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# Phylogenetic Study of Plant Q-type C2H2 Zinc Finger Proteins and Expression Analysis of Poplar Genes in Response to Osmotic, Cold and Mechanical Stresses

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## Abstract

**Plant Q-type C2H2 zinc finger transcription factors play an important role in plant tolerance to various environmental stresses such as drought, cold, osmotic stress, wounding and mechanical loading. To carry out an improved analysis of the specific role of each member of this subfamily in response to mechanical loading in poplar, we identified 16 two-fingered Q-type C2H2-predicted proteins from the poplar Phytozome database and compared their phylogenetic relationships with 152 two-fingered Q-type C2H2 protein sequences belonging to more than 50 species isolated from the NR protein database of NCBI. Phylogenetic analyses of these Q-type C2H2 proteins sequences classified them into two groups G1 and G2, and conserved motif distributions of interest were established. These two groups differed essentially in their signatures at the C-terminus of their two QALGGH DNA-binding domains. Two additional conserved motifs, MALEAL and LVDCHY, were found only in sequences from Group G1 or from Group G2, respectively. Functional significance of these phylogenetic divergences was assessed by studying transcript accumulation of six poplar C2H2 Q-type genes in responses to abiotic stresses; but no group specificity was found in any organ. Further expression analyses focused on *PtaZFP1* and *PtaZFP2*, the two genes strongly induced by mechanical loading in poplars. The results revealed that these two genes were regulated by several signalling molecules including hydrogen peroxide and the phytohormone jasmonate.**

**Key words:** C2H2; phylogenetic analysis; abiotic stress; mechanical loading

## 1. Introduction

Transcription factors play a key role in modulating the acclimation response of plants to various internal

or external cues. A subset of these transcription factors belongs to the zinc finger proteins (ZFPs) characterized by zinc finger domains (ZFs) enabling protein interaction with DNA. The term “zinc finger” refers to a protein motif that binds a zinc ion in order to stabilize its three-dimensional structure consisting of a two-stranded antiparallel  $\beta$ -sheet and

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$\alpha$ -helix.<sup>1</sup> ZFPs are classified according to the number and the order of the Cys (cysteine) and His (histidine) residues that bind the zinc ion. Among these different ZFPs types, C2H2-ZFPs are one of the most abundant and often studied transcription factors in eukaryotes.<sup>2</sup> *In silico* analysis has shown that ~3% of all genes in mammals, ~2.3% in *Diptera*, ~0.8% in *Saccharomyces cerevisiae* and ~0.7% in *Arabidopsis thaliana* encode C2H2-ZFPs.<sup>3</sup> In *A. thaliana*, more than 2000 potential transcription factors have been identified,<sup>4,5</sup> of which ~9% belong to the C2H2-ZFP family.

C2H2-ZFPs are characterized by a conserved sequence CX<sub>2-4</sub>CX<sub>3</sub>FX<sub>5</sub>LX<sub>2</sub>HX<sub>3-5</sub>H (where underlined C and H represent Cys and His interacting with the zinc ion). Plant C2H2-ZFPs possess one to five ZFs. Two main features distinguish most of them from other eukaryote C2H2-ZFPs. First, most plant C2H2-ZFPs contain an invariant QALGGH motif inside the ZF, whereas yeast and animals do not. Second, for multiple-fingered C2H2-ZFPs, ZFs are separated by longer spacers than in other eukaryotes.<sup>1,6</sup> Both the QALGGH motif and length of sequences between two successive ZFs are thought to be important for the affinity of the protein to its DNA target.<sup>6-8</sup> C2H2-ZFPs can be divided into three classes according to the number, types and arrangement of ZFs.<sup>3,9</sup> According to an *in silico* genome-wide comparative analysis performed in *A. thaliana*,<sup>3</sup> two classes, A and B, encompass ZFPs that contain tandem ZFs in one or in more than one array, respectively (as proposed earlier for yeast genome). Class C corresponds to ZFPs containing a single ZF or dispersed ZFs (ZFs being considered dispersed when more than 10 amino acid (AA) residues separate two consecutive ZFs). Most C2H2-ZFPs in animals and yeast can be classified in Classes A and B, but plant C2H2-ZFPs are found mainly in Class C. This third class can be divided into three subclasses according to the number of AA residues separating the two invariant His of the ZFs: three, four or five residues for Subclasses C1, C2 and C3, respectively. Inside Subclass C1, 85% of the C2H2 proteins contain strictly the QALGGH motif (or with few modifications),<sup>3</sup> also called Q-type ZFs in rice.<sup>3,10</sup> *In silico* studies on ZFPs have identified 64 Q-type C2H2-ZFPs in *A. thaliana*,<sup>3</sup> 99 in rice (*Oryza sativa*)<sup>10</sup> and 47 in bread wheat (*Triticum aestivum*).<sup>11</sup>

First considered exclusively as transcription factors with sequence-specific binding to DNA, ZFPs are now known to recognize RNA or other proteins.<sup>12</sup> However, their role as transcription factors is still most often studied. In plants, the C2H2 proteins belonging to Class C are the largest family. They are involved in a wide range of processes including development and organogenesis along with response to stress and defence pathways. These proteins have

been shown to be involved in salt stress, cold, dehydration and light stress in *A. thaliana*,<sup>13-16</sup> the switch from vegetative to floral development and drought in rice,<sup>11,17</sup> flower development and drought in *Petunia*,<sup>18,19</sup> pathogen defence in *Capsicum annuum*<sup>20</sup> and water and salt stress in *Thellungiella halophila*.<sup>21</sup> These studies suggest that C2H2-ZFPs may not be specific to one particular stress but may regulate responses to several stresses.

In *Populus tremula*  $\times$  *alba*, by studying the first molecular stages of the mechanosensing response, we recently identified a C2H2 Q-type gene called *PtaZFP2*. Its expression is induced as fast as 5 min after the bending of a poplar stem.<sup>22</sup> As in other plant species,<sup>6</sup> this gene is also regulated by other stresses such as cold and salt stress.<sup>22</sup> Apart from this *PtaZFP2* gene, little is known about the Q-type C2H2 gene subfamily in poplar (and in woody species more generally) or their specific responses to different abiotic stresses, in particular to mechanical loadings. In this study, we performed a genome-wide identification of Q-type ZFP genes in poplar, using *PtaZFP2* protein sequence as a query in the NR protein and Phytozome databases. In all, 310 sequences were identified, belonging to more than 50 plant species. We then focused on two-fingered Q-type ZFPs encompassing 168 non-redundant sequences from different plant species, including 16 *Populus trichocarpa* sequences. Expression analyses of different members of the poplar Q-type subfamily were performed by designing primers according to the different poplar phylogenetic groups. We determined their mRNA distribution in different organs with or without abiotic stresses. Finally, poplar cell cultures were used to study the effect of calcium, reactive oxygen species (ROS) and phytohormones on the expression of the two genes previously described as showing the strongest response to stem bending.

## 2. Materials and methods

### 2.1. Identification of C2H2 plant proteins

A BLAST search (blastp with default parameters)<sup>23</sup> against the NR protein database of NCBI and *P. trichocarpa* Phytozome database was performed, using the AA sequence of the *PtaZFP2* gene from *P. tremula*  $\times$  *P. alba* (GenBank: FM172949.1) as query sequence. All hits below an *E*-value of  $10^{-4}$  were retrieved. We found 310 and 26 sequences in NCBI and *P. trichocarpa* Phytozome databases, respectively. These 336 sequences were analysed using the Multiple Expectation Maximization for Motif Elicitation (MEME)<sup>24</sup> program with default settings to detect ZF motifs. Only two-fingered C2H2 sequences were further analysed (243 sequences).

## 2.2. Sequence alignment and phylogenetic analysis

Protein sequences were aligned using MUSCLE.<sup>25</sup> Identical and incomplete sequences were excluded from the alignment. The final alignment was composed of 168 sequences. Due to the high variability of sequences and length of spacers between the two ZFs, phylogeny was carried out only on the ZF motifs. The phylogenetic tree of 100 bootstrapped samples was constructed using the maximum likelihood (ML) method implemented PhyML<sup>26</sup> using the GTR + I + G model, chosen after using the program ProtTest<sup>27</sup> on the sequence set.

## 2.3. Identification of conserved two-fingered C2H2 plant protein-associated motifs

The program MEME<sup>24</sup> was used with default settings, except for the maximum number of motifs to find, which was set to 15, to detect potential conserved motifs including the known ZF motifs.

