in height increment (Henry and Thomas 2002, Anten et al. 2005), dry mass increment (Niklas 1998) and seed production (Niklas 1998), and increases in diameter growth in stems (Boyer et al. 1986), root allocation (Crook and Ennos 1996, Coutand et al. 2008) and root growth (Jaffe and Forbes 1993). However, the nature and the extent of the responses vary between species and environmental conditions. When plants, mainly woody plants, are exposed to mechanical stimuli, generated by internal self-loading induced by growth or during fruit bearing, the morphogenetic response consisted of stem reorientation or orientation keeping in view the production of reaction wood (Almeras et al. 2004, Telewski 2006, Coutand et al. 2007).

The signal transduction pathway leading to thigmomorphogenesis is not clearly understood, although: (i) a unified hypothesis of mechanoperception in plants has been proposed (Telewski 2006) and (ii) the role of calcium as the second messenger in mechanical signal transduction is generally admitted (Knight et al. 1992, Depege et al. 1997). An increase in the intracellular calcium concentration is observed several minutes after wind treatment in *Arabidopsis thaliana* L. plants (Knight et al. 1992) and several calmodulin-like (TCH2 and TCH3) proteins are involved in mechanical response in *Arabidopsis* (for review, see Braam 2005). Moreover, a lot of reports support that mechanical stress responses in plants involved the reactive oxygen species signaling pathway (Depege et al. 1998, Apel

and Hirt 2004) and are hormonally mediated (Erner and Jaffe 1982, Boyer et al. 1983, Biro and Jaffe 1984). The plant hormone ethylene has received more attention because similar modifications of growth have been shown to result from either touch or exogenous applications of ethylene (Mitchell 1996). Nevertheless, the nature of ethylene's involvement in touch-induced responses remains unclear (Johnson et al. 1998, Chotikacharoensuk et al. 2006).

Recent studies have encouraged the identification of the common molecular strategy involved in the stress signaling networks, and among the several candidate molecules, the transcription factors are the promising candidates (Fujita et al. 2006). Numerous transcription factors involved in the regulation of many physiologic processes have been identified in plants and are classified according to their DNA-binding domains (Montiel et al. 2004). Transcription factors such as MADS-box, homeodomain, zinc finger (ZF), basic Leu-zipper, basic helix-loop-helix, MYB and MYC proteins have been found in both animals and plants (Riechmann et al. 2000). Among these transcription factors, Cys2/His2 (C2H2) zinc finger proteins (ZFPs) in plants are involved not only in DNA binding but also in RNA binding or protein-protein interactions (Gamsjaeger et al. 2007). They are involved in a wide range of developmental processes such as floral organogenesis, leaf initiation, lateral shoot initiation, gametogenesis and stress response (Takatsuji 1998, Englbrecht et al. 2004). A gene family of C2H2-type two zinc finger proteins (ZFP2) has been identified in *Petunia hybrida* L. (Kubo et al. 1998). Using systematic analysis of plant genomes, 176 proteins that contain one or more ZFs were described in *A. thaliana* (Englbrecht et al. 2004), and 189 members in *Oryza sativa* L. (Agarwal et al. 2007). The first ZFP2 protein was found in *Petunia* and named ZPT2-1. Takatsuji et al. (1992) had showed that ZPT2-1 interacts with the promoter region of the 5-enolpyruvylshikimate-3-phosphate synthase gene. More recently, Sakamoto et al. (2004) had reported that four *Arabidopsis* ZPT2-related proteins (AZF1, AZF2, AZF3 and STZ) were localized in nuclei and bound to DNA in a sequence-specific manner (A(G/C)T repeat sequences). These four ZPT2-related proteins were shown to act as transcriptional repressors that down-regulate other transcription factors. Among the four genes, *AZF2* and *STZ* showed strong induction by dehydration, high salt, cold stress and abscisic acid (ABA) treatment.

We have recently isolated a ZFP gene from *Juglans regia* L. (*JrZFP2*) encoding a Cys2/His2-type ZFP2 (Leblanc-Fournier et al. 2008). In this study, RT-PCR analysis indicated that *JrZFP2* mRNA accumulation is rapidly and transiently induced in response to the controlled stem bending. The aim of the present study was to isolate the homolog of *JrZFP2* in *Populus tremula* L. × *Populus alba* L. and to further clone the promoter to identify potential regulators of the gene. The expression of *PtaZFP2* was

studied in response to several abiotic factors (bending, cold, salt, wounding and gravity) to check its involvement in crosstalk between stress-signaling pathways. In *Arabidopsis*, 50% of touch-induced genes are also regulated by darkness (Lee et al. 2005). Therefore, we also tested the effect of darkness on *PtaZFP2* mRNA accumulation.

Materials and methods

Plant material and culture conditions

Hybrid poplars (*P. tremula* × *P. alba*, clone INRA 717-1B4) were multiplied clonally in vitro on Murashige and Skoog medium (Murashige and Skoog 1962). When they reached a height of about 4 cm, poplar micropropagated shoots were gradually acclimatized on hydroponic solution (Morizet and Mingeau 1976). Then, they were kept in a controlled environment room: 16 h daylight, at 24 °C and 40 μmol m⁻² s⁻¹ (day) and 20 °C (night) with 70 ± 10% relative humidity. Experiments were done on poplars that reached a height of about 35 ± 4 cm with 20 internodes. Four days before each treatment, poplars were transferred on a new device in which they were attached a few centimeters above the collar, and some basal leaves were removed.

Environmental stress treatments

Mechanical stimulation was performed by rolling the stem basal part (10 cm above the collar) on a plastic tube of 32 mm diameter. The bent portion of the stem (3 cm long and of 4 ± 0.5 mm diameter) was collected at different times (5, 10, 30 min and 1 h) after the mechanical stimulation for time course expression analysis. For *PtaZFP2* expression analysis in different poplar organs after stem bending, six types of organs were collected 1 h after stimulation, quickly frozen in liquid nitrogen, and stored at -80 °C until analysis. Expression was studied on roots, basal leaves (2 cm above the bent part of the stem), apical leaves (below the apex), basal internodes (10 cm above the collar), apical internodes (1 cm below the apex), and apex of control and bent plants.

