The zinc finger protein PtaZFP2 negatively controls stem growth and gene expression responsiveness to external mechanical loads in poplar

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Summary

- Mechanical cues are essential signals regulating plant growth and development. In response to wind, trees develop a thigmomorphogenetic response characterized by a reduction in longitudinal growth, an increase in diameter growth, and changes in mechanical properties. The molecular mechanisms behind these processes are poorly understood. In poplar, PtaZFP2, a C2H2 transcription factor, is rapidly up-regulated after stem bending.
- To investigate the function of PtaZFP2, we analyzed PtaZFP2-overexpressing poplars (Populus tremula × Populus alba). To unravel the genes downstream PtaZFP2, a transcriptomic analysis was performed.
- PtaZFP2-overexpressing poplars showed longitudinal and cambial growth reductions together with an increase in the tangent and hardening plastic moduli. The regulation level of mechanoresponsive genes was much weaker after stem bending in PtaZFP2-overexpressing poplars than in wild-type plants, showing that PtaZFP2 negatively modulates plant responsiveness to mechanical stimulation. Microarray analysis revealed a high proportion of down-regulated genes in PtaZFP2-overexpressing poplars. Among these genes, several were also shown to be regulated by mechanical stimulation.
- Our results confirmed the important role of PtaZFP2 during plant acclimation to mechanical load, in particular through a negative control of plant molecular responsiveness. This desensitization process could modulate the amplitude and duration of the plant response during recurrent stimuli.

Introduction

In their natural environment, plants undergo continuous exposure to various mechanical signals such as stresses and strains (Mouulia et al., 2011; Hamant, 2013). These mechanical cues are produced intrinsically during tissue or cellular expansion (Ingber, 2005; Hamant et al., 2008) or are triggered by environmental mechanical loads mainly as a result of wind (Mouilia et al., 2011). As for other abiotic factors, plants perceive these stimuli and trigger a network of signaling events, resulting in a generic syndrome of growth responses (Potters et al., 2007, 2009). In the case of external mechanical loads such as wind, this syndrome has been called thigmomorphogenesis (Boyer, 1967; Jaffe, 1973). In woody species, the thigmomorphological response of the stem is generally characterized by a reduction in stem elongation (Telewski & Pruyn, 1998; Anten et al., 2005; Coutand et al., 2008; Voelker et al., 2011), a local stimulation of radial growth (Telewski & Pruyn, 1998; Pruyn et al., 2000; Coutand et al., 2009) and a modification of the mechanical properties of the stem (Telewski & Jaffe, 1986; Kern et al., 2005), thereby reducing their exposure and increasing their resistance to wind loads. To study early kinetics of plant growth responses to mechanical loadings, continuous monitoring of primary and secondary growth has been previously carried out in tomato and poplar, respectively (Coutand et al., 2000, 2009). In those two species, a single transient bending was sufficient to induce a complete growth arrest for a few hours. In tomato, the longitudinal growth continued to be reduced for up to 24 h after stem bending, depending on the amount of mechanical strain induced in the tissues (Coutand & Mouilia, 2000). By contrast, local diameter growth in poplar stopped for 4 h and then increased for 3–5 d before plant growth went back to a normal rate (Coutand et al., 2009).

In natural conditions, wind induces repeated bending at various frequencies (Rodriguez et al., 2008). In temperate climates, windy and still periods alternate on a typical timescale of several days (Stull, 1988). The effect of daily recurring mechanical loads...
on poplar diameter growth has thus been studied (Martin et al., 2010). When one bending was followed by another 24 h later, the magnitude of molecular and growth responses observed after the second bending were lower than those observed after a single bending. About 7 d without any bending were necessary to recover full mechanosensitivity. This accommodation of the mechanosensitivity is believed to be crucial to avoid over-response to wind (Moulia et al., 2011).

Mechanosensitive responses are controlled by a complex network of regulatory genes. Based on analyses performed on different species, a tentative general flow chart of physiological and molecular responses to mechanosensation has been set up (Telewski, 2006). Two classes of putative mechanosensors have been proposed: mechanosensitive channels and receptor-like kinases inserted into the cell wall–plasma membrane–cytoskeleton network (Monshausen & Gilroy, 2009; Monshausen & Haswell, 2013). Yet the signal transduction pathway leading to growth responses is not clearly understood and the mechanosensors have not been identified so far. Transcriptional analysis conducted on Arabidopsis 30 min after a touch stimulus on rosette leaves showed that the expression of up to 700 genes was rapidly modified (Lee et al., 2005). Among the induced genes were the previously described TOUCH genes encoding proteins involved either in calcium binding (Braam & Davis, 1990; Sistrunk et al., 1994) or cell wall modifications (Xu et al., 1995). Genes encoding protein kinases, disease resistance protein and transcription factors were also widely represented (Lee et al., 2005). Among these transcription factors, ZAT10 and ZAT12, two genes encoding Q-type C2H2 zinc finger proteins (ZFP) belonged to the 15 most highly touch induced genes.

With their large dimensions, trees are among the most highly exposed plant organisms to environmental mechanical stresses such as wind. Thigmomorphogenesis is crucial to their mechanical stability and longevity (Moulia et al., 2006). Yet, in trees, the molecular events occurring after the application of external mechanical loads are even less well elucidated than in herbs. Studies on Juglans regia and Populus tremula × Populus alba revealed rapid and local induction of expression of JrZFP2 and PtaZFP2, two close homologs of ZAT12, after a transient stem bending (Leblanc-Fournier et al., 2008; Martin et al., 2009). Interestingly, the abundance of PtaZFP2 transcripts was linearly correlated with the amount of mechanical strain induced in the tissues (Coutand et al., 2009; Moulia et al., 2011). Furthermore, repeated stem bending differentially regulated the expression of PtaZFP2, its induction level being highly reduced after the second bending (Martin et al., 2010). These observations suggest that PtaZFP2 may play a key role in the cascade of mechanical signal transduction.