## 2.4. Plant material and culture conditions

Young poplars (*P. tremula* × *P. alba* INRA clone 717-1B4) were obtained by *in vitro* micropropagation and grown on nutrient solution<sup>28</sup> after acclimation (for more details, see Martin *et al.*<sup>22</sup>). Trees were grown in a growth chamber (16 h/8 h light/dark at 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at 24°C/20°C with relative humidity of 60 ± 10%). Two months after micropropagation, the poplars were ready to be used in experiments; stems were about 35 cm tall at this stage. The tested stems ranged in diameter from 4.3 to 6.3 mm, with an average of 5.18 ± 0.51 mm.

## 2.5. Cell culture conditions

Poplar suspension cells were initiated by transferring 2–3 g of fresh callus to 20 ml of liquid MB5 medium containing 2 mg ml<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 g l<sup>-1</sup> L-glutamine and 1 mg l<sup>-1</sup> vitamins (nicotinic acid, pyridoxine hydrochloride and thiamine hydrochloride). Cell cultures were maintained in the dark at 120 rpm on a gyratory shaker in a culture room at 23°C for at least 8 days. Cell cultures were then maintained in the same environmental conditions by putting 10 ml into 40 ml of fresh MB5 medium each week containing 2 mg ml<sup>-1</sup> 2,4-D, 0.2 g l<sup>-1</sup> L-glutamine and 1 mg l<sup>-1</sup> vitamins (nicotinic acid, pyridoxine hydrochloride and thiamine hydrochloride).

## 2.6. Plant treatments

Plants were subjected to salt, osmotic, cold, wounding, or bending stresses, or were unstressed (controls). Salt stress was applied by adding NaCl to the hydroponic solution to a final concentration of 50, 100 or 200 mM. Osmotic stress was applied by adding

polyethylene glycol (PEG) to the hydroponic solution to a final concentration of 20% (w/v). In cold treatment, plants were left at 4°C for 1 h. Plants were wounded by crushing their laminae (leaves without the petiole and central vein) with a pestle. Sampling was carried out 1 h after the treatments. Finally, stem bending was performed by rolling stems on a plastic tube according to Martin *et al.*<sup>29</sup> taking care that the applied mechanical loading was the same for all stems. The bent portion (3 cm long) was collected 0, 10, 15, 30 min, 1, 3, 5 or 24 h after bending. Samples were quickly frozen in liquid nitrogen after sampling and stored at -80°C until analysis.

## 2.7. Cell treatments

Aliquots of 5 ml of 4-day-old cell cultures were placed in 15 ml sterile tubes and left with stirring at 120 rpm in the dark at 23°C for 2 h to prevent aliquoting stress. The culture cells were then treated with calcium 10 mM (CaCl<sub>2</sub>), hydrogen peroxide 500  $\mu\text{M}$  (H<sub>2</sub>O<sub>2</sub>), abscisic acid 100  $\mu\text{M}$  (ABA), ethephon 1 mM (Ete), gibberellic acid 100  $\mu\text{M}$  (GA), methyljasmonate 100  $\mu\text{M}$  (MeJA) or cycloheximide 50  $\mu\text{M}$  (Chx). Stirring, darkness and temperature were maintained throughout the experiment. After different times, the medium was removed and cells were frozen in liquid nitrogen and stored at -80°C until analysed.

## 2.8. RNA isolation and cDNA synthesis

Total RNAs were extracted from 5 ml of culture cells using CTAB extraction buffer as described by Chang *et al.*<sup>30</sup> and then treated with RNase-free RQ1 DNase (Promega, Charbonnières-les-Bains, France). RNA was quantified spectrophotometrically and checked by agarose gel electrophoresis. First-strand cDNA was synthesized from 1  $\mu\text{g}$  total RNAs using oligodT and SuperScript III (Invitrogen, Cergy-Pontoise, France) following the supplier's protocol.

## 2.9. RT-PCR analysis

RT-PCR analyses were performed on 4  $\mu\text{l}$  of 1:40 dilution of the first-strand cDNA. After a heat step at 95°C for 5 min, PCR cycling conditions were: denaturation (95°C, 30 s), annealing (temperature according to primers, Supplementary data 1, 30 s) and elongation (72°C, 45 s), ending with a final elongation step at 72°C for 5 min. Transcripts of each studied gene and of reference genes *EF-1 $\alpha$*  (Elongation Factor-1 $\alpha$ ) and ubiquitin (*Ubq*) were amplified using the primers described in Supplementary data 1. The PCR products were separated on a 2% agarose gel.



### 2.1.10. Real-time quantitative RT-PCR experiments

The real-time quantitative RT-PCR amplifications were performed using an iCycler IQ (Bio-Rad) with SYBR green as a fluorescent dye. Each PCR reaction (25 µl) contained the following: cDNA (4 µl of 1:40 dilution of the first cDNA strands), MESA GREEN qPCR MasterMix Plus for SYBR® Assay w/ fluorescein (Eurogentec, Angers, France) (1×) and primers (200 nM of each). After a heat step at 94°C for 3 min, PCR cycling conditions were 40 cycles of denaturation (94°C, 15 s), annealing (temperature according to primers, Supplementary Table S1, 15 s) and elongation (72°C, 20 s), ending with a final elongation step at 72°C for 5 min. Transcripts of each studied gene and of reference gene *EF-1α* were amplified using the primers described in Supplementary data 1.

Relative quantitative abundance (Qr) of each gene transcripts was calculated by comparison with the expression of *EF-1α* using the delta-delta method mathematical model:<sup>31</sup>

$$Qr = \frac{2^{(C_{\text{control}} - C_{\text{treated}})_{\text{targetgene}}}}{2^{(C_{\text{control}} - C_{\text{treated}})_{\text{referencegene}}}}$$

where C is the threshold cycle number of PCR. The specificity of amplification was confirmed by determining the melt curves for the PCR products at the end of each run and by gel electrophoresis. The real-time PCR amplifications were performed in at least two independent experiments, and each run was carried out in triplicate. Statistically different groups were obtained with a Tukey's honestly significantly different (HSD) test.