For gravitropic studies, poplars were artificially inclined at 35° from the vertical axis for 10, 20, 30, 45 min, 1, 3 or 6 h. The five internodes above the collar were then harvested from tilted and non-tilted plants, frozen in liquid nitrogen, and stored at -80 °C until RNA extraction.

In salt stress experiments, plants were treated with 200 mM NaCl. RNAs were extracted from poplar roots, basal internodes, basal leaves and apex 30 or 75 min after NaCl addition. To assess the effects of cold and dark stresses, plants were incubated at 4 °C in the light or at 24 °C in the dark, respectively, for 1 h and then the stem portions were removed and frozen in liquid nitrogen. In wounding experiments, poplar leaves were slashed with a razor blade and then incubated on a wet paper for 1 h.

RNA isolation and cDNA synthesis

Total RNAs were extracted from about 150 mg of bent stems using CTAB extraction buffer as described by Chang et al. (1993) and then treated with RNase-free RQ1 DNase (Promega, Charbonnières-les-Bains, France). RNA was quantified spectrophotometrically and checked out by agarose gel electrophoresis. First-strand cDNA was synthesized from 1 µg total RNA using oligodT and SuperScript III (Invitrogen, Cergy Pontoise, France) following the protocol of the supplier.

cDNA cloning and promoter isolation

For cDNA cloning, two degenerated primers 5'-TTTCA (A/G)GC(A/G/T/C)TT(A/G)GG(A/G/T/C)GGTCA-3' and 5'-AGTGG(A/G/T/C)GT(A/G/T/C)AA(A/G)TT (A/G/T/C)AAATC-3' were designed in two Cys2/His2 ZF conserved regions. A 332 bp fragment was obtained after PCR amplification from bent stem cDNA. Using this sequence, two antisense-specific primers were designed: PZF1 5'-CAAGTTCCCAGTTGATTCCATGAGTTTTG G-3' and PZF2 5'-TTGGCTTTGAAGCGAATTAG GCAGCTTC-3' to clone *PtaZFP2* promoter sequence. Promoter isolation was performed using Universal Genome Walker kit (Clontech, St-Germain-en-Laye, France) on DNA that was isolated from poplar leaves using a CTAB technical extraction (Ziegenhagen et al. 1993). The products were then cloned in pGEM-T easy vector according to the manufacturer's conditions (pGEM-T easy vector system II, Promega) and sequenced by MilleGen company (Labège, France). The cloning sequence was submitted to PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE/>). This allowed us to reach 5'-untranslated region (UTR) of *PtaZFP2* cDNA also. The end of the coding sequence in 3' was obtained by RT-PCR using the specific primer PZF3 5'-ATGAAGA-GAGATAGAGAACAG-3' and an oligodT.

Northern blot hybridization

For Northern blot analysis, a *PtaZFP2* gene-specific probe of 287 bp was generated by PCR with a specific pair of primers Pe1S 5'-CGTGCGAGTCACAA GAAACC-3' and Pe1AS 5'-CACAGAACTCTCTTG CTGCT-3', labeled with α^{32} P-dCTP using Ready-To-Go DNA labeling Beads kit (Amersham, Orsay, France) and then purified with Cleanmix kit (Euromedex, Mundolsheim, France) according to the manufacturer's recommendations. Total RNAs (15 µg) were separated by 1% agarose gel electrophoresis after denaturation with formaldehyde and blotted onto a nylon membrane Hybond-N + (Amersham). The Northern hybridization was carried out with NorthernMax kit (Ambion, Huntingdon, UK). Membranes were then exposed to a phosphoimager screen (Kodak, Rochester, USA) for 4 h. Signal quantification was made using Quantify One software (Bio-Rad, Marnes-la-Coquette, France). Student's *t* test was used to determine statistical significance.

Real-time quantitative RT-PCR experiments

The real-time quantitative RT-PCR amplifications were done using an iCycler IQ (Bio-Rad) and using SYBR green as a fluorescent dye. Each PCR reaction (30 µl) contained the following: cDNA (4 µl of 1:40 dilution of the first cDNA strands), PCR buffer (1X), MgCl₂ (1.66 mM), dNTP mix (200 µM of each), primers (0.3 µM of each), Platinum Taq DNA polymerase (0.5 unit, Invitrogen) and SYBR green I (1/1000, Sigma, St Quentin Fallavier, France).

After a heat step at 94 °C for 5 min, PCR cycling conditions were 40 cycles of denaturation (94 °C, 15 s), annealing (61 °C, 15 s) and elongation (72 °C, 20 s), ending by a final elongation step at 72 °C for 5 min.

PtaZFP2 transcripts were detected by amplifying 287 bp with primers Pe1S and Pe1AS. The reference gene *EF-1α* (Elongation factor-1α) transcripts were amplified using the primers EF1S 5'-GACAACACTAGGTACTACTGC ACTGTC-3' and EF1AS 5'-TTGGTGGACCTCTCGAT CATG-3'. Relative quantitative abundance (Qr) of *PtaZFP2* transcripts was calculated by comparison to the expression of *EF-1α* using the delta-delta method mathematical model (McMaugh and Lyon 2003)

$$Qr = 2^{(C_{\text{control}} - C_{\text{treated}})_{\text{target}}} / 2^{(C_{\text{control}} - C_{\text{treated}})_{\text{reference}}}$$

Here, C is the cycle number of PCR. Specificity of amplification was confirmed by determining the melt curves for the PCR products at the end of each run and by using a gel electrophoresis. The real-time PCR amplifications were done in at least three independent experiments, and every run was carried out in triplicate. Student's *t* test was used to determine statistical significance.