PtaZFP2 belongs to the Q-type C2H2 zinc finger proteins, which represent a large family of eukaryotic transcription factors (Englbrecht et al., 2004; Gourcilleau et al., 2011). The PtaZFP2 amino acid sequence contains all the structural features well characterized in Q-type C2H2 proteins (Martin et al., 2009): two canonical C2H2 zinc fingers (ZF) containing the invariant QALGGH motif essential for their DNA-binding activity (Kubo et al., 1998) and an Ethylene responsive element binding factor-associated Amphiphilic Repression (EAR) motif (Ohta et al., 2001). In Arabidopsis, transcription factors containing this repression motif were reported to play important roles in modulating plant growth, development and response to biotic and abiotic stresses (Ohta et al., 2001; Kazan, 2006; Ciftci-Yilmaz & Mittler, 2008). Arabidopsis transgenic plants overexpressing different isoforms of C2H2 transcription factors were more tolerant to various abiotic stresses, such as high light, salt, oxidative stress and cold (Rizhsky et al., 2004; Davletova et al., 2005; Vogel et al., 2005; Ciftci-Yilmaz et al., 2007). Interestingly, ZAT12, an early cold-responsive gene, is involved in a negative feedback control of the CBF (C-repeat Binding Factor) regulon in the cold transduction pathway (Vogel et al., 2005). Because of the presence of the EAR repression motif in the PtaZFP2 protein sequence, these data suggest a putative involvement of PtaZFP2 in a negative control during the mechanical signaling pathway. In Arabidopsis, the roles of the Q-type C2H2 ZFP in plant responses to mechanical loads have been poorly studied. In poplar, such proteins have not yet been functionally characterized.

We hypothesized that the in vivo function of PtaZFP2 in a plant submitted to mechanical loads was dual. It could control the growth reduction, but at the same time, it could repress genes involved in the mechanotransduction pathway, thereby inducing a negative control on the mechanosensitivity of the plant to subsequent mechanical loads. To assess these two hypotheses, transgenic poplars overexpressing PtaZFP2 were produced. As several authors have previously reported the difficulty of generating Arabidopsis transgenic plants constitutively over- or underexpressing Q-type C2H2 genes (Sakamoto et al., 2004; Devaiah et al., 2007), we investigated the in vivo function of PtaZFP2 by fusing the PtaZFP2 coding sequence to an inducible promoter activated by 17β-estradiol (Zuo et al., 2000). In this study, we report that overexpression of PtaZFP2 mimics part of the wind-induced physiological responses. Microarray analysis revealed that the expression of 195 genes is modified in transgenic poplars. A weaker bending-dependent induction of various mechanoresponsive genes was also observed. Altogether, our results suggest that PtaZFP2 not only is involved in the transduction pathway leading to changes in poplar growth, but is also part of a negative regulatory circuit controlling poplar responsiveness to mechanical loads.

Materials and Methods

Vector construction and plant transformation

The estrogen-inducible LexA-46::PtaZFP2 construct (Supporting Information, Fig. S1) was assembled by subcloning the full-length PtaZFP2 cDNA (Martin et al., 2009) into the PMDC7 vector using the GATEWAY technology (Invitrogen). This plasmid is a derivative of pEr8 (Zuo et al., 2000), which was made GATEWAY-compatible by Curtis & Grossniklaus (2003). The construct was introduced into poplar (P. tremula × P. alba cv 717-1B4) by Agrobacterium tumefaciens-mediated transformation on internodal stem explants cut from in vitro plantlets as described by Leplé et al. (1992). Transgene orientation and
Plant material, culture conditions and 17β-estradiol treatments

Transgenic and wild-type (WT) poplars (P. tremula × P. alba cv 717-1B4) were obtained by in vitro micropropagation on MS medium (Murashige & Skoog, 1962). After acclimation, plants were grown in liquid nutrient solution (Morizet & Mingeau, 1976) in a growth chamber at 22°C, with a relative air humidity of 60% and a 16:8 h, light:dark cycle with photosynthetic active radiation (PAR) of 50 μmol m⁻² s⁻¹.

Transgene expression was induced by adding fresh stock solutions of 17β-estradiol directly to the nutrient solution. 17β-estradiol was prepared as a 10 mM fresh stock solution in dimethyl sulfoxide (DMSO). The same treatments were applied to WT poplars. The untreated condition corresponded to plants treated with the same volume of DMSO.

Growth measurements and mechanical tests were carried out on 4-month-old poplars. The 17β-estradiol treatment was started on 2-month-old trees, which were 20 cm (± 1) high and 2.77 mm (± 0.1) wide, and lasted for 2 months. The molecular analyses were conducted on 3-month-old poplars which were 35 cm high and 5.37 mm (± 0.3) wide on average. The treatment duration varied between 1 and 144 h.

Bending treatments

Poplars were set vertically and fixed on the bending device as described in Coutand et al. (2009). Plants were left undisturbed for 5 d so that they could settle down after the uncontrolled mechanical disturbance from the installation. Each stem basal part was bent against a plastic tube allowing a homogeneous bending curvature. The tube diameter was chosen depending on each stem diameter, thus imposing a controlled amount of flexural strain (Coutand et al., 2009).

Growth measurements and mechanical behavior

Plant longitudinal growth was determined by measuring stem length from the shoot apex to the stem base. The stem diameter at 25 cm above the collar was measured using a caliper.

Both the elastic (reversible) and plastic (irreversible) behaviors of the wood were characterized on 20-cm-long fresh stem segments. The diameter was measured with a laser beam micrometer. Three-point bending tests were performed using a mechanical testing machine (Instron 5565; Instron, Norwood, MA, USA). The displacement limit was fixed in such a way that the sample experienced elastic and large plastic deformations. The elastic limit separating elastic and plastic behavior was characterized as the minimum strain, \( \varepsilon_{el} \), that generates a permanent plastic deformation. The elastic behavior of the tissue for strains below \( \varepsilon_{el} \) was characterized by the longitudinal Young’s modulus \( E_l \). The plastic behavior of the tissue above \( \varepsilon_{el} \) was characterized by the tangent modulus (\( E_t \)) and by the hardening modulus (\( H \)).