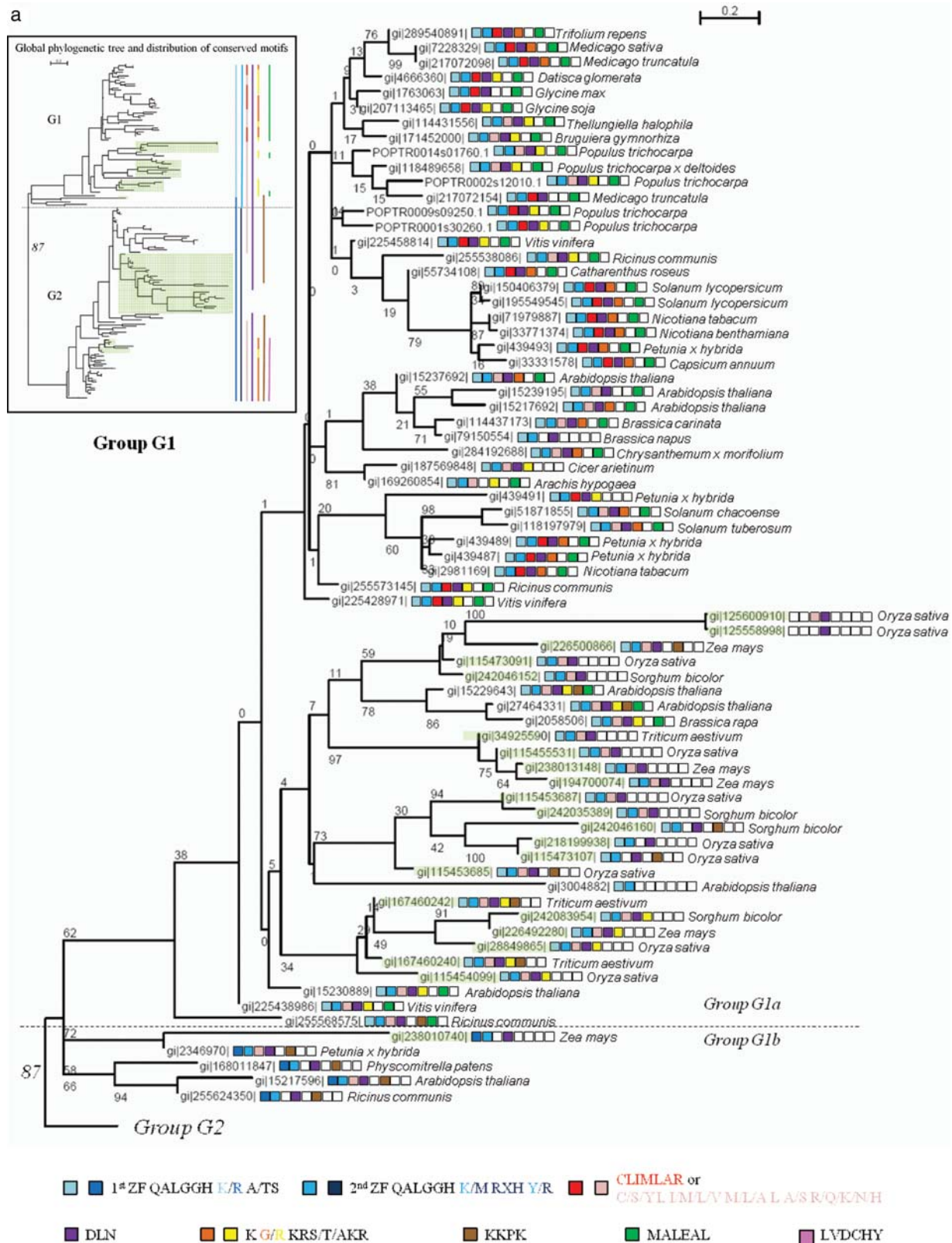
## 3. Results and discussion

### 3.1. Identification of Q-type C2H2-ZFPs in

#### *P. trichocarpa* and their phylogenetic relationships with Q-type C2H2-ZFPs of other plant species

The mechanosensitive *PtaZFP2* gene isolated in *P. tremula* × *P. alba* encodes a C2H2-ZFP with two Q-type ZFs, i.e. containing an invariant plant QALGGH motif.<sup>22</sup> To gain a better understanding of the putative function of this gene subfamily in poplar in response to mechanical loading, the *PtaZFP2* protein sequence was used for BLAST searches against the NR protein and *P. trichocarpa* Phytozome databases. The search with the NR protein database resulted in a total of 310 Q-type C2H2-ZFPs sequences belonging to more than 50 species (data not shown). A first search with the MEME program on these sequences enabled us to determine Q-type ZF domains and manually to distinguish 16 sequences with only one ZF, 226 with two ZFs, 53 with three ZFs, 13 with four ZFs and two sequences with five ZFs. The same was done with the

*P. trichocarpa* Phytozome database and 26 C2H2-ZFPs sequences from *P. trichocarpa* genome were identified. Among these sequences, 16 contained two ZFs, 6 had three ZFs, 3 had four ZFs and 1 had five ZFs. No one-fingered ZFPs were identified in poplar with our method. However, when only one ZF motif was used for supplementary BLAST analysis against the *P. trichocarpa* Phytozome database, five poplar ZFPs with one QALGGH finger were detected. Because they are known to be implicated in abiotic stress responses,<sup>6</sup> in this study we focused the phylogenetic analysis on two-fingered sequences, corresponding to the C1-2i subclass of ZFPs described in *A. thaliana*.<sup>3</sup> AA sequence alignment enabled us to discriminate redundant sequences, and the final tree was constructed with 168 sequences (152 from NCBI and 16 from *P. trichocarpa* Phytozome database). Among the 16 *P. trichocarpa* sequences, the POPTR-1833s00200 is a very short sequence (partial EST), corresponding to a predictive peptide sequence of only 77 AA, whereas the other Q-type ZFP protein contains between 193 AA and 318 AA. In the case of POPTR-0008s03220, the N-terminus part of the predictive sequence seems shorter than would be expected for a complete sequence. Analysis of the *P. trichocarpa* genome<sup>32</sup> revealed that 15 of these sequences could be localized on nine different chromosomes (1,2,4,6,8,9,10,14,16) out of the 19 chromosomes of the poplar genome. Examination in the *P. trichocarpa* genome browser showed that in the same chromosome, these Q-type C2H2 genes are not particularly close to each other. To illustrate phylogenetic relationships between these sequences, the ML tree (unrooted) was constructed (Supplementary data 2) using PhyML.<sup>26</sup> Bootstrap values from 100 replicates were used to indicate the robustness of the result. This analysis clearly identified two major groups with a strong bootstrap value of 87: Group G1 and Group G2 (Fig. 1a and b, respectively). Sequences from monocots and eudicots were found in both, but in each group, the monocot sequences were grouped. This finding suggests that the separation of these two groups was ancestral to the monocot/eudicot separation. Within each group, the bootstrap values are weak. As most C2H2-ZFPs share little homology with highly variable spacer length outside the conserved C2H2-ZFs, the phylogeny was performed on their C2H2-ZFs domains, justifying these lower bootstrap values. Interestingly, some motifs other than the ZF motifs were strictly associated with a group (e.g. MALEAL with Group G1 and LVDCHY with Group G2). These data reinforce the phylogenetic separation of the two groups and may reveal different functions between the corresponding proteins. An ML tree was also constructed with the 16 *P. trichocarpa* sequences (Fig. 2a). Here again two major independent groups



**Figure 1.** Phylogenetic study of two-fingered Q-type C2H2-ZFPs in plants using NCBI and poplar Phytozome blast against the PtaZFP2 AAs sequence. The global phylogenetic tree is shown in insets. The distribution of conserved AAs motifs is symbolized with coloured lines. (a and b) Detail of Group G1 and Group G2 of the global phylogenetic tree. The poplar sequences begin with POPTR. The monocot sequences are framed in light green. Presence of motifs described in (c) is symbolized with coloured squares just after the sequence name; white squares symbolize absence of motif. (c) Consensus sequences of conserved motifs among two-fingered Q-type C2H2-ZFPs in plants and associated colours for motif symbolization.