RT-PCR analysis

To study the time course accumulation of *PtaACS*, *PtaTCH2* and *PtaZFP2* transcripts, PCR amplification was performed on 4 µl of 1:40 dilution of the first-strand cDNA. *PtaACS* is the ortholog of *Arabidopsis ACS6* gene encoding ACC synthase (Arteca and Artica 1999). For *PtaACS* time course expression study, specific primers PtaACSS 5'-AAGCCTCCATTTGCANTCCTG-3' and PtaACSAS 5'-TTCAATATCTTCCTGTAGTAT-3' were used. Amplification consisted of 30 cycles with the following conditions: 94 °C, 30 s; 50 °C, 30 s; and 72 °C, 30 s. *PtaTCH2* is the ortholog of *Arabidopsis TCH2* gene (Braum and Davis 1990). Specific primers PtaTCH2F 5'-TGAT CAAGATGGTGTGATGGTAATG-3' and PtaTCH2R 5'-CGCAAAAACATCAATGGAAA-3' were used for *PtaTCH2* time course expression study. Amplification was made with an annealing temperature of 60 °C, during 26 cycles. The same cDNA reactions were used to amplify *PtaZFP2* and the control *EF-1α* with the specific primers described above. These amplifications were made with an annealing temperature of 61 °C, during 28 cycles for

PtaZFP2 and 21 cycles for *EF-1 α* . The PCR products were separated on a 2% agarose gel.

Results

Isolation and sequence analysis of *PtaZFP2*, a poplar ZFP encoding gene

To identify a C2H2-type ZFP2 encoding a gene homologous to *JrZFP2* which is involved in mechanosensing in *J. regia* (Leblanc-Fournier et al. 2008), we designed the

degenerated primers corresponding to the two conserved regions of different C2H2-type ZFP2. These primers allowed us to amplify a partial cDNA highly accumulated in poplar bent stems. After sequencing, specific primers were used to obtain the full-length cDNA, named *PtaZFP2* (Accession No. FM172949). Sequence analysis showed that this cDNA is 801 bp long from the ATG and contains a 3'-untranslated sequence of 210 bp including the poly A tail. It encodes a polypeptide of 197 amino acids (Figure 1A). Analysis of the deduced amino acid sequence indicated that this protein contained two canonical Cys2/

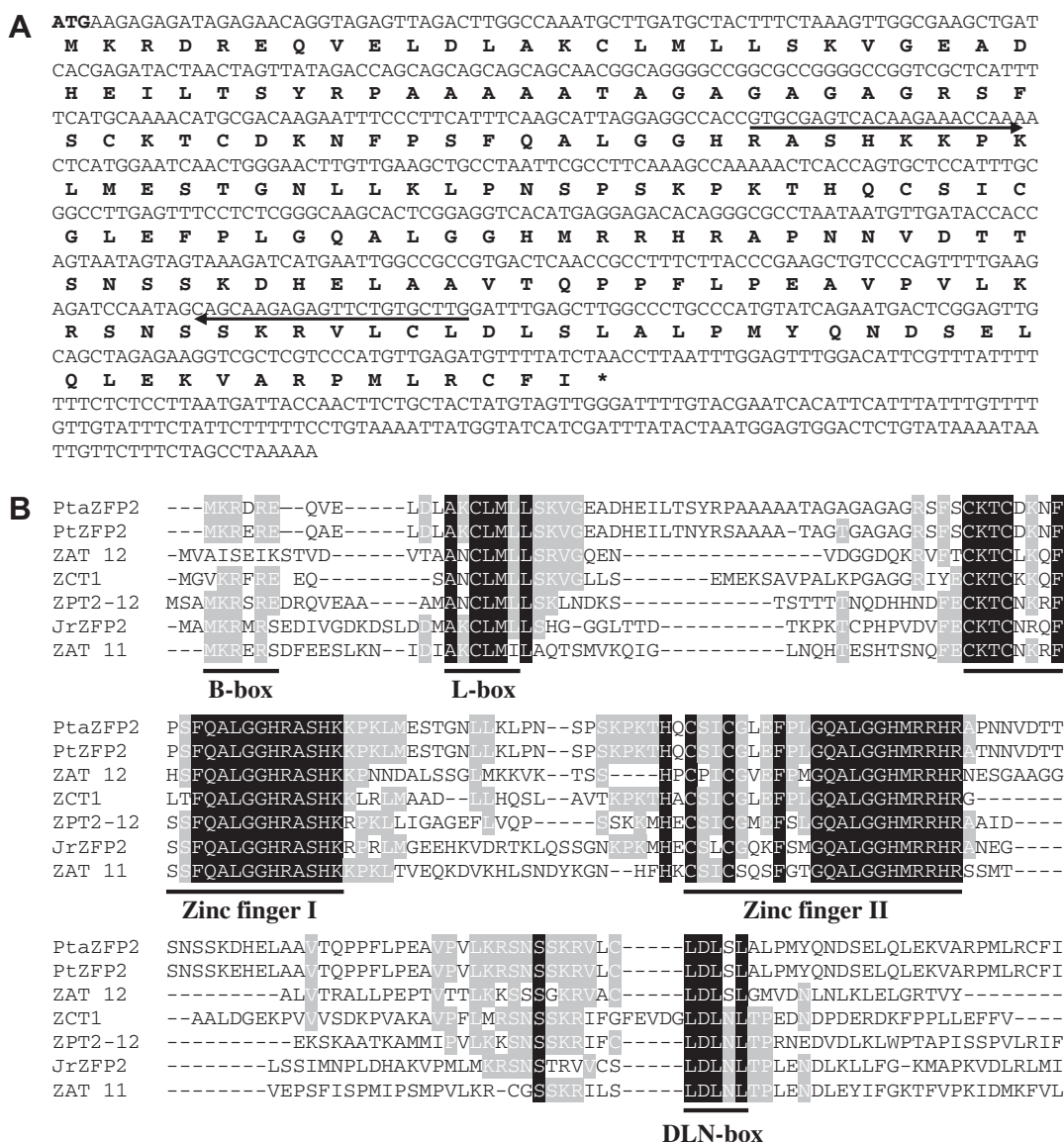


Figure 1. (A) Nucleotide sequence of *PtaZFP2* and its deduced amino acid sequence. Primers used for quantitative PCR are indicated by arrows. Codon stop is shown by an asterisk. (B) Comparison of the deduced amino acid sequence of the *P. tremula* \times *P. alba* ZFP PtaZFP2 with ZFPs from *P. trichocarpa* (PtZFP2, corresponding to the model gene estExt_Genewise1_v1.C_LG_I1393), *C. roseus* (ZCT1, Accession No. AJ632082), *P. hybrida* (ZPT2-12, Accession No. AB006599), *J. regia* (JrZFP2, Accession No. AJ937310), and *A. thaliana* (ZAT11, Accession No. NM129298 and ZAT12, Accession No. NM125374). Residues conserved among all seven sequences or at least four of seven are highlighted in black or gray, respectively. The two zinc finger motifs, partial B-box, L-box and DLN-box are underlined.