Details of the analysis of the load–displacement curves are given in Fig. S2. Statistical significance was determined by Student’s \( t \)-test.

Histological analysis

Histological observations were made on the poplars used for growth measurements. Small pieces of stem were cut in a zone above the initial height of the plants (30 cm), and were therefore formed during 17β-estradiol treatment. They were fixed, dehydrated and gradually infiltrated with medium-grade LR White resin (Sigma Aldrich) according to Azri et al. (2009). Semithin sections were stained with toluidine blue, dried, mounted in Eukitt and examined under a Zeiss Axioplan 2 microscope. Data were recorded using a digital camera (AxioCam HR; Zeiss) and image analysis was undertaken using the ImageJ software (Schneider et al., 2012). After image segmentation, cell walls and lumens were segregated. The ratio between the cumulated lumen surface and the total surface was referred to as the ‘vessel lumen fraction’. The ratio between the cumulated cell wall surface and the total surface was referred to as the ‘cell wall fraction’. Statistical significance was determined by Student’s \( t \)-test.

RNA isolation and cDNA synthesis

For the real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) experiments, RNAs were extracted from 150 mg of bent stems using CTAB extraction buffer (Chang et al., 1993), and treated with RNase-free RQ1 DNase (Promega). RNA was quantified spectrophotometrically and checked by agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg total RNA using oligo(dT) and SuperScript III (Invitrogen).

For the microarray experiment, RNA was extracted from plants preliminarily treated with 10 μM 17β-estradiol for 96 h, in order to attain a PtaZFP2 transcript accumulation similar to the one observed after a stem bending (Fig. 1c). RNA was isolated from 100 mg of bent stem using a Rneasy Plant Mini kit followed by a DNase1 treatment (Qiagen). Before labeling, RNA was quantified using the RiboGreen RNA Quantification Reagent (Invitrogen) and integrity was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Microarray analysis

Three independent biological experiments were analyzed by Affymetrix (Santa Clara, CA, USA) GeneChip Poplar Genome Array oligonucleotide microarrays. For each genotype (WT and PtaZFP2-OE line #39), each independent experiment was hybridized with complementary RNA obtained from 2 μg of total RNA made of a pool from two individuals. The amplification, labeling, hybridization, and imaging procedures were performed at the URGV Transcriptomic Plateform (Evry, France) according to the manufacturer’s instructions (Affymetrix, http://www.affymetrix.com). Arrays were scanned with the GeneChip Scanner 3000-7G piloted by the GeneChip Operating Software.
Raw data were normalized using the GC-RMA algorithm (Irizarry et al., 2003). To determine which genes were differentially expressed between WT and PtaZFP2-OE line #39, a two-group t-test assuming equal variance between groups was performed. To fit the assumption of equal variance of gene expression between groups, genes displaying extreme variation were excluded from the analysis. The raw P-values were adjusted by the Bonferroni method (Ge et al., 2003). A gene was declared differentially expressed if the Bonferroni P-value was below 0.05. Microarray raw, normalized data and further details of the samples are available through both the CATdb database (AFFY_POP_2011_08_POPLAR ESTRADIOL STUDY) and the Gene Expression Omnibus repository at the National Center for Biotechnology Information (GEO submission GSE43533; http://www.ncbi.nlm.nih.gov/geo/). Enrichment of gene ontology (GO) terms was evaluated with the AgriGO Gene Ontology annotation search tool (http://www.arabidopsis.org/tools/bulk/go/) with the Arabidopsis matches of the differentially expressed poplar genes.

Real-time quantitative RT-PCR experiments

Real-time RT-PCR amplifications were carried out using an iCycler IQ (Bio-Rad). Each PCR reaction (15 μl) contained 2 μl of 1:40 dilution of the first cDNA strands, primers (0.3 μM of each), and MESA GREEN qPCR MasterMix Plus (Eurogentec, Seraing, Belgium). After a heat step at 94°C for 5 min, PCR conditions were as follows: 40 cycles consisting of denaturing (94°C, 10 s), annealing (60°C, 15 s), and elongation (72°C, 15 s), ending with a final elongation step at 72°C for 5 min.

Elongation factor-1α (EF-1α) was retained as the reference gene (Martin et al., 2009). The specificity of the primers (listed in Table S1) was checked by sequencing the PCR products (Beckman Coulter Genomics, Takeley, UK).

The relative quantitative abundance (Q) was calculated by comparison with the expression of EF-1α using the delta–delta method mathematical model (McMaugh & Lyon, 2003). The specificity was confirmed by determining the melt curves for the PCR products and by gel electrophoresis. Real-time PCR amplifications were carried out in triplicate on at least three independent experiments. A Kruskal–Wallis test was used to determine
overall statistical significance. Statistically different groups were obtained with a Newman–Keuls test.

**Results**

Production of transgenic poplars overexpressing *PtaZFP2* in physiological amounts

Among the 24 *PtaZFP2*-overexpressing lines (*PtaZFP2*-OE) recovered from independent transformation events, seven contained a single copy of the transgene (lines #9, #22, #23, #26, #30, #39 and #49; e.g. Fig. S1). The *PtaZFP2* gene expression was tested 24 h after the addition of 5 μM of 17β-estradiol in nutrient solution, corresponding to the optimal 17β-estradiol concentration used in *Arabidopsis* (Zuo et al., 2000). As shown in Fig. 1(a), *PtaZFP2* expression was 15–140 times higher in *PtaZFP2*-OE lines than in WT poplars grown under controlled conditions. In the absence of an inducer, the *PtaZFP2* expression level of *PtaZFP2*-OE lines was identical to that of WT plants (Fig. S1). In WT poplars, 17β-estradiol treatment had no effect on the expression of the *PtaZFP2* endogenous gene (Fig. S1). Under these conditions, #30 and #39 *PtaZFP2*-OE lines exhibited levels of *PtaZFP2* expression comparable to that observed in WT poplars after a transient stem bending (Martin et al., 2010), and were selected for further phenotypic characterization. For these two lines, the maximum induction was observed for 10 μM of 17β-estradiol, higher concentrations having no additive effects (data not shown). This concentration was then used for all experiments.