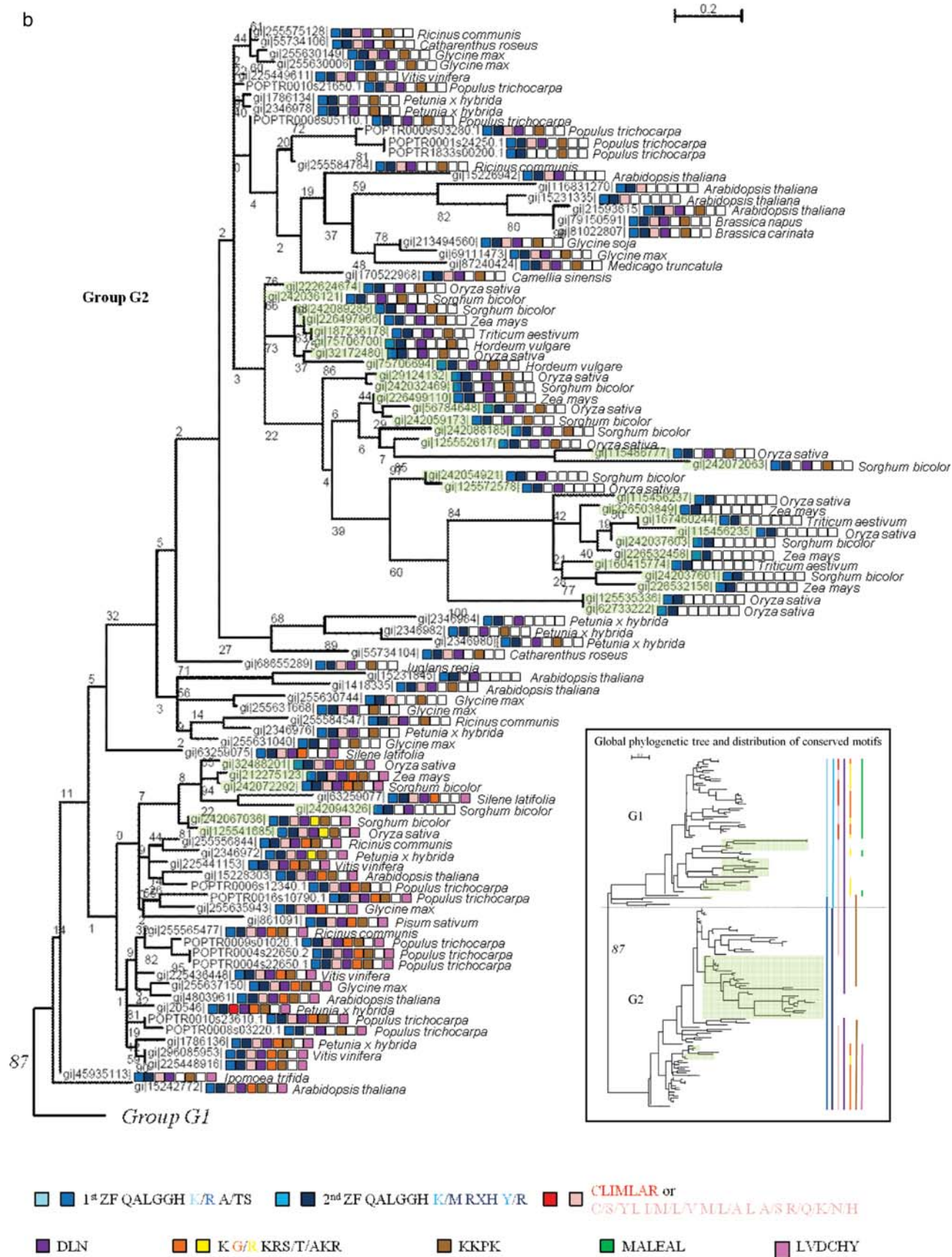
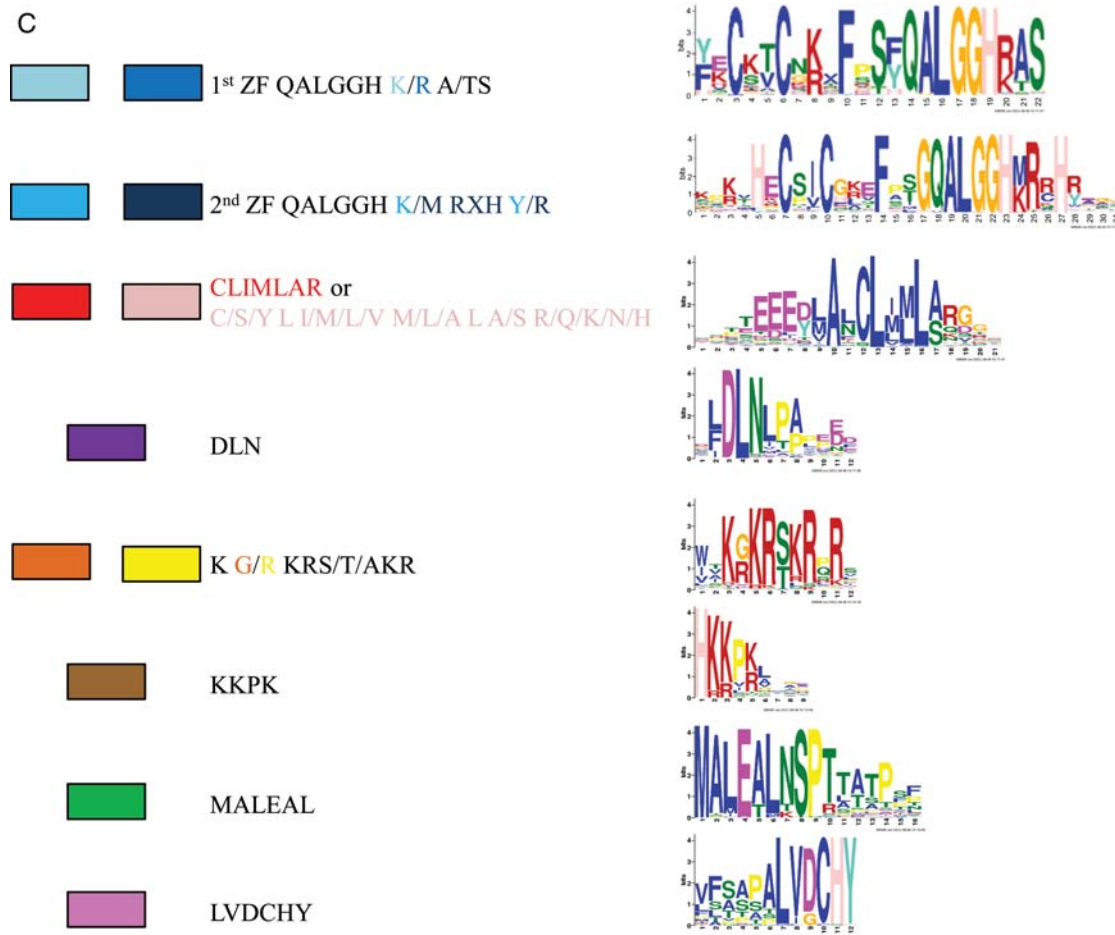


Figure 1. (Continued)



**Figure 1.** (Continued)

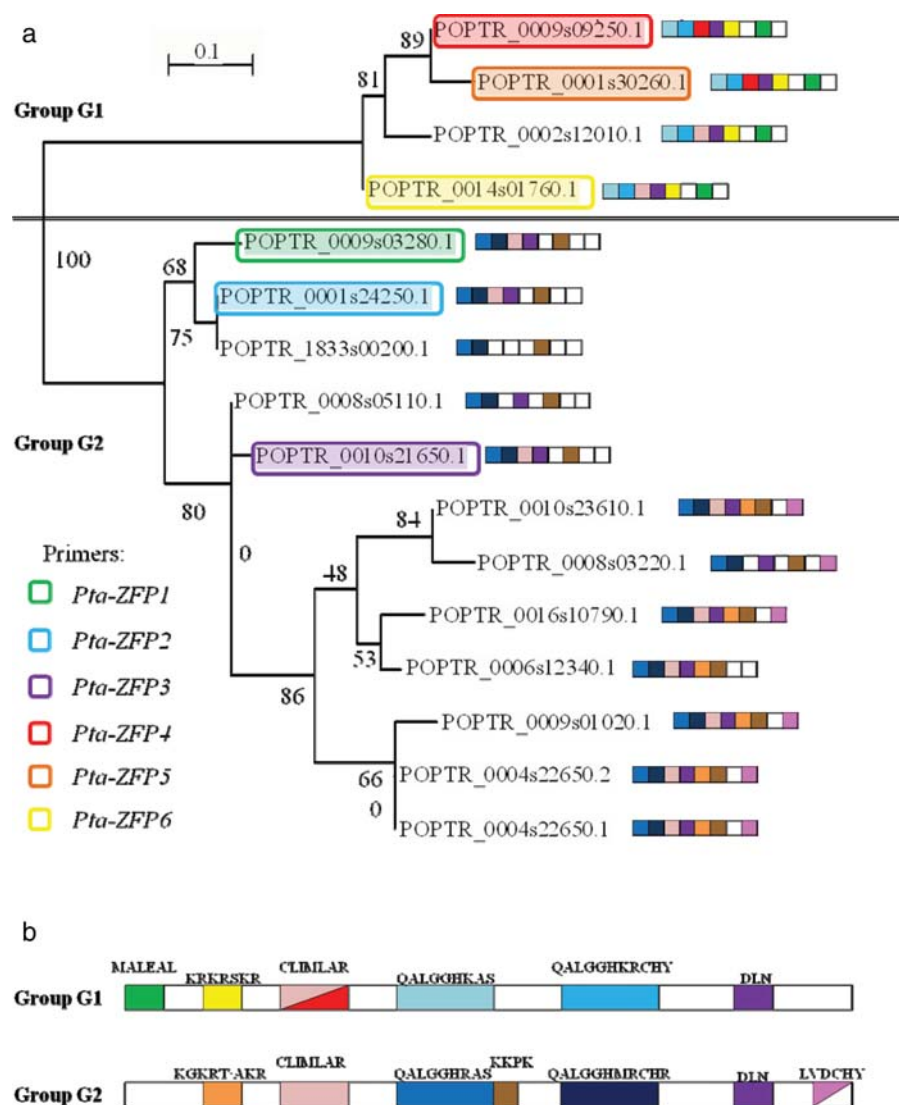
were strongly differentiated, with a bootstrap value of 100, and a different distribution of conserved motifs could be observed between the two groups (Fig. 2b). Group G1 comprised four of these poplar sequences (POPTR\_0009s09250, POPTR\_0001s30260, POPTR\_0002s12010 and POPTR\_0014s01760). Inside Group G2, POPTR\_0001s24250 corresponded to the *P. trichocarpa* orthologous gene of *P. tremula* × *P. alba* mechanosensitive gene *PtaZFP2*. When we compared these phylogenetic relationships with the classification proposed for the *A. thaliana* C1-2i subclasses,<sup>3</sup> Group G1 comprised *STZ/ZAT10* (gi.15217692),<sup>33</sup> *ZAT6* (gi.15237692)<sup>34</sup> and *AZF3* (gi.15239195)<sup>33</sup> genes of *A. thaliana*, previously grouped in the C1-2i subclass by Englbrecht *et al.*<sup>3</sup> In our study, the other *A. thaliana* subclasses (C1-2iB and C1-2iC) were found in Group G2. The *P. trichocarpa* sequences POPTR\_0009s03280, POPTR\_0001s24250, POPTR\_1833s00200, POPTR\_0008s05110 and POPTR\_0010s21650 had closest similarity with *ZAT7* (gi.15231335), *ZAT12* (gi.21593615) and *At2g28710* (gi.15226942) *A. thaliana* genes (C1-

2iB subclass), whereas the other poplar sequences of group G2 shared similarity with *ZAT 5* (gi.15242772) and *At3g10470* (gi.15228303) genes of *A. thaliana* (subclass C1-2iC). No poplar sequence corresponding to the *A. thaliana* C1-2iA (including the *ZAT11* gene-gi.1418335) was found in this analysis.