His2 ZF motifs of 21 amino acids separated by 26 AA. The two ZF motifs contain the QALGGH motif, a highly conserved domain localized in the N-terminal region of the recognition helix for DNA binding in C2H2 proteins.

Comparisons of the amino acid sequences between *PtaZFP2* and C2H2 ZFPs of other plants are presented in Figure 1B. The putative protein encoded by the *PtaZFP2* gene shares 91% identity with a deduced protein *PtZFP2* (corresponding to the model gene estExt_Gene-wise1_v1.C_LG_I1393) from *P. trichocarpa* JGI database. Compared with C2H2 ZFPs of other species, *PtaZFP2* had 46% identity with *Catharanthus roseus* ZCT1 (Pauw et al. 2004), 45% identity with *Petunia* ZPT2-12 (Kubo et al. 1998), 43% identity with *J. regia* JrZFP2 (Leblanc-Fournier et al. 2008), and 44% and 41% identity with ZAT12 and ZAT11 from *A. thaliana*, respectively (Meissner and Michael 1997). ClustalW alignment analysis of putative amino acid sequences revealed that these proteins shared the two zinc finger motifs. Apart from these two well-conserved motifs, there are three other more or less complete

regions: a partial putative nuclear localization signal (B-box), a hydrophobic leucine-rich region (L-box) probably involved in protein-protein interaction, and a C-terminal region DLN-box. In *PtaZFP2*, the B-box and DLN-box are not entirely conserved.

Isolation and sequence analysis of PtaZFP2 promoter

To identify putative cis-elements that are responsible for *PtaZFP2* regulation, a 1500 bp region upstream of the start codon of the *PtaZFP2* gene was isolated by a genome walking strategy on *P. tremula* × *P. alba* genomic DNA. As shown in Figure 2, a PLACE Web Signal Scan analysis (Higo et al. 1999) revealed regulatory motifs common to other eukaryotic promoters.

In the first 1000 bp region of the *PtaZFP2* promoter, three GARE elements (TAACAA/GA-box) and two Pyrimidine boxes were identified, cis-elements related to gibberellin response in the case of the α -amylase encoding gene promoters (Jacobsen et al. 1995). We also detected

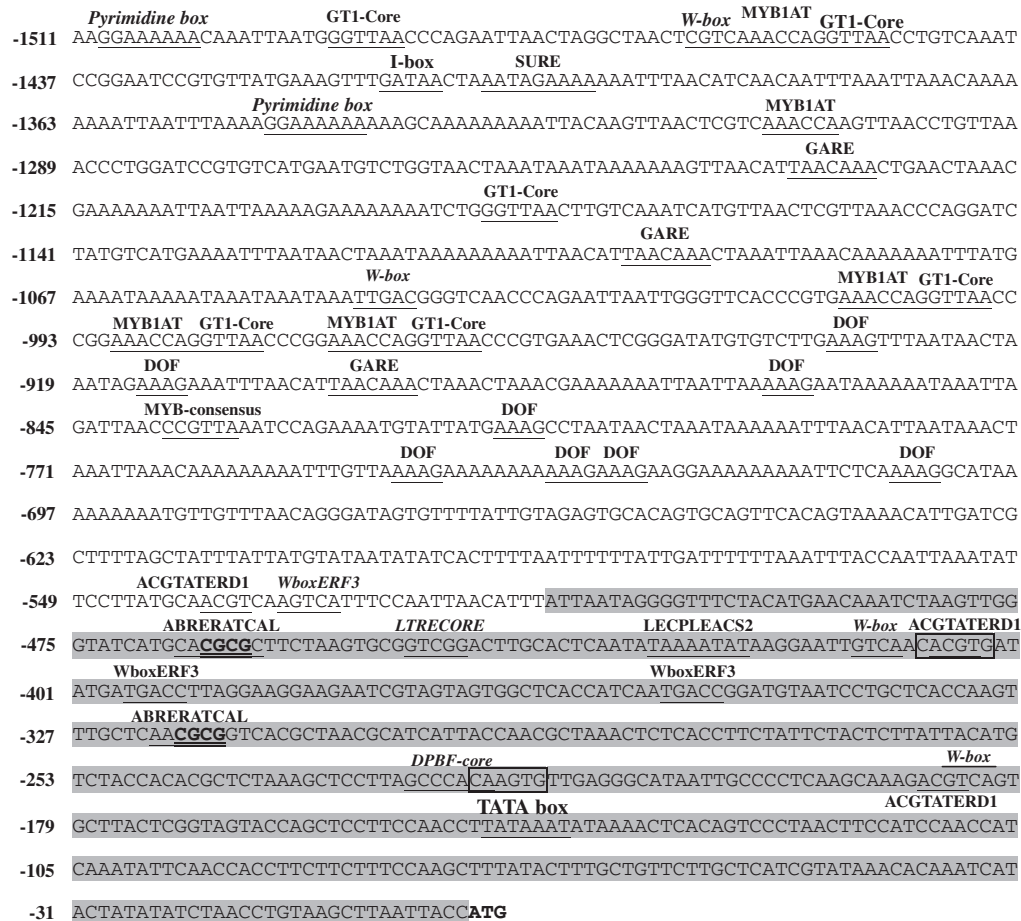


Figure 2. Nucleotide sequence of the 5'-flanking region containing the promoter and putative cis-acting elements of the *PtaZFP2* gene. The gray-colored sequence corresponds to the region showing high homology with the regulatory sequence of a homologous gene in *P. trichocarpa* genome. Motifs that are homologous to known regulatory cis-elements are underlined and their names are written in bold or in italic, when the motif was found in sense or antisense orientation, respectively. MYC-consensus motifs are framed, and sequences representing the GCGC-box are in bold. The numbering refers to distance relative to the ATG codon (+1).