As this type of inducible construction had never been used in poplar before, we examined changes in the abundance of *PtaZFP2* transcripts in different organs 24 h after 17β-estradiol addition in #30 and #39 *PtaZFP2*-OE lines. As shown in Fig. 1(b), *PtaZFP2* overexpression was observed in all organs tested, indicating that the inducible system had been activated in the whole plant. For both *PtaZFP2*-OE lines, the highest overexpression was observed in leaves, probably resulting from the accumulation of 17β-estradiol through the xylem flow. However, the overexpression found in the lamina showed that 17β-estradiol was not confined to vascular tissues. A significant overexpression of *PtaZFP2* was also observed in both the bark and xylem. In stems, the time course accumulation of *PtaZFP2* transcripts after addition of 17β-estradiol was also analyzed. Similar transcript induction profiles were observed in the treated #30 and #39 *PtaZFP2*-OE lines (Fig. 1c). In these lines, *PtaZFP2* expression was detectable 3 h after the treatment and rose to a peak at 4 d after induction. While in *Arabidopsis* the transgene expression decreased after 96 h (Zuo et al., 2000), in *PtaZFP2*-OE poplar lines it reached a plateau at 96 h, then remaining steady.

Overexpression of *PtaZFP2* induces diminution of poplar growth and modifications of stem histological and mechanical properties

To examine whether *PtaZFP2* overexpression affects poplar growth and wood mechanical properties, *PtaZFP2*-OE and WT poplars were treated for a period of 2 months with 10 μM 17β-estradiol (Fig. 2a). *PtaZFP2* overexpression led to a 15%
reduction of longitudinal growth (Fig. 2b) and an 8% reduction of diameter growth (Fig. 2c), whereas 17β-estradiol had no significant effect on WT plants. This growth reduction was higher in the #39 line than in #30 and seemed to be correlated with that of PtaZFP2 expression.

In comparison with untreated transgenic plants, the 17β-estradiol-treated #39 line had a significantly higher Young’s modulus (+12%, P = 0.049) (Table 1). In the plastic regime, they also displayed a significantly higher $E_t$ (+33.3%, $P = 0.018$) and $H$ (+79.4%, $P = 0.031$), so that their tissues were less prone to plastic irreversible deformations under large mechanical loads (plastically stiffer). However, the elastic strain limit $\varepsilon_{el}$ was not significantly affected by the treatment. None of these mechanical properties were modified in WT plants after addition of 17β-estradiol (data not shown). To test whether the Young’s modulus modification was the result of changes in the wood anatomy, xylem characteristics were measured in stem cross-sections. Overexpression of PtaZFP2 induced a significant increase (+15%) of the vessel lumen fraction. This was mainly the result of the formation of bigger vessels (Table 2). However, the cell wall fraction was not significantly affected by the treatment. Therefore the previously reported changes in mechanical properties can be attributed to rheological changes of the cell walls. These results indicated that overexpression of PtaZFP2 modified poplar growth as well as wood anatomy and cell wall mechanical properties, especially in the range of large plastic strains.

**Poplar sensitivity to bending is modified by PtaZFP2 overexpression**

We have previously shown that, after a first bending, poplars become less sensitive to subsequent ones (Martin et al., 2010). To evaluate the involvement of PtaZFP2 overexpression on plant sensitivity to mechanical loads, we compared the transcriptional patterns of four mechanoresponsive genes in WT and PtaZFP2-OE #30 and #39 lines treated for 96 h with 17β-estradiol (i.e. when the amount of PtaZFP2 transcript in PtaZFP2-OE is similar to that observed in the WT after a single stem bending). Two of these genes (PtaTCH4 and PtaXET6) encode xyloglucan endotransglycosylases/hydrolases shown to be regulated by mechanical loads in both Arabidopsis and poplar (Lee et al., 2005; Martin et al., 2010). The other two are PtaZFP2 and its closest Q-type C2H2 homolog, PtaZFP1 (Gourcilleau et al., 2011).

### Table 1 Stem mechanical properties of PtaZFP2-overexpressing (PtaZFP2-OE) poplars (Populus tremula × Populus alba) (#39 line) treated or not with 10 µM of 17β-estradiol for 2 months

<table>
<thead>
<tr>
<th>Mechanical traits</th>
<th>Untreated #39</th>
<th>17β-estradiol-treated #39</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young modulus (Mpa)</td>
<td>1920 ± 88</td>
<td>2150 ± 58</td>
<td>0.049</td>
</tr>
<tr>
<td>Elastic limit</td>
<td>$7.71 \times 10^{-3} \pm 0.29 \times 10^{-3}$</td>
<td>$6.89 \times 10^{-3} \pm 0.33 \times 10^{-3}$</td>
<td>0.082</td>
</tr>
<tr>
<td>Tangent modulus (Mpa)</td>
<td>1025 ± 56</td>
<td>1366 ± 111</td>
<td>0.018</td>
</tr>
<tr>
<td>Hardening modulus (Mpa)</td>
<td>2333 ± 294</td>
<td>4185 ± 700</td>
<td>0.031</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE of seven plants per treatment. P-values ($t$-test) are indicated. Values in bold were significantly different ($P < 0.05$) from controls.