### 3.2. C2H2 zinc finger domains and other conserved motifs

For a better analysis of these phylogenetic relationships, we searched all the sequences for conserved motifs. Eight motifs including the ZFs were identified using the MEME algorithm and MUSCLE multiple sequence alignments (Fig. 1c). For each sequence of the tree, we manually annotated the absence or presence of each motif (Figs 1a, b and 2; Supplementary data 1). Interestingly, in each protein, the first and second ZF differed in their sequences and in particular by AAs localized in the C-terminus of the conserved QALGGH motif. The first ZF was characterized by a QALGGH R/K A/T S motif, whereas the second was characterized by QALGGH M/K RXH R/Y. These





**Figure 2.** (a) Phylogenetic tree of the poplar two-fingered Q-type C2H2-ZFPs gene family using poplar Phytozome blast against the *PtaZFP2* AA sequence and corresponding primers used for expression analyses. Presence of motifs described in Fig. 1c is symbolized with a coloured square just after the sequence name; white squares symbolize absence of motif. (b) Distribution of conserved motifs along the poplar sequences.

different signatures in the two ZFs had already been detected in *A. thaliana* and *Petunia* genomes. The first ZF was called Q2-2 or type A ZF for *A. thaliana* and *Petunia*, respectively, and the second ZF was called Q2-3 or type B.<sup>3,8</sup> Also, the signatures in the different ZFs varied appreciably between the two phylogenetic groups G1 and G2. In Group G1, the first ZF was QALGGHKA/TS, whereas in Group G2, it was QALGGHRA/TS. For the second ZF, the signature was QALGGHKRXHY in Group G1, but corresponded to QALGGHMRXHR in Group G2. Inside Group G1, five sequences out of the 72 sequences possess the first ZF of Group G2. These several proteins possessed a “mixed” signature forming a monophyletic group distinct from the other Group G1 proteins. Thus two

subgroups, G1a and G1b, could be defined (Fig. 1a). For poplar sequences, the discrimination between the first and second ZF and between Group G1 and Group G2 was strict (Fig. 2a). In *Petunia*, *in vitro* binding analysis revealed that the conserved QALGGH motif played a critical role in DNA binding activity.<sup>7</sup> Other work showed that the flanking residues at the C-terminus of QALGGH motif were important for the specific DNA recognition.<sup>8</sup> Also, the optimal binding sites of the *Petunia* ZPT2-2 protein corresponded to the AGC(T) sequence for the first ZF and to the CAGT core for the second ZF,<sup>35</sup> confirming the role of the residues flanking the QALGGH motif in the specificity of the DNA target-sequence recognition and in DNA binding. The function of the

different isoforms of Q-type ZFPs proteins inside the same species is not well understood. It is debatable whether the different signatures in the C-terminus of the ZF between Group G1 and Group G2 confer an ability to regulate different sets of genes and different plant regulation pathways.

Outside of the ZF motifs, a conserved DLN motif was found to be present at the C-terminus of all the sequences except in 11 sequences of Group G2, all belonging to monocot species. Two of these monocot sequences corresponded to TaZFP2 and TaZFP34, transcription factors of Q-type C2H2 subfamily in *T. aestivum*.<sup>11</sup> In their study, the authors reported that these two sequences of 135 AA and 176 AA, respectively, were full length and effectively lacked a DLN motif, like 20 other *T. aestivum* Q-type sequences out of the 27 full-length two-fingered proteins identified. Apart from POPTR\_1833s00200, which is probably a partial EST, all poplar sequences contain this domain (Fig. 2a). This DLN motif was first reported as the DLN box in *A. thaliana* Q-type C2H2 gene subfamily.<sup>33,34</sup> This DLN-box domain has been shown to confer a repression activity to these transcription factors.<sup>16,36,37</sup> Also, the DLN box corresponds to the EAR domain (ethylene-responsive element-binding factor (ERF)-associated amphiphilic repression domain) found in the ethylene-responsive element binding factor and auxin/indole-3-acetic acid families of transcriptional regulators.<sup>38</sup> Recent *in silico* analysis revealed two distinct conservation patterns of the EAR domain: LxLxL and DLNxxP. Interestingly, in all the poplar sequences, a proline (P) was found three or four AAs after the DLN motif, and a leucine (L) was present before the aspartate (D). The plant proteins containing these motifs are involved in developmental, hormonal and stress signalling pathways.<sup>38</sup> Although it should be tested experimentally, these results suggest that most of the poplar two-fingered Q-type C2H2 proteins could also be transcriptional repressors.

Two lysine-rich motifs were found: the KG/RKRS/TKR domain was localized in the N-terminal part of the proteins before the first ZF and the KKPK domain at the end of the first ZF (Fig. 1c; Supplementary data 3). The first lysine-rich motifs has already been described in *A. thaliana* C2H2 proteins (named the B box) and may function as targeting signals for the translocation of proteins to the nucleus (NLS-nuclear localization signal).<sup>33</sup> As the C2H2-ZFPs are known mainly as transcription factors, nuclear localization is necessary for their function. Most of the Q-type proteins of the phylogenetic tree (Supplementary data 1) contained either the first motif (Group G1) or the second motif (Group G2). However, part of the proteins from Group G2, including the proteins homologous to the C1-2iC subclass of *A. thaliana*, possessed both motifs.

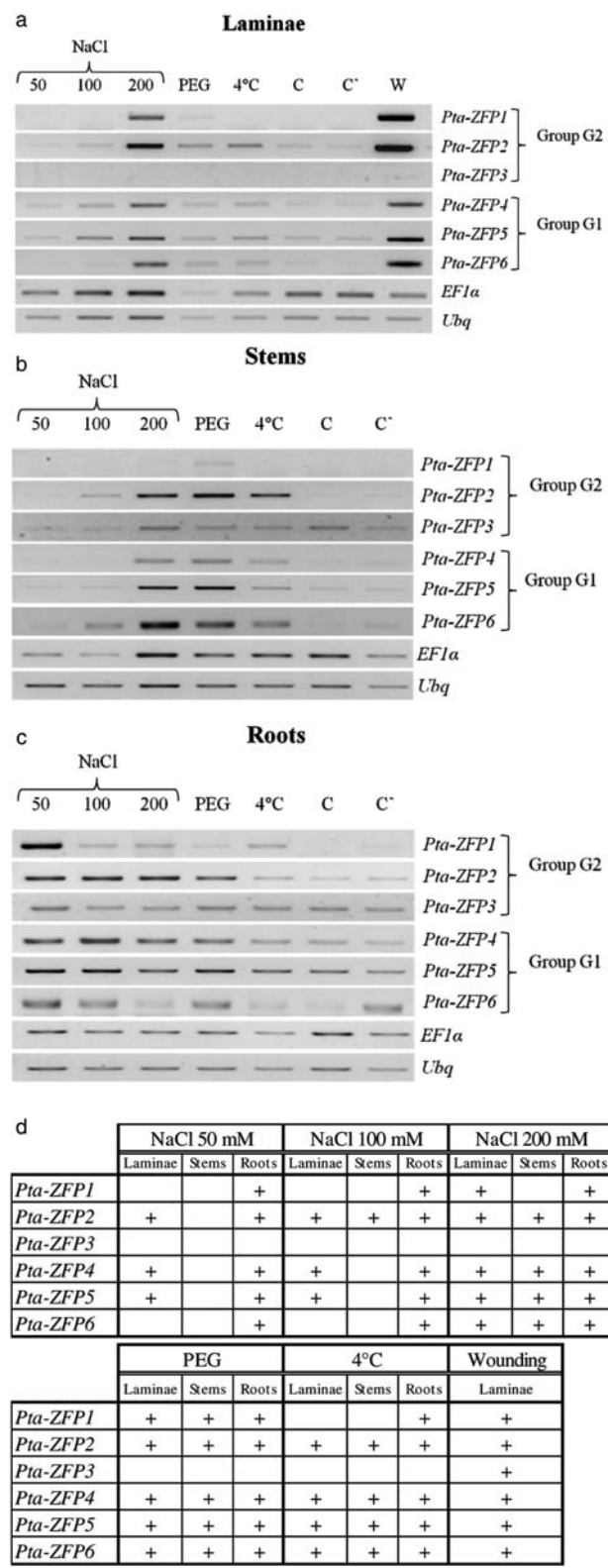
Some monocot proteins of both Group G1 and Group G2 lacked these motifs, in particular the proteins that also lacked the DLN box. However, some plant transcription factors may lack an NLS, and they are thought to be imported into the nucleus by dimerizing with proteins that possess these signals.<sup>39</sup> Alternatively, these proteins could be involved in interaction with RNA or other proteins as described for some of the ZFPs<sup>2</sup> and do not need nuclear localization.