eight DOF motifs ((A/T)AAAG) corresponding to the recognition core of Dof proteins (DNA binding with one finger), regulating the expression of plant genes in response to light, developmental stage and hormone treatment such as gibberellin (Washio 2001, Yanagisawa 2002). Another particular region corresponds to the three consecutive repetitions of MYB1(AT) and GT1 sites. The GT1-element was first identified as a light up-regulated element (Zhou 1999) but has also been found in the promoter regions of other genes such as the defence-related *PR-1a* tobacco gene where it may function as a repressor of gene expression (Buchel et al. 1999). MYB1(AT) sites are MYB recognition sites found in the promoters of the dehydration-responsive gene *rd22b* and many other genes in *Arabidopsis* (Abe et al. 1997). Nagano (2000) described that the important residues in the DNA-binding of GT1 factors and MYB proteins are well conserved. These results suggested that GT-factors arose in evolution from MYB proteins and could explain the vicinity of their DNA recognition site.

Blast analysis showed that the 500 bp region immediately upstream of the ATG codon shared more than 90% of homology with the upstream region of the model gene *estExt_Genewise1_v1.C_LG_I1393* of *P. trichocarpa* genome (data obtained from JGI database), whereas no significant similarity was found for the more distal part of this sequence. This 500 bp sequence immediately upstream of the ATG codon of *PtaZFP2* contains several cis-elements implicated in response to abiotic stress. The three ACG-TERD1 motifs detected in positions -539, -409 and -187 bp were described in the up-regulation of *erdl* gene in *Arabidopsis* by dehydration stress and dark-induced senescence (Simpson et al. 2003). At position -409 bp, this motif matches at the same position with an E-box element (CANNTG) and a MYC-consensus (AT) motif. This MYC-consensus site is the binding site of AtMYC2, a protein involved in cold responsiveness (Chinnusamy et al. 2003). Another MYC-binding site is detected at position -222 bp; this site is partially overlapping with a DPBF-core motif shown to be the binding core of bZip transcription factors whose expression is normally embryo-specific and can be also induced by ABA (Kim et al. 1997). The LTRECORE motif detected at position -449 bp in anti-sense orientation is the core of low temperature responsive element found in the *cor15a* gene (Baker et al. 1994).

The several W-box and WBOXNTERF3 elements are important motifs in response to wounding and pathogen response and show specific interaction with the transcription factors WRKYs (Nishiuchi et al. 2004, Ulker and Somssich 2004).

Two ABRE-like motifs (ABRERATCAL) are detected in positions -321 and -467 bp from the ATG. Kaplan et al. (2006) showed that a tetramer of this ABRE cis-element is sufficient to confer transcriptional activation in response to cytosolic Ca^{2+} transients. Furthermore, this cis-element overlaps with a CGCG-box [(A/C/G)CGCG(G/T/C)] identified as a specific binding site of

AtSR1, a protein containing a CaM-binding domain in the C-terminus (Yang and Poovaiah 2002). This CaM-regulated *AtSR* gene family responds differentially to multiple physical and chemical stimuli.

At position -431 bp from the ATG, the LECPLE-ACS2 element corresponds to a DNA sequence in tomato which is required for elicitor EIX responsiveness of 1-aminocyclopropane-1-carboxylic acid synthase gene expression (*LeACS2*). A Cys protease that specifically binds to this cis-element in vitro was isolated (Matarasso et al. 2005).

Time course accumulation of PtaZFP2 transcripts and localized expression after controlled stem bending

The accumulation of *PtaZFP2* mRNAs after controlled bending in poplar stems was investigated by semi-quantitative RT-PCR analysis. With our amplification conditions, no *PtaZFP2* transcripts were detected in the control plant stems (Figure 3). Bending induced the accumulation of *PtaZFP2* transcripts within 5 min. *PtaZFP2* transcripts highly accumulated 10–30 min after the stem bending, and the level of *PtaZFP2* transcripts slowly decreased 1 h after bending. Next, we compared the time course accumulation of *PtaZFP2* transcripts to that of *PtaACS* and *PtaTCH2*. *ACC synthase* encoding gene (*ACS*) is a primary response gene to mechanical stress involved in ethylene biosynthesis. *ACC synthase* mRNAs accumulate 10 min after the bending of *Vigna radiata* leaves (Botella et al. 1995). As shown in Figure 3, *PtaACS* transcripts accumulated 10 min after stem bending and reached a peak after 30 min. *TCH2* gene encoding calmodulin-like protein is induced 10 min after touch stimulation in *Arabidopsis* (Braam and Davis 1990). In poplar, bending induced the accumulation of *PtaTCH2* transcripts within 5 min.

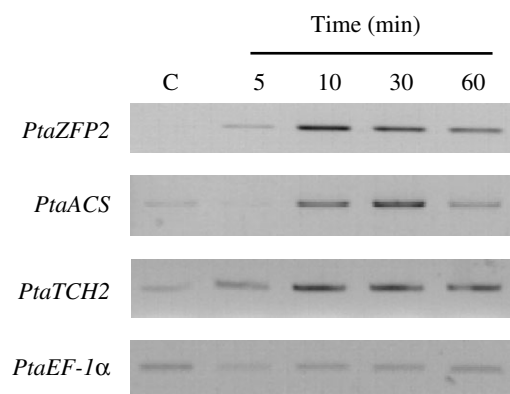


Figure 3. Time course accumulation of *PtaZFP2*, *PtaACS* and *PtaTCH2* transcripts after stem bending. Total RNAs were extracted from the stems of control plants (C) and from bent stems at different times (5, 10, 30 and 60 min). Changes in the abundance of *PtaZFP2*, *PtaACS* and *PtaTCH2* mRNAs were analyzed by RT-PCR using specific primers. As a control, the expression of *EF-1α* gene is also shown.

PtaTCH2 transcripts highly accumulated 10–30 min after bending as we observed for *PtaZFP2* but remained at the same level 1 h later (Figure 3). In contrast, no similar trend was detected for the *PtaEF-1α* mRNA level.