As shown in Fig. 3(a–c), when no bending was applied, no or little basal expression of PtaTCH4, PtaXET6 and PtaZFP1 was observed in stems of WT and transgenic lines previously treated with 17β-estradiol. As expected, a high transcript accumulation was observed 30 min after stem bending in WT plants for these three genes. By contrast, the increase in mRNA levels after bending was much reduced in PtaZFP2-OE lines, although a quantitatively identical mechanical stimulus was applied. These results suggest that the overexpression of PtaZFP2 triggers a reduction of poplar molecular responses to bending.

Consistent with what was shown previously, when no bending was applied, PtaZFP2 transcript accumulation was 40 and 90 times higher in PtaZFP2-OE #30 and #39 lines, respectively, than in WT plants (Fig. 3d). After bending in PtaZFP2-OE lines, a weak increment in PtaZFP2 expression was detected, probably corresponding to the mechano-induced expression of the endogenous PtaZFP2 gene. However, the induction level of endogenous PtaZFP2 gene expression was much lower in PtaZFP2-OE lines than that observed in bent WT plants. These observations strongly support the hypothesis that under mechanical stress conditions, PtaZFP2 negatively modulates, indirectly or directly, its own expression and the expression of several mechanoresponsive genes.

### Table 2 Xylem characteristics of PtaZFP2-overexpressing (PtaZFP2-OE) poplars (Populus tremula × Populus alba) (#39 line) treated or not with 10 µM of 17β-estradiol for 2 months

<table>
<thead>
<tr>
<th>Xylem traits</th>
<th>Untreated #39</th>
<th>17β-estradiol-treated #39</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall fraction (%)</td>
<td>69.43 ± 0.64</td>
<td>67.23 ± 0.88</td>
<td>0.101</td>
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<tr>
<td>Vessel lumen fraction (%)</td>
<td>20.94 ± 0.40</td>
<td>24.14 ± 0.35</td>
<td>0.004</td>
</tr>
<tr>
<td>Vessel density (no. of vessels mm$^{-2}$)</td>
<td>141 ± 3.17</td>
<td>149 ± 1.8</td>
<td>0.129</td>
</tr>
<tr>
<td>Mean vessel lumen area (µm$^2$)</td>
<td>1480 ± 11</td>
<td>1625 ± 25</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE of five plants per treatment. P-values ($t$-test) are indicated. Values in bold were significantly different ($P < 0.001$) from controls.
lines treated with 17β-estradiol for 96 h with that of WT plants. In the PtaZFP2-OE plants, a total of 132 genes matching 138 probesets were down-regulated and a total of 63 genes matching 76 probesets were up-regulated (Table S2). This disproportion between down- and up-regulated genes points towards PtaZFP2 being mainly part of a negative regulatory pathway.

In order to identify over-represented gene categories in our data set when compared with their proportion on the Affymetrix GeneChip Poplar Genome Array, we explored the functional categorization of the deregulated genes using the AgriGO SEA tool (Singular Enrichment Analysis). Up-regulated genes belonged to two major over-represented categories: ‘sequence-specific DNA binding’ and ‘response to biotic stimulus’. The categories ‘response to other organism’ and ‘multi-organism process’ were also over-represented, but contained identical genes to the category ‘response to biotic stimulus’, to which we will solely refer hereafter (Table 3). Among the four genes belonging to the ‘sequence-specific DNA binding’ category were three WRKYs, a group of transcription factors known for their involvement in senescence regulation and plant defense against both biotic and abiotic stresses (Rushton et al., 2010). Genes down-regulated by PtaZFP2-OE belonged to three major over-represented functional categories, namely ‘cellular response to chemical stimulus’, ‘catalytic activity’ and ‘tetrapyrrole binding’. The group of

<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>Number in input list</th>
<th>Number in background1</th>
<th>P-value</th>
<th>FDR</th>
</tr>
</thead>
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<tr>
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<tr>
<td>GO:0051707</td>
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<td>GO:0009607</td>
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<td>GO:0051704</td>
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<td>GO:0043565</td>
<td>Sequence-specific DNA binding</td>
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<td>Down-regulated</td>
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<td>GO:0003824</td>
<td>Catalytic activity</td>
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<td>12 398</td>
<td>0.0009</td>
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<td>GO:0046906</td>
<td>Tetrapyrrole binding</td>
<td>6</td>
<td>370</td>
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<td>0.043</td>
</tr>
</tbody>
</table>

FDR, false discovery rate.
The table displays significantly over-represented GO categories as determined by AgriGO SEA using a Fisher test and a Bonferroni multi-test correction.

1The chosen background is the Populus Affymetrix Genome Array, which consists of 35 906 annotations.
‘catalytic activity’ genes made up 39% of the down-regulated genes and participated mainly in phosphate (phosphatases and kinases), carbon and fatty acid metabolism. Among the seven genes belonging to the ‘cellular response to chemical stimulus’ category, five were involved in plant hormonal regulation, especially ethylene and auxin. The over-representation of hormonal-related processes was also evident in the ‘catalytic activity’ category, as six genes out of 54 were assigned a function in hormone biosynthesis, degradation or transport (Table 3).

The incompleteness of poplar GO annotations prompted us to conduct a second analysis by interrogating the TAIR GO annotation search tool with the Arabidopsis matches of the differentially expressed poplar genes. This approach confirmed globally the results obtained with the AgriGO SEA tool (Table S3), highlighting the prevalence of genes responding to various stimuli and stresses. Overall, our GO data are in accordance with the previously identified involvement of C2H2 from other species (e.g. ZAT12 in Arabidopsis) in response to biotic and abiotic stimuli (Rizhsky et al., 2004; Vogel et al., 2005; Kilian et al., 2007; Sun et al., 2010; Luo et al., 2012).

