Genome-wide identification and evolutionary analyses of the PP2C gene family with their expression profiling in response to multiple stresses in Brachypodium distachyon

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Abstract

Background: The type-2C protein phosphatases (PP2Cs), negatively regulating ABA responses and MAPK cascade pathways, play important roles in stress signal transduction in plants. Brachypodium distachyon is a new model plant for exploring the functional genomics of temperate grasses, cereals and biofuel crops. To date, genome-wide identification and analysis of the PP2C gene family in B. distachyon have not been investigated.

Results: In this study, 86 PP2C genes in B. distachyon were identified. Domain-based analyses of PP2C proteins showed that they all contained the phosphatase domains featured as 11 conserved signature motifs. Although not all phosphatase domains of BdPP2C members included all 11 motifs, tertiary structure analysis showed that four residues contributing to magnesium/manganese ions (Mg²⁺/Mn²⁺) coordination were conserved, except for two noncanonical members. The analysis of their chromosomal localizations showed that most of the BdPP2C genes were located within the low CpG density region. Phylogenetic tree and synteny blocks analyses among B. distachyon, Arabidopsis thaliana and Oryza sativa revealed that all PP2C members from the three species can be phylogenetically categorized into 13 subgroups (A–M) and BdPP2Cs were evolutionarily more closely related to OsPP2Cs than to AtPP2Cs. Segmental duplications contributed particularly to the expansion of the BdPP2C gene family and all duplicated BdPP2Cs evolved mainly from purifying selection. Real-time quantitative reverse transcription PCR (qRT-PCR) analysis showed that BdPP2Cs were broadly expressed in disparate tissues. We also found that almost all members displayed up-regulation in response to abiotic stresses such as cold, heat, PEG and NaCl treatments, but down-regulation to biotic stresses such as Ph14, Guy11 and F0968 infection.

Conclusions: In the present study, a comprehensive analysis of genome-wide identification and characterization of protein domains, phylogenetic relationship, gene and protein structure, chromosome location and expression pattern of the PP2C gene family was carried out for the first time in a new model monocot, i.e., B. distachyon. Our results provide a reference for genome-wide identification of the PP2C gene family of other species and also provide a foundation for future functional research on PP2C genes in B. distachyon.

Keywords: B. distachyon, Protein phosphatases 2C (PP2C), Phylogenetic analysis, Gene expression profiling
Background

Wild plants are usually consistently exposed to various environmental challenges such as drought, high salinity, extreme temperature, heavy metals and pathogen infections, which affect their growth and development. To adapt to these unpredictable environmental stresses, plants have evolved signaling mechanisms to transmit stimuli to different cellular compartments and then respond to these stresses. Accumulating evidence indicates that reversible protein phosphorylation catalyzed by protein kinases and phosphatases plays important roles in cellular stresses signal transduction in plants [1]. In the past decades, several protein kinases have been extensively investigated and proved to be positive regulation factors responding to a diversity of abiotic and biotic stresses [2–5]. In contrast to the protein kinases, protein phosphatases are not well investigated.

Protein phosphatases modify protein function by removing the phosphate group from phosphorylated proteins. They are divided into three major classes: tyrosine phosphatases, serine/threonine phosphatases and dual-specificity phosphatases (DSPTPs). According to distinct amino acid sequences, different dependencies on metal ions and sensitivities to inhibitors such as cyclosporine A and okadaic acid, protein serine/threonine phosphatases can be classified into the phosphor-protein phosphatases (PPP), phosphoprotein metallophosphatases (PPM) and aspartate-based protein phosphatases [6]. The PPP family includes type 1 (PP1), type 2A (PP2A), type 4 (PP4), type 5 (PP5), type 6 (PP6), type 7 (PP7) and type 2B (PP2B), whereas the PPM family is represented by the Mg2+- or Mn2+- dependent type-2C protein phosphatases (PP2Cs) [7].