Another motif found to be widely distributed among Q-type C2H2 proteins corresponds to the CLIMLAR domain (Fig. 1c), localized between the first lysine-rich motif and the first ZF (Supplementary data 3). This motif was present in all sequences except in some of the Group G2 monocot proteins. However, the CLIMLAR *sensu stricto* (CLIMLAR) domain was present only in a part of Group G1, again excluding any monocots. Such distribution of the motif *sensu lato* (C/S/Y L I/ M/L/V M/L/A L A/S R/Q/K/N/H) or *sensu stricto* may reflect a different evolutionary story. This motif has been described as the L-Box domain (EXEXXAXCLXXL) in some C2H2 *A. thaliana* proteins and has been thought to be involved in protein–protein interactions or in maintaining the protein folding.<sup>33</sup>

Finally, two other motifs were identified: the MALEAL domain localized in the N-terminal part of some G1 proteins and the LVDCHY domain located at the C-terminus end of some G2 proteins (Fig. 2b). The MALEAL motif was restricted to 42 out of the 50 eudicot proteins of Group G1 and the LVDCHY domain in 24 out of the 60 eudicot proteins of Group G2 (Fig. 1a and b). Interestingly, the MALEAL motif was completely absent in monocot sequences of Group G1, whereas the LVDCHY domain was detected in five monocot sequences of Group G2. The distribution between Group G1 and Group G2 of each motif was very strict: no Group G1 sequence possessed the LVDCHY motif and no Group G2 sequence possessed the MALEAL motif. This strict distribution reinforces the phylogenetic separation already shown with ZFs. These motifs have not been described before in other Q-type C2H2 proteins and no function has been assigned to these domains. However, such cluster distribution may indicate a distinct role for the corresponding proteins in regulation or response to specific stresses.

### 3.3. Expression pattern of poplar two-fingered Q-type C2H2-ZFPs in various organs and in response to different stresses

Numerous studies have shown a role for C2H2 Q-type genes in response to different environmental



**Figure 3.** Accumulation of *PtaZFP1*, *PtaZFP2*, *PtaZFP3*, *PtaZFP4*, *PtaZFP5* and *PtaZFP6* transcripts after different treatments in laminae (a), stems (b) and roots (c) of *P. tremula* × *P. alba* plants. Total RNAs were extracted from control plants (C and C') or from treated plants (NaCl 50, 100 or 200 mM, PEG 20% (w/v), cold treatment at 4°C or wounding (for laminae only) 1 h after the treatment. Changes in the abundance *PtaZFP1*, *PtaZFP2*,

stimuli.<sup>6</sup> In poplar, *PtaZFP2* mRNAs accumulated strongly and rapidly after stem bending, i.e. mechanical loading.<sup>22</sup> For a better characterization of this gene subfamily in poplar, we studied the expression of different poplar two-fingered C2H2-ZFPs in different organs (leaf laminae, stems, roots) and in response to various treatments [salt, osmotic stress (PEG), cold stress (4°C), wounding (W) and bending]. As our poplar model species corresponded to the *P. tremula* × *P. alba* hybrid, we searched for homologous sequences of *P. trichocarpa* genes in the PopulusDB database, where cDNA libraries were built from *P. tremula* or *P. tremula* × *P. tremuloides* (*Populus* section) samples. We then designed specific primers outside the conserved regions to amplify, by RT-PCR, several *P. tremula* × *P. alba* Q-type C2H2 sequences corresponding to different groups in the phylogenetic tree. Three of these genes are included in Group G2 of the poplar phylogenetic tree (Fig. 2a): *PtaZFP2*, isolated earlier,<sup>22</sup> is orthologous to POPTR-0001s24250, its closest homologous gene *PtaZFP1* homologous to POPTR-0009s03280 and *PtaZFP3* corresponding to POPTR-0010s21650 (Fig. 2a). Three other sequences were chosen in Group G1 (*PtaZFP4*, *PtaZFP5* and *PtaZFP6*) that correspond, respectively, to POPTR\_0009s09250, POPTR\_0001s30260 and POPTR\_0014s01760 (Fig. 2a). As shown in Fig. 3, all mRNAs isoforms were detected in laminae, stems and roots, except for *PtaZFP3* which were not detected in the laminae. *PtaZFP3* was found to be poorly regulated in all organs, whatever the applied treatments, and will not be described further. When poplar plants were not subjected to any treatment (Fig. 3 lines C and C'), mRNA levels of all genes were higher in roots except for *PtaZFP1*. mRNA levels of the 5 Q-type C2H2 genes increased strongly in laminae after wounding treatment (Fig. 3a). The higher salt treatment (200 mM) also induced mRNA accumulation for all genes tested in all organs. In laminae, this mRNA induction was also observed for two Group G1 genes (*PtaZFP4* and *PtaZFP5*) and for one Group G2 gene (*PtaZFP2*) for the smaller salt concentration (50 and 100 mM) (Fig. 3a). In roots, mRNA levels were high whatever the salt concentration tested (Fig. 3c). The various treatments were directly applied in the nutritive solution where roots were directly and continuously in stress conditions. In the case of osmotic stress with PEG treatment, mRNA levels increased for all genes in all organs. The osmotic stress induced by PEG

*PtaZFP3*, *PtaZFP4*, *PtaZFP5* and *PtaZFP6* mRNAs were analysed by RT-PCR using specific primers (Supplementary data 1). As a control, the expression of *EF1α* and ubiquitin (*Ubq*) genes are also shown. (d) Summary of the inductions of mRNA accumulation (+) observed in different agarose gels (a–c) according to each gene, organ and treatment.



treatment was close to the NaCl 200 mM treatment (hydraulic potential measurements:  $-1.3$  and  $-1$  MPa, respectively). mRNA induction was similar for the two treatments. Finally, the cold treatment induced all genes in laminae and stems except for *PtaZFP1* in laminae and stems. This study suggests that expression patterns are not clearly different between the different Q-type C2H2 genes in poplar whatever their position in the two phylogenetic groups. These results are in accordance with the role described for the *A. thaliana* Q-type C2H2 subfamily. For example, *ZAT10* gene (included in phylogenetic group G1) has been shown to be involved in the regulation pathway of osmotic, salinity and cold stresses.<sup>14</sup> Other transcriptomic analyses have described an important function of *A. thaliana* *ZAT12* gene (included in phylogenetic group G2) in strong light, osmotic, oxidative and also cold stresses.<sup>15,40</sup> However, in our study, all stresses (NaCl, PEG and cold) had an osmotic and therefore mechanical component. This may explain similarities in gene responses.

*PtaZFP2* has already been shown to be implicated in mechanical stress.<sup>22</sup> To test the putative role of other poplar Q-type C2H2 genes in response to mechanical loading, we subjected poplar plants to stem bending and studied the expression of the six genes in the stem at different times after treatment. As shown in Fig. 4, only the expression of *PtaZFP2* and *PtaZFP1* genes was strongly induced by mechanical loading in stems. The maximum mRNA accumulation occurred between 15 and 30 min after bending. Except for *PtaZFP3*, by increasing the cycle number of PCR, a small mRNA increase was also detected for the other poplar genes but to a much lower extent (data not shown). These variations were difficult to detect experimentally, but the plant might be able

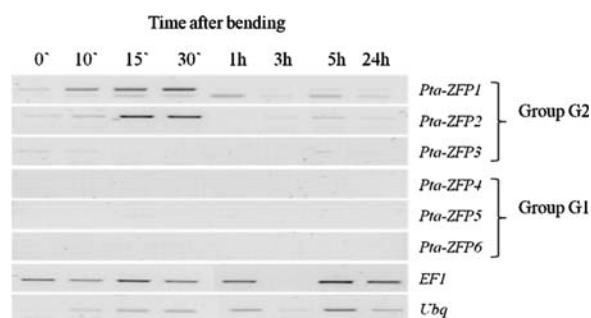
to perceive these slight inductions. Interestingly, *PtaZFP1* mRNA accumulation occurred earlier than for *PtaZFP2*. A previous work on *A. thaliana* suggested a possible cascade between the different C2H2 genes during a signalling pathway. For example, *ZAT12* is necessary for the expression of *ZAT7* in response to oxidative stress.<sup>41</sup> In the same way, a C2H2-ZFPs cascade might exist in poplar.