PtaZFP2 modulates the expression of various genes involved in mechanotransduction

In WT poplar, a single transient stem bending triggers a rapid induction of PtaZFP2 expression (Martin et al., 2009; Fig. 3d), suggesting that, besides its role in the desensitization process, this transcription factor might be a short-term inducer of early mechanical responses. To investigate whether the most induced and repressed genes identified in PtaZFP2-OE poplars were effectively mechanoresponsive, we compared the expression of 10 genes, 30 min and 2 h after a single transient stem bending in PtaZFP2-OE and WT poplars (Figs 4, 5). In PtaZFP2-OE poplars, the transcript profiles of the 10 genes characterized by qRT-PCR assays were consistent with those in microarray analysis (Table S2). Apart from a gene encoding a putative alpha-fucosidase (PtaGDSL), nine of the 10 genes misregulated in PtaZFP2-OE poplars also appeared to be rapidly deregulated by a transient bending in WT plants. The four PtaZFP2-down-regulated genes, encoding a Clavata-like peptide (PtaCLE1-like), a peroxidase (PtaPO2), a putative auxin ABC transporter (PtaABC), an ethylene receptor (PtaETR1), were rapidly repressed by bending in WT poplars (Fig. 4). Among the PtaZFP2-up-regulated genes, two different expression profiles could be distinguished. The basal expression level of genes encoding an alpha-amyrase (PtaAMY1), a transmembrane protein kinase (PtaFERONIA), a calmodulin-like protein (PtaCML42) and an 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (PtaACO4) was higher in PtaZFP2-OE plants than in WT plants without bending, but lower than the mRNA accumulation observed in WT plants after bending (Fig. 5). Considering PtaNIMIN-1, a gene involved in the regulation of the pathogen defense in Arabidopsis (Hermann et al., 2013), its expression is up-regulated by stem bending, but the basal level detected in PtaZFP2-OE plants is higher than the expression level observed in WT poplars after bending (Fig. 5).

Fig. 4 Expression levels of four genes down-regulated in PtaZFP2-overexpressing (PtaZFP2-OE) poplars (#39) without or after a transient stem bending. PtaZFP2 expression was monitored by real-time PCR (qPCR). Plants (Populus tremula × Populus alba) were treated with 17β-estradiol (10 μM) for 4 d. The bent zone of stems was collected 30 and 120 min after a single transient bending. In each line and condition, the relative transcript abundance was determined by comparison with the gene expression level in 17β-estradiol-treated wild-type (WT) plants using EF-1α transcript abundance as a reference. Closed circles, WT; open circles, #39. Represented values are means of five to seven biological replicates ± SE. Asterisks indicate statistically significant differences (Student’s t-test) between WT and PtaZFP2-OE poplars: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Of particular interest, the regulation by bending of these nine mecha

...mechanoresponsive genes appeared to be strongly reduced in

...PtaZFP2-OE poplars (Figs 4, 5), revealing again the role of

...PtaZFP2 in a desensitization mechanism. Moreover, our results

...indicate that some of the genes misregulated in PtaZFP2-OE poplars are actually early molecular actors of mechanotransduc

...tion and that overexpression of PtaZFP2 mimics part of the short-term effects induced by a transient bending.

To assess whether these conclusions may be conserved across species, we then compared the transcriptome profiling of PtaZFP2-OE poplars with an Arabidopsis ‘touch dataset’ obtained from rosettes 30 min after a touch stimulus (Lee et al., 2005). In the Arabidopsis ‘touch dataset’, ZAT12/RHL41 (AT5G59820), the putative Arabidopsis homolog of PtaZFP2, was one of the most highly induced genes among the 589 up-regulated ones. Additionally, several PtaZFP2-OE deregulated genes were also deregulated in the touched Arabidopsis (shown in bold in Table S2). Deregulated genes included PEPKRI (Potri.015G136900), MURUS4 (Potri.001G459700) and RVE1 (Potri.017G144800) for the PtaZFP2-OE down-regulated genes, and Potri.010G251000 (uncharacterized protein), an EP3 chitinase encoding gene (Potri.013G125000), WRKY53 (Potri.014G096200), PLAZA (Potri.007G040400), a phytosulfokine two precursor encoding gene (Potri.002G116300), and WRKY40 (Potri.018G019800) for the PtaZFP2-OE up-regulated genes. Interestingly, WRKY40 was one of the most highly induced genes in the touch dataset. RVE1 was down-regulated both in our dataset and in the touch dataset. These data suggest that PtaZFP2 and its Arabidopsis homolog ZAT12 could regulate part of the transcriptional network triggered by mechanical stimuli.
**Discussion**

**PtaZFP2 role in stem growth**

In response to sublethal abiotic stress conditions, herbaceous plants exhibit a generic ‘stress-induced morphogenic response’ (inhibition of cell elongation, localized stimulation of cell division and alteration in cell differentiation status) that could be part of a general acclimation strategy to diminish plant exposure to stress (Potters et al., 2007). Such morphogenic responses are also described in woody plants after wind exposure (Telewski, 2006). However, the molecular mechanisms triggering this thigmomorphogenetic syndrome have still not been fully elucidated.

Here, we describe the first direct evidence of the role of a C2H2-type zinc finger protein in the control of tree growth and development. The phenotype of the PtaZFP2-OE lines cannot be attributed to some indirect deleterious effect, as it was observed in two independent lines with different transgene insertion sites (Fig. S1) and 17β-estradiol had no effect on WT poplar. Furthermore, no physiological effect of the XVE system itself was observed in transgenic Arabidopsis plants (Zuo et al., 2000). PtaZFP2 overexpression led to a 15% reduction in longitudinal growth and an 8% diminution in radial growth (Fig. 2b,c), which is reminiscent of the early growth responses of the thigmomorphogenetic syndrome. Indeed, the first responses to a transitory bending are a cessation of both subapical and cambial growth (Coutand et al., 2000, 2009). In poplar, the 4 h radial growth arrest observed in the bent zone of the stem (Coutand et al., 2009) coincided with the time of local PtaZFP2 induction (Martin et al., 2009). PtaZFP2 is thus likely to be directly involved in the establishment of the first inhibitory stage of the growth responses to mechanical loads.