To study the *PtaZFP2* expression in several poplar organs after local stem bending, we examined changes in the abundance of *PtaZFP2* transcripts by Northern blot analysis (Figure 4A). As shown in Figure 4B, *PtaZFP2* mRNAs were strongly accumulated in bent internodes and slightly in the roots. However, *PtaZFP2* transcripts were not detected in basal and apical leaves, apical

internodes, and apex. These results show that bending induced a rapid but transient accumulation of *PtaZFP2* transcripts, which is only localized in the stressed part of the plants.

Time course accumulation of PtaZFP2 transcripts in response to gravitational stimulation

According to the recent hypotheses, internal and external mechanical signals are produced during plant responses to gravity (for review, see Telewski 2006). To study the time course accumulation of *PtaZFP2* transcripts during gravitational response, young poplars were inclined at 35° from the vertical axis. The real-time quantitative RT-PCR analysis showed that *PtaZFP2* mRNAs increased as soon as 10 min and accumulated 26-fold in the basal part of poplar stems 20 min after inclination (Figure 5A). *PtaZFP2* mRNA abundance remained high at 30 min and suddenly decreased at 45 min after the treatment, indicating again a rapid regulation of *PtaZFP2* and a transient accumulation of the transcripts during gravitational response. In comparison, the relative transcript abundance of *PtaZFP2* is 25-fold lower 30 min after gravitropic stimulus than that observed half an hour after a single stem bending (Figure 5B).

Expression pattern of PtaZFP2 in response to other abiotic factors

To examine whether *PtaZFP2* was involved in other abiotic stresses, we investigated its expression under several stresses by the real-time quantitative RT-PCR analysis.

As shown in Figure 6, the treatment of poplars with NaCl by adding salt in hydroponic solution to 200 mM resulted in a slight but significant induction in roots. This 4-fold induction remained the same at 30 and 75 min after treatment. In basal leaves and internodes, a rapid and significant induction of *PtaZFP2* expression of 9- and 35-fold, respectively, was observed 30 min after treatment. Then, the abundance of *PtaZFP2* transcripts slowly decreased 75 min after treatment in these two types of organs but remained 10- and 4-fold higher than that in control plants. In apex, the accumulation of *PtaZFP2* transcripts was not detectable 30 min after treatment but a 70-fold relative abundance of transcripts was observed 75 min later. However, this induction by salt treatment remained 10- to 100-fold lower than that in bent plants (Figure 5B). Physiologically, 75 min after salt treatment, poplars carried out sloping leaves and wilt apex, showing that salt stress has been perceived by plants (data not shown).

Incubation of plants at 4 °C for 1 h induced an 85-fold induction of *PtaZFP2* expression in poplar stems (Figure 7A). After wounding, a 900-fold relative abundance of transcripts was observed in detached leaves 1 h after slashing (Figure 7B). On the contrary, no significant changes in mRNA levels could be observed in stems after the incubation of poplars in the dark for 1 h (Figure 7C).

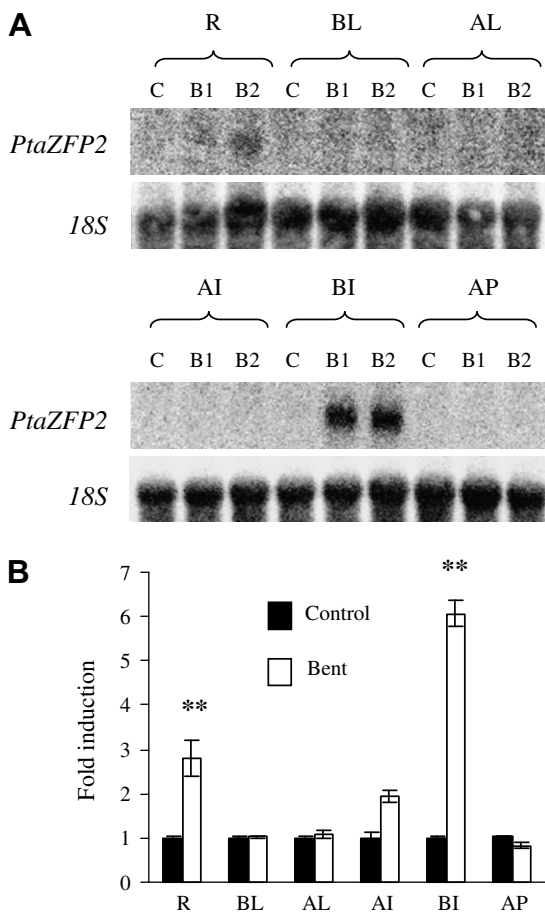


Figure 4. Northern blot analysis of the induction of the accumulation of *PtaZFP2* transcripts 1 h after basal internodes bending in several poplar organs. (A) Total RNAs were extracted from roots (R), basal leaves (BL) localized 2 cm above the bent part of the stem, apical leaves (AL) below the apex, apical internodes (AI) localized 1 cm below the apex, basal internodes (BI) located 10 cm above the collar and apex (AP) of control (C) and bent (B1, B2) plants. Total RNAs (15 µg) were separated on formaldehyde gel, blotted and probed with ³²P-labeled *PtaZFP2* gene-specific probe. The expression of 18S rRNA was used as a loading and transfer control and is shown below the specific gene. (B) The amount of *PtaZFP2* transcripts was obtained by quantifying each band. Quantifications were corrected by 18S rRNA and expressed as a relative value of control plants. Double asterisks (**) represent Student's *t* test significant at *P* < 0.01.