PP2Cs, evolutionarily conserved and found in archaea, bacteria, fungi, plants and animals, are implicated in regulating stress-signaling pathways and act as negative modulators of protein kinase cascades activated by diverse stresses. For instance, a PP2C phosphatase, RsbP, in Bacillus subtilis dephosphorylates RsBV (an anti-anti-σ factor) and mediates the regulation during energy stress [8]. In Saccharomyces cerevisiae, PP2Cs play a negative role in the signal-transduction process responding to osmotic stress by counteracting the mitogen-activated protein kinase (MAPK) pathway [9]. Furthermore, one of the human PP2Cs was shown to inactivate the AMP-activated protein kinase (AMPK), a central component of the protein kinase cascade activated by ATP depletion [10]. In higher plants such as Arabidopsis, PP2C genes were demonstrated to regulate signaling pathways negatively by opposing the action of particular protein kinases. At least eight PP2Cs from subgroup A in Arabidopsis have been characterized as key factors in ABA signaling transduction. Briefly, subgroup A PP2Cs inactivate SnRK2 via dephosphorylation, and this inactivation is inhibited by ABA receptors, PYR/PYL/RCRA, in an ABA-dependent manner [11]. The subgroup B PP2C, AP2C1, interacts with MPK4 or MPK6 and subsequently suppresses MAPK activities during wounding as well as pathogen stresses [12]. The subgroup C PP2C POL or PLL1 interacts with the receptor kinase CLV1, inducing and maintaining stem cell polarity [13, 14]. The subgroup E PP2C AtPP2C6-6 interacts with Arabidopsis histone acetyl transferase GCN5 and controls the activation of stress-responsive genes in the stomatal signaling network [15–17]. The subgroup F PP2C WIN2 may interact with the bacterial effector HopW1-1 and regulate HopW1-1-induced plant resistance [18]. The unclustered PP2C KAPP interacts with different receptor-like protein kinases (RLKs) and is predicted to control plant immunity responses or hormone signaling [19, 20]. To date, there are 80 and 90 genes coding for PP2C proteins identified using bioinformatics surveys in Arabidopsis and rice, respectively [21–24]. However, few PP2C genes in monocots have been functionally investigated.

B. distachyon is a new model monocot for exploring the functional genomics of temperate grasses, cereals and biofuel crops. In 2010, the complete genome of the B. distachyon Bd21 was sequenced and the sequence data can be easily obtained via the Brachypodium Genome Resource (http://www.brachypodium.org) [25]. To our knowledge, a genome-wide analysis of the PP2C gene family in B. distachyon has not been reported so far. Here, we first identified 86 PP2C genes based on phosphatase domains analysis. Then, we further examined whether all phosphatase domains of BdPP2C members harbored magnesium/manganese ions (Mg2+/Mn2+) coordination residues through protein structural analysis. We also investigated BdPP2C genes chromosomal localization, constructed the phylogenetic tree of all BdPP2C genes based on their PP2C domains and categorized them into 13 subgroups. Subsequently, we analyzed the duplication events contributing to the expansion and functional divergences of the BdPP2C gene family. In addition, we examined the expression profiles of BdPP2C genes in different tissues and their responses to different phytohormone treatments as well as various abiotic and biotic stresses. Our results provide a foundation for future functional analysis of the PP2C gene family in stress responses in B. distachyon.

Results and discussion

Genome-wide identification and characterization of BdPP2C genes

Protein phosphatase PP2Cs are evolutionarily conserved [26]. During the evolution from prokaryotes to multicellular eukaryotes, the number of PP2C genes increased from one member to as many as 130 members. In previous reports, one PP2C member in Thermococcus, three
in *Synechococcus*, four in *Bacillus*, seven in *Saccharomyces*, 34 in *Chlamydomonas*, 51 in *Physcomitrella*, 57 in *Selaginella*, 80 in *Arabidopsis*, 90 in *Oryza* and ~130 in *Zea mays* were characterized [26]. The increase and expansion of PP2C genes from Archaea to higher plants may correlate with adaptations to complex environmental conditions for plants. To identify PP2C candidate genes in *B. distachyon*, the InterPro PP2C domain “IPR001932” was used to search the Plaza2.5 database (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v2_5/) and 88 putative PP2C genes were found. By using Pfam and SMART domain identification tools, we found that 2 of the 88 putative PP2C genes lacked PP2C catalytic domains. Therefore, 86 genes in *B. distachyon* were identified as PP2C family members. The 86 BdPP2C genes identified in this study encode proteins varying from 281 to 1087 amino acids in length, with wide variations in isoelectric point (pI) values from 4.24 to 9.31 and molecular weight from 30 kDa to 120 kDa. The subcellular localization prediction indicated that most of the BdPP2C proteins might be located in cytoplasm, chloroplast or nucleus, while only a few might be located in cytoskeleton or mitochondria (Table 1). A total of 86 PP2C genes were anchored to corresponding chromosomes and designated as *BdPP2C1-BdPP2C86* according to their order on the chromosomes.

The PP2C phosphatase catalytic domain has been reported to harbor 11 conserved motifs, in which 4 conserved residues contribute to Mg$^{2+}$/Mn$^{2+}$ coordination [27, 28]. So we further checked if BdPP2