One of the recurrent phenotypes in Arabidopsis plants overexpressing Q-type C2H2 genes such as ZAT12 (Vogel et al., 2005), STZ (Sakamoto et al., 2004) or ZAT6 (Devaiah et al., 2007) is growth and development retardation. RNAi suppression of ZAT6 is lethal (Devaiah et al., 2007), and, interestingly, our attempts to suppress PtaZFP2 by constitutive RNAi in poplars were also unsuccessful (personal data), again suggesting a key role of such C2H2 transcription factors in regulating plant growth and development. Whereas the impact of these genes on tolerance to various abiotic stresses was well studied in Arabidopsis, little is known on how these genes regulate growth responses.

Using transcriptome analysis of PtaZFP2-OE plants, PtaZFP2 was found to control directly or indirectly the expression of 195 genes with widespread putative functions. Almost 70% of these genes were down-regulated, which shows PtaZFP2 could be part of a negative regulatory circuit that impedes the response of mechanically responsive genes. Part of the transcriptional network operating downstream PtaZFP2 could explain the growth reduction phenotype. The most highly PtaZFP2-OE down-regulated gene PtaCLE1-like (Potri.001G376100) matches an A-type peptide of the Clavata3/Endosperm Surrounding Region (ESR)-related (CLE) peptide family. PtaCLE1-like is also down-regulated in the stem of WT poplar shortly after mechanical loads (30 min) and up-regulated thereafter (2 h after bending; Fig. 4) Although A-type CLE peptide function in vascular development has been poorly characterized, especially in trees, some A-type CLE peptides were shown to enhance the vascular cell proliferation-stimulating activity of B-type CLE (Etchells & Turner, 2010).

In trees, auxin and ethylene responsiveness is known to control cambial cell activity (Nilsson et al., 2008; Love et al., 2009). In our dataset, the deregulation of many genes related to hormonal signaling, especially ethylene and auxin, was striking. Probesets matching genes involved in auxin response, the auxin response factors ARF1 and ARF6, and the auxin signaling F-box AFB2, were down-regulated in the PtaZFP2-OE plants. ABCB15, a putative auxin transport protein encoding gene, was also down-regulated. Such a negative regulation of auxin responsiveness by C2H2 zinc finger proteins has also been identified in Arabidopsis, but for distantly related homologs of PtaZFP2 (AZF1 and AZF2), and in other stress conditions (Kodaira et al., 2011).

We also found that many genes related to ethylene biosynthesis and signaling were deregulated by PtaZFP2 overexpression. For example, ETR1, ERS1, two of the ethylene receptors, and EIN3, the transcription factor on which ethylene signaling is supposed to converge, were down-regulated. This may indicate a decrease in ethylene sensitivity. At the same time, the ACC oxidase gene ACO4 involved in ethylene biosynthesis was strongly up-regulated. This is not necessarily contradictory, as mutations resulting in hormone insensitivity often lead to a positive feedback on biosynthesis (Vandenbussche et al., 2012).

Overall, these data could make PtaCLE1-like, auxin and ethylene responsiveness good candidates to explain part of the decreased radial growth of PtaZFP2-OE poplars.

**PtaZFP2 affects stem mechanical properties**

Mechanical loads modified the mechanical properties of the stems. Multiple stem bending in poplar induced an increase in the cell wall fraction (higher specific density) of the wood but a decrease in its elastic stiffness (Young’s modulus) and flexural strength (module of rupture). Therefore the specific cell wall properties decreased even more (Pruyne et al., 2000; Kern et al., 2005). In our study, PtaZFP2-OE poplars did not show significant changes in the cell wall fraction but they displayed a slightly higher Young’s modulus (+11.9%). More dramatic changes occurred regarding plastic properties, with an increase in the tangent modulus $E_T$ (+33.3%, $P = 0.018$) and in the hardening modulus $H$ (+79.4%, $P = 0.031$). This suggests that for large mechanical stresses, the plastic nonrecoverable strain was reduced and the wood of the stem was less flexible. These results reveal changes in the cell wall structure/composition. Plastic hardening is driven by macromolecular slidings. Although the molecular mechanisms controlling wall polymer sliding have not yet been studied in green wood, they probably depend on modifications of cell wall polymer quality/reciprocal arrangements. The changes in plastic hardening could be orchestrated by PtaZFP2, as supported by some of the transcriptional modifications observed in the PtaZFP2-OE plants. Indeed, the most highly up-regulated
gene (PtaGDSL) in the PtaZFP2-OE plants is similar to an Arabidopsis alpha-fucosidase (AT3G26430), an enzyme breaking down fucose residues (Del Bem & Vincentz, 2010). Previous studies have suggested that the presence of the L-fucose-containing trisaccharide side-chain on xyloglucan chains is a key component in the association with cellulose (Vanzin et al., 2002). Interestingly, mnr2, an Arabidopsis mutant almost completely devoid of fucosyl residues on xyloglucans, displayed reduced cell wall strength (Vanzin et al., 2002). Xyloglucans, and especially (fucogalacto)xyloglucans, are key wall hemicelluloses that bind to cellulose microfibrils by hydrogen bonds. In the cell wall, xyloglucan endotransglycosylase/xyloglucan endo-transglycosylase/hydrolase (XET/XTH) specifically catalyze the endolytic cleavage and re-ligation of xyloglucan chains. Cleavage of xyloglucans by XET/XTH would facilitate the sliding while re-ligation would allow the cell wall to return to a stabilized state. In our dataset, genes encoding Pt-XTRA.1 (Potri.003G097300) and Pt-EXT.15 (Potri.014G140300), two XET/XTH proteins similar to the Arabidopsis XTRA4/XTH30 and XTRA5, were up- and down-regulated, respectively. Furthermore, polars over-expressing PtxXET6-34 tend to have significantly wider vessel elements than the WT (Nishikubo et al., 2011), a phenotype that was also observed in the PtaZFP2-OE plants. 