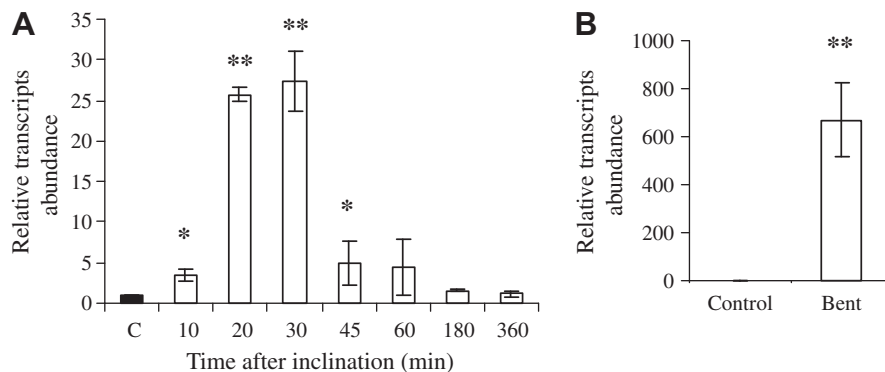


Figure 5. Time course accumulation of *PtaZFP2* after gravitational stimulus. (A) Total RNAs were extracted from the bottom part of the stems (2 cm above the collar) of control plants (C) and at 10, 20, 30, 45, 60, 180 and 360 min after plant inclination at 35°. (B) Total RNAs were extracted from control plant stems or from bent plant stems 30 min after bending. The accumulation of relative transcripts was determined by the real-time quantitative PCR and it corresponds to the mean values of three independent experiments. Asterisks (*) represent Student's *t* test significant at $P < 0.05$. Double asterisks (**) represent Student's *t* test significant at $P < 0.01$.

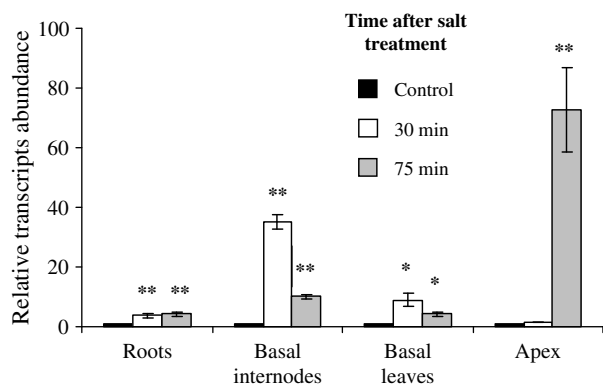


Figure 6. Abundance of relative transcripts of *PtaZFP2* after salt stress. Two-month-old poplars were transferred on nutrient solution or on nutrient solution with 200 mM NaCl. Total RNAs were extracted from roots, basal internodes (localized 10 cm above the collar), basal leaves (localized 2 cm above the basal internodes), and apex of control plants and plants treated with 200 mM NaCl for 30 or 75 min. The abundance of relative transcript was determined by the real-time quantitative RT-PCR and it corresponds to the mean values of three independent experiments. Asterisks (*) represent Student's *t* test significant at $P < 0.05$. Double asterisks (**) represent Student's *t* test significant at $P < 0.01$.

Discussion

The C2H2 ZFPs constitute one of the largest transcription factor families in plants (Takatsuji 1999, Englbrecht et al. 2004). In the database of *P. trichocarpa* transcription factors, the C2H2 transcription family contains more than 80 gene models (Zhu et al. 2007). In poplar, such proteins have not yet been functionally characterized. In this study, we identified one member of the C2H2 family in *P. tremula* × *P. alba*. This protein contains the plant-specific QALGGH motif in both ZF domains. According to the systematic classification of C2H2 proteins based on their ZF types proposed in

Arabidopsis (Englbrecht et al. 2004), *PtaZFP2* can be classified under family C1, earlier described as the EPF-family in *Petunia*. The detailed comparisons of the amino acid around the QALGGH motif suggest that *PtaZFP2* shows highest homology with C1-2iA (At2g37430, ZAT11) and C1-2iB (At3g46070, ZAT7 and At5g59820, ZAT12) sub-classes in *Arabidopsis*.

In *Petunia*, the QALGGH motif has already been demonstrated to be a sequence-specific DNA-binding domain (Takatsuji et al. 1994, Takatsuji and Matsumoto 1996, Yoshioka et al. 2001). Apart from QALGGH motifs, the B-box, the L-box and the DLN-box are partially conserved in *PtaZFP2*. The conserved L/F DLN L/F(X) P motif (DLN-box) found in several C2H2 proteins and ERF proteins can confer the capacity for repression on a heterologous DNA-binding domain and the motif itself is essential for such repression (Ohta et al. 2001). As in *Arabidopsis* ZAT12 protein, this motif is not completely conserved in *PtaZFP2* (LDLSLALP). Recently, the LxLxL motif found within domain I of Aux/IAA proteins was shown to be important to confer repression of auxin response genes via ARF-Aux/IAA dimerization on auxin-responsive promoters (Tiwari et al. 2004). Taken together, these structural features are consistent with *PtaZFP2* being an active repressor but experimental validation is required.

In this work, we showed that *PtaZFP2* is up-regulated by mechanical stress, wounding, and cold and salt treatments. These results are in line with the bioinformatic analysis of the regulatory region of *PtaZFP2*, revealing numerous putative cis-elements implicated in response to abiotic stresses. It is also consistent with the reported role of other C2H2-type ZFPs in regulating the defence response of plants to biotic and abiotic stress conditions (Sakamoto et al. 2004, Ciftci-Yilmaz and Mittler 2008). In particular, in *Arabidopsis*, ZAT11 was shown to be highly induced by wounding (Cheong et al. 2002) and ZAT12 is involved in light, cold and oxidative stresses (Rizhsky et al. 2004,