### 3.4. Upstream regulation of the two mechanosensitive genes *PtaZFP1* and *PtaZFP2*

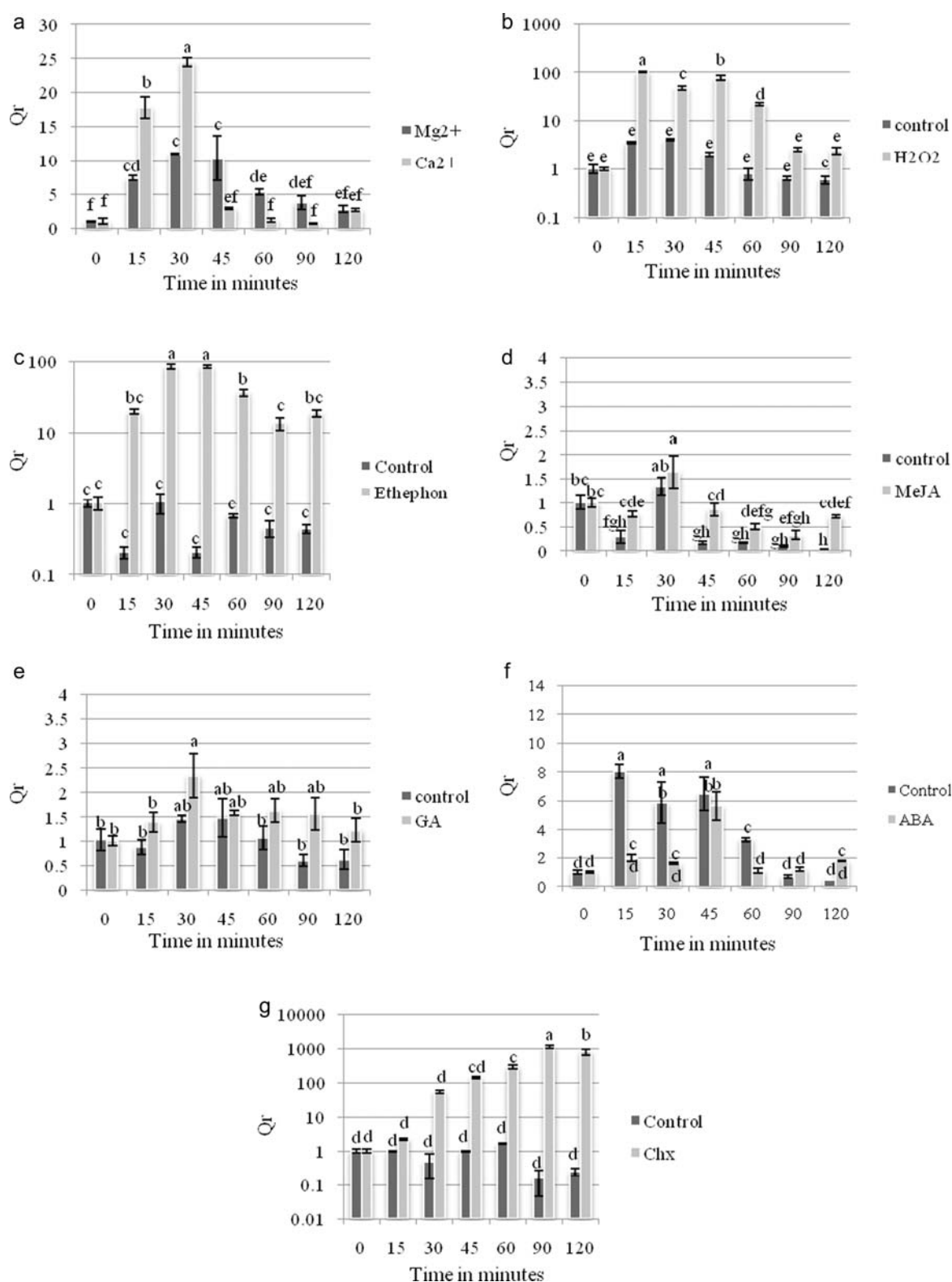
Whereas physiological responses to mechanical stress are well described,<sup>42–46</sup> little is known about the regulation of molecular actors of the mechanical signalling pathway. Calcium is known to be an important second messenger involved in numerous transduction pathways including mechanoperception,<sup>47</sup> and several calmodulin-like (TCH2 and TCH3) proteins are involved in mechanical response in *A. thaliana* (for review, see Braam<sup>44</sup>). Other signalling molecules such as ROS and phytohormones (including jasmonates and ethylene) have been implicated in the mechanical transduction pathway.<sup>48</sup> As shown in Fig. 4, *PtaZFP1* and *PtaZFP2* Q-type C2H2 genes are the most responsive to mechanical stress in *P. tremula* × *P. alba*. The corresponding orthologous genes of *P. trichocarpa* are very close in the phylogenetic tree, the proteins sharing 83.8% similarity. Also, the promoter sequence of *PtaZFP2* contains putative *cis*-elements related to these signalling molecules.<sup>22</sup> We therefore studied putative signals involved in the regulation of these two mechanical responsive genes by testing the effect of calcium, ROS, ethylene, MeJA, ABA, GA and Chx on the expression of both genes in poplar cell cultures (Figs 5 and 6 for *PtaZFP1* and *PtaZFP2*, respectively).

As shown in Fig. 5a, external calcium treatment (10 mM) seems to significantly induce *PtaZFP1* mRNA accumulation as little as 15 min after the treatment. In the case of *PtaZFP2*, the mRNA accumulation was weaker and not significantly different from the  $Mg^{2+}$  treatment used as control (Fig. 6a). In *A. thaliana*, some mechanosensitive genes have been shown to be regulated by calcium.<sup>49</sup> However, the calcium concentrations necessary to detect a significant increase in *TOUCH* genes expression were higher (50 mM, 100 mM) than those used in our study. With stronger but non-physiologic concentrations of  $Ca^{2+}$  (50 or 100 mM), *PtaZFP2* gene expression was also strongly induced (data not shown).

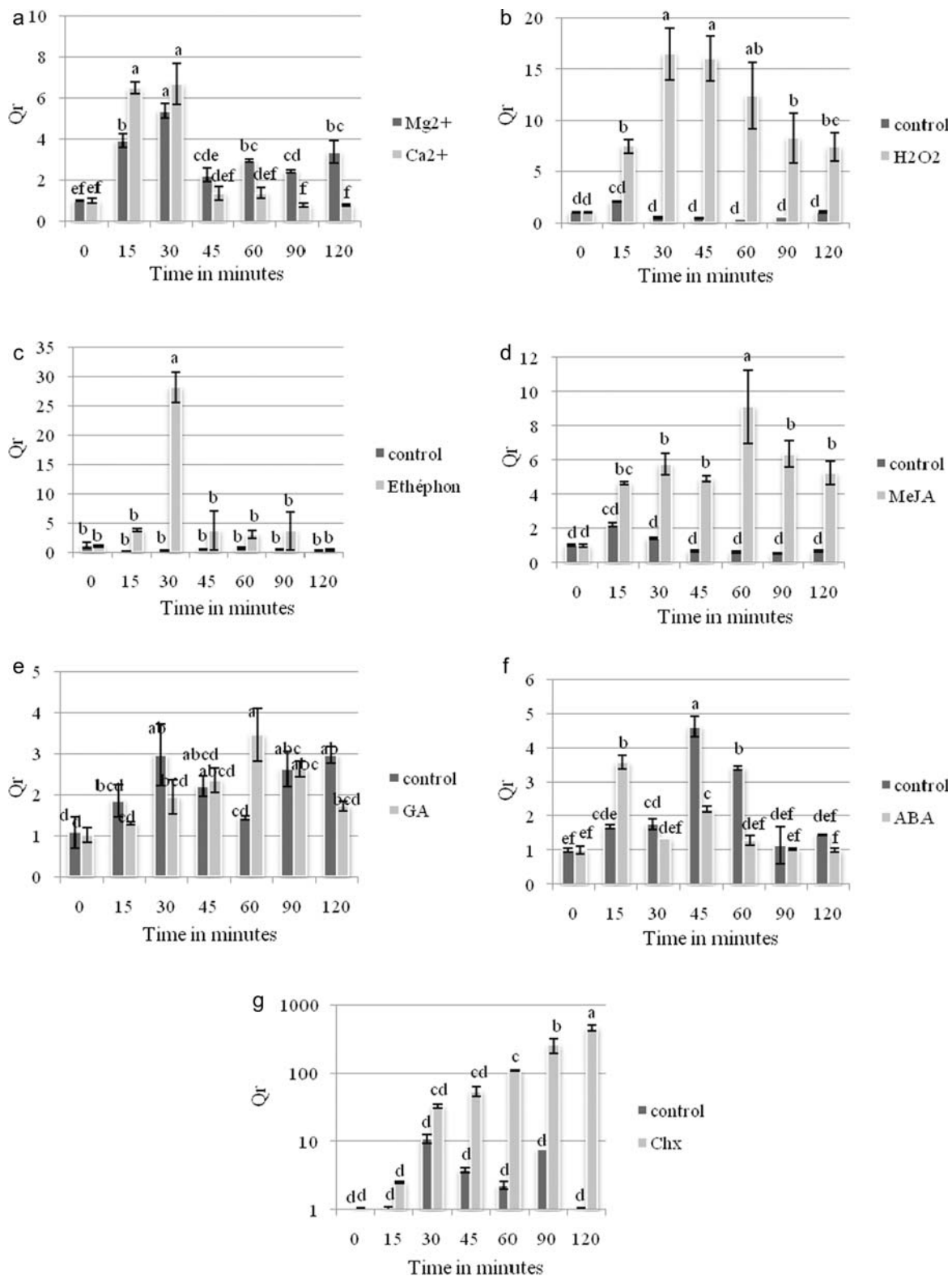
Concerning the effect of ROS, hydrogen peroxide ( $H_2O_2$ ) increased mRNA accumulation for both genes, with higher levels for *PtaZFP1* than for *PtaZFP2* (Figs 5b and 6b, respectively). Several Q-type C2H2 genes in other species have been shown to be responsive to  $H_2O_2$ .<sup>15,20</sup> Interestingly, transcriptome and