Thus, the up-regulation of Pt-XTRA.1 and PtaGDSL-like in response to PtaZFP2 overexpression may be partly responsible for the PtaZFP2-OE mechanical plastic behavior changes and vessel diameter phenotype. But PtaZFP2 does not seem to control the whole thigmomorphogenetic response of wood anatomical and mechanical properties (especially specific density and cell wall elastic stiffness).

As a functional benefit of a higher hardening modulus controlled by PtaZFP2, our results suggest an adaptive process in the wind at the whole-plant level, as the amount of unrecoverable strain after large mechanical loads should be reduced.

PtaZFP2 reduces the induction of molecular responses to bending: a driver of mechanosensitivity?

In polars, the effects of stem bending on growth and molecular responses depend on the mechanical history of the plant (Martin et al., 2010), with a weaker induction of several mecanoresponsive genes as well as of secondary growth responses after successive bendings (Martin et al., 2010). Desensitization of growth responses was also suggested in experiments on Ulmus americana: no increment of the secondary growth response was detected when increasing the number of loadings from five to 80 bendings a day (Telewski & Pruyn, 1998). In Arabidopsis, Arteca & Arteca (1999) demonstrated that multiple touch stimulations were progressively less effective in promoting ACS6 expression, a gene encoding ACC synthase. However, by following early cellular events, such as calcium or pH modification in Arabidopsis, a desensitization phenomenon (potentially as a result of a refractory period of the channels) was only observed when intervals between two touch stimulations were smaller than 20–45 s (Knight et al., 1992; Monshausen et al., 2009). Therefore, it is more likely that the long-term desensitization observed in our study involves a change in the amount of mechanoreceptors and/or of key controlling actors of responsiveness rather than being an effect on the kinetic properties of each mechanosensor.

In our work, the expression level of numerous mecanoresponsive genes after a transient stem bending was much weaker in PtaZFP2-OE poplars than in the WT (Figs 3–5), showing that PtaZFP2 negatively modulates plant responsiveness to mechanical load. Two hypotheses can be suggested. First, the perception capacities could be modified. Our knowledge of plant mechanosensors is not good enough to evaluate the role of PtaZFP2 in modifying perception capacities. However, two genes, FERONIA (Potri.017G097500) and WAKL2 (Potri.004G192500), encoding transmembrane protein kinases described as putative mechanosensors of wall status (Monshausen & Gilroy, 2009), are up-regulated in PtaZFP2-OE plants. The members of the receptor-like kinase (RLK) family play an important role in cell wall integrity surveillance (Humphrey et al., 2007; Cheung & Wu, 2011) and are supposed to feedback-regulate cell wall properties (Guo et al., 2009). Their regulation by PtaZFP2 or external mechanical loads has never been reported before. The link between an increase in their expression and the desensitization process, however, is not direct.

The second hypothesis is that some early components of the mechanotransduction could be modified after the first stimulation, thus limiting the response to subsequent stimulations. The study of PtaZFP2 structural features led to the hypothesis that this protein may act as a transcriptional repressor (Gourcilleau et al., 2011), through the presence of an EAR motif (Ohta et al., 2001; Kazan, 2006). Thus, PtaZFP2 could be directly involved in the desensitization phenomenon, through a negative feedback regulation of the early molecular actors involved after mechanostimulation. Indeed, when we studied the expression of 10 genes derived from the transcriptomic analysis, nine of these genes were also regulated by bending in WT poplars (Figs 4, 5), the majority being regulated as early as 30 min after the mechanical load.

The negative effect of PtaZFP2 on plant responsiveness to bending could also be explained by a less direct mechanism involving PtaZFP2 deregulation of other transcription factors. Interestingly, among the PtaZFP2 up-regulated genes, four genes have been described as negative regulators of plant response to biotic or abiotic stresses in Arabidopsis. CML42 is a Ca^{2+} sensor having multiple functions in biotic and abiotic stress responses (Vadassery et al., 2012). Arabidopsis WRKY18, -40, and -60 negatively regulated resistance to Pseudomonas syringae (Xu et al., 2006). Arabidopsis NIM1-INTERACTING (NMIN1) suppressed NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) activity, a central regulator of the pathogen defense reaction (Hermann et al., 2013). Furthermore, we demonstrated that these genes are effectively early molecular actors of the mechanical transduction pathway in poplar. PtaNIMIN-1 (Potri.002G190800) and PtaCML42 (Potri.006G112500) gene expressions are induced early by mechanical load in poplars (Fig. 5). PtaWRKY53 (Potri.014G096200) and PtaWRKY40 (Potri.018G019800) were up-regulated in PtaZFP2-OE and also up-regulated in response to touch in Arabidopsis (Lee et al., 2005). Thus, after a first mechanical load, PtaZFP2, in concert
with these negatively acting molecular actors, could prevent or reduce the reactivation of the mechanical signaling pathway at the time of a subsequent mechanical load.

This functional study confirmed the important role of PtaZFP2 during plant acclimation, being involved both in growth rate reduction and plant desensitization to mechanical load. This desensitization process is essential during recurrent mechanical stimulations, as it could modulate the magnitude and duration of the plant response in order to prevent significant costs for reduced plant growth. One challenge will now be to understand how this state of desensitization to mechanical loads could last for several days.

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References


**Fig. S2** Schematic representation of the stress vs strain curves during a three-point bending test.

**Table S1** Sequences of primers used for qRT-PCR

**Table S2** List of up- and down-regulated genes in *PtaZFP2-OE* poplars

**Table S3** List of over-represented GO terms in the *Arabidopsis* matches of the differentially expressed genes in *PtaZFP2-OE* poplars

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