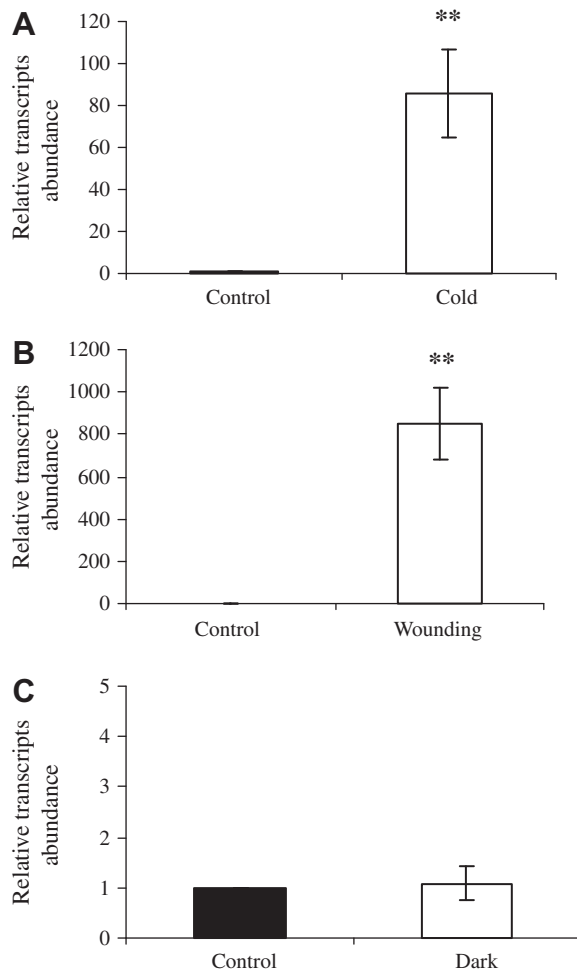


Figure 7. Abundance of relative transcripts of *PtaZFP2* under various abiotic factors. Two-month-old poplars were incubated at 4 °C (cold) (A) or in the dark (C) for 1 h, or their leaves were slashed (B) and collected 1 h later. Total RNAs were extracted from stems (A) and (C) or leaves (B) of control and stressed plants. Relative transcript abundance was determined by the real-time quantitative RT-PCR and corresponds to the means of three independent experiments. Double asterisks (**) represent Student's *t* test significant at $P < 0.01$.

Vogel et al. 2005, Davletova et al. 2005). *ZAT12* belongs to the 15 genes that are most inducible by touch, and it is also induced by darkness (Lee et al. 2005). Under our conditions, the accumulation of *PtaZFP2* transcripts is higher after wounding and bending stress than after cold and salt treatments.

Whereas many efforts were made recently to understand the function of the C2H2-type ZFPs in plant tolerance to light, drought, cold and high-salinity stress conditions, the regulation of this gene family by mechanical stimulus is not well understood. In our conditions, increase of *PtaZFP2* transcripts after bending is as rapid as the increase observed for other primary mechanical-response genes, such as *TOUCH* genes (Figure 3; Braam and Davis 1990) or *ACC synthase* (Figure 3; Botella et al. 1995), implying this gene is involved in the first stages of

mechanosensing in plants. These results are confirmed by the fact that the accumulation of *PtaZFP2* transcripts is detected only where the stress was applied after bending and as if it was following a gradient of dehydration along the stem caused by salt treatment. Our work suggested the involvement of *PtaZFP2* also in plant response to gravity applied on poplar stems with similar kinetics. This result is in favour of a unique mechanosensory network in plants perceiving the numerous mechanical signals of the environment (gravitropism, thigmomorphogenesis, self-loading, turgor pressure, and so on) as suggested by Telewski (2006). Recently, a C2H2-type *Arabidopsis* protein (SGR5), belonging to a distinct family (named as set A) that contains four ZFs without the QALGGH motif, has been involved in gravity perception after amyloplast sedimentation (Morita et al. 2006). As suggested by Ciftci-Yilmaz and Mittler (2008), it could be interesting to test if a cascade of ZFPs could be activated in response to various stresses. For example, during oxidative stress, *ZAT12* is required for the accumulation of *ZAT7* transcripts (Rizhsky et al. 2004).

No consensus for regulatory cis-elements for mechanical response has been clearly defined. Iliev et al. (2002) had described the regulatory region of *TCH4*, a gene encoding a xyloglucan endotransglucosylase/hydrolase (XET), and up-regulated by touch stimulus in *Arabidopsis*. In their study, they demonstrated that the induction of *TCH4* by mechanical stimulus can be conferred by a 102 bp 5'-untranscribed region to a reporter gene. The same 102 bp is also sufficient for the induction of *TCH4* by other factors such as darkness, and heat and cold shocks. The authors described that this region contains an E-box/Mybore. Such elements are also found in the *PtaZFP2* regulatory region at positions -218 and -405 bp before the ATG codon. This consensus DNA sequence has also been described to correspond to the binding site of MYC2, a transcription factor involved in numerous abiotic stresses in other species. Apart from this box, no other similarities between the *TCH4* 102 bp regulatory region and *PtaZFP2* promoter sequence are found. However, in *Arabidopsis*, in the 1 kb context of the *TCH4* upstream region, this 102 bp motif is not necessary for the regulated expression, suggesting an involvement of other additional E-boxes and MYC motifs found in the distal regions.

Calcium and reactive oxygen species (ROS) function as versatile messengers in mediating the mechanical responses. Even though no known DNA-binding site for ROS responses was identified, two cis-elements that are involved in calcium response are found in the *PtaZFP2* promoter. *TCH2*, *TCH3* and *TCH4* promoter regions also contain one or two of these elements in sense or antisense orientation inside the 300 bp immediately upstream of the ATG codon but the *TCH1* promoter does not (TAIR database). Braam (1992) had demonstrated that all *TOUCH* genes except *TCH1* are regulated by calcium in cultured *Arabidopsis* cells. These three *TOUCH* genes were also identified in a transcriptional approach studying genes responsive to cytosolic Ca^{2+}

transients in *Arabidopsis* (Kaplan et al. 2006) and the *Arabidopsis* C₂H₂-type ZFP ZAT10. Functional analysis of the deleted parts of the *PtaZFP2* promoter will help to define if such Ca²⁺ putative cis-element is involved in the up-regulation of the *PtaZFP2* by mechanical solicitation.

Recently, a global analysis studying the common aspects of abiotic stress responses demonstrated that at an early point, nine genes were up-regulated during all stress responses in *Arabidopsis* (Kilian et al. 2007). Among these genes, two C₂H₂ zinc finger transcription factors were described. The authors proposed that these nine early genes might represent basic but nonspecific master regulators and the stress-specific reactions would take place later (1–3 h after the stress). However, as observed by Kilian et al. (2007), according to the applied stress, differential kinetic expression of these genes will be observed suggesting a plant-specific response. As noticed by Telewski (2006), a number of these abiotic stimuli like cold, drought, or osmotic stress induce deformation via turgor pressure variation. Thus, they can also be considered as mechanical stimuli similar to wind and gravity. To elucidate the first stages of mechanoperception in plants, we are currently using *PtaZFP2* promoter-GFP fusions to study the *PtaZFP2* promoter regulation in transgenic poplars.

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