**Figure 4.** Time course accumulation of *PtaZFP1*, *PtaZFP2*, *PtaZFP3*, *PtaZFP4*, *PtaZFP5* and *PtaZFP6* transcripts after stem bending of *P. tremula* × *P. alba* plants. Total RNAs were extracted from the stems of control plants (C) and from bent stems at different times (10, 15, 30 min, 1, 3, 5 and 24 h). Changes in the abundance of *PtaZFP1*, *PtaZFP2*, *PtaZFP3*, *PtaZFP4*, *PtaZFP5* and *PtaZFP6* mRNAs were analysed by RT-PCR using specific primers (Supplementary data 1). As a control, the expression of *EF-1α* and ubiquitin (*Ubq*) genes are also shown.



**Figure 5.** Time course accumulation of *PtaZFP1* transcripts after different treatments of *P. tremula* × *P. alba* cells cultures. (a) Calcium 10 mM ( $Ca^{2+}$ ; magnesium was used as osmotic control,  $Mg^{2+}$ ), (b) Hydrogen peroxide 500  $\mu$ M ( $H_2O_2$ ), (c) Ethephon 1 mM, (d) MeJA 100  $\mu$ M, (e) GA 100  $\mu$ M, (f) ABA 100  $\mu$ M and (g) Chx 50  $\mu$ M. Total RNAs were extracted at different times (0, 15, 30, 45, 60, 90 and 120 min). The accumulation of relative transcripts ( $Q_r$ ) was determined by real-time quantitative PCR and it corresponds to the mean values of three independent experiments  $\pm$  SE. Significant differences ( $P < 0.05$ ) of responses between different times or treatment are indicated by different letters (Tukey's HSD test). For a better understanding, a semi-logarithmic scale has been used for b, c and g.



**Figure 6.** Time course accumulation of *PtaZFP2* transcripts after different treatments of *P. tremula* × *P. alba* cells cultures. (a) Calcium 10 mM (Ca<sup>2+</sup>; magnesium was used as osmotic control, Mg<sup>2+</sup>), (b) Hydrogen peroxide 500 μM (H<sub>2</sub>O<sub>2</sub>), (c) Ethephon 1 mM, (d) MeJA 100 μM, (e) GA 100 μM, (f) ABA 100 μM and (g) Chx 50 μM. Total RNAs were extracted at different times (0, 15, 30, 45, 60, 90 and 120 min). The accumulation of relative transcripts ( $Q_r$ ) was determined by real-time quantitative PCR and it corresponds to the mean values of three independent experiments ± SE. Significant differences ( $P < 0.05$ ) of responses between different times or treatment are indicated by different letters (Tukey's HSD test). For a better understanding, a semi-logarithmic scale has been used for g.



transgenic plant analysis has shown that the *A. thaliana* *ZAT12* and *ZAT7* genes, included in the same phylogenetic group as *PtaZFP1* and *PtaZFP2*, are associated with the response of plants to ROS during abiotic stresses.<sup>15,41</sup>

In hormonal treatments, to test the effect of ethylene, ethephon (known to release ethylene in solution)<sup>50</sup> was added to cell culture medium. Both *PtaZFP1* and *PtaZFP2* mRNA levels increased 15 min after ethephon application (Figs 5c and 6c). Whereas the mRNA accumulation was very transient for *PtaZFP2*, it was stronger and longer for *PtaZFP1*. Such induction of Q-type C2H2 genes by ethylene has already been reported in *C. annuum*<sup>20</sup> and an ethylene burst was observed in response to mechanical stress.<sup>45,51</sup> However, some reports suggest that ethephon could release both ethylene and H<sub>2</sub>O<sub>2</sub> into the medium<sup>52</sup> and so further analysis is needed to confirm the regulation of these genes by ethylene.

Among the other hormones tested (Figs 5d–f and 6d–f), only MeJA had a slight effect on *PtaZFP2* mRNA accumulation 30 min after the treatment (Fig. 6d). It has been demonstrated that wind and touch can induce the expression of lipoxygenase, the first enzyme of the biosynthesis pathway of jasmonate, in *T. aestivum*.<sup>53</sup> Also, the *STZ/ZAT10* gene of *A. thaliana* has recently been demonstrated to be involved in the jasmonic signalling pathway.<sup>54,55</sup> To our knowledge, no study has revealed the involvement of abscisic and GAs in the mechanical signalling pathway, in accordance with the results in this study.

As *PtaZFP1* and *PtaZFP2* gene expressions are induced very rapidly after stem bending, we tested whether they were primary genes by testing the effect of Chx, an inhibitor of protein synthesis in eukaryotic cells, alone or associated with ethephon. *PtaZFP1* and *PtaZFP2* mRNAs levels strongly increased with Chx treatment (Figs 5g and 6g) and by Chx associated with ethephon (data not shown). It thus seems that these genes may be primary genes whose expression may be regulated by the presence of a repressor on their promoters, as it has been demonstrated in other signalling pathway such as for genes responsive to auxin<sup>56</sup> or wounding.<sup>57</sup>

#### 4. Conclusion

Numerous studies have shown that Q-type C2H2 ZF proteins play a key role in plant acclimation to biotic and abiotic stresses.<sup>6</sup> However, the mode of regulation of C2H2 genes is complex and the specific role of each isoform in the different signal transduction pathways is still difficult to define. This study allowed the detection of two phylogenetic groups in

the Q-type C2H2 ZFPs in plants that are distinguished essentially in their different signatures at the C-terminus of their two QALGGH domains. As these protein domains are essential for DNA binding, these results suggest that proteins inside these two groups may regulate different sets of genes. Although the phylogenetic study was conducted only on the ZF-conserved motifs, two new motifs have been shown to be clearly associated with each group. These data confirm a phylogenetic relationship inside these groups. Concerning these two additional motifs MALEAL and LVDCHY revealed by this analysis, biochemical analyses of these proteins would help to define their importance in protein–protein interaction, protein folding or protein localization. The expression analysis of six poplar Q-type C2H2 ZFPs confirms their involvement in response to abiotic stresses such as cold, salt, osmotic and mechanical stresses. However, no real specificity of expression was detected between these genes towards these different stresses. However, as noted by Telewski,<sup>45</sup> a number of these abiotic stimuli induce plasma membrane deformation *via* turgor pressure variation and they can also be considered as mechanical stimuli similar to wind and gravity. Finally, despite the high protein similarity between *PtaZFP1* and *PtaZFP2* and the similar response of corresponding genes to mechanical stress, some differences have been revealed in their expression in response to signalling molecules such as calcium, H<sub>2</sub>O<sub>2</sub> and MeJA, suggesting their implication in different regulation pathways. It remains to be clarified how such signalling molecules could regulate these two genes in response to mechanical stress.

**Supplementary Data:** Supplementary data are available at [www.dnaresearch.oxfordjournals.org](http://www.dnaresearch.oxfordjournals.org).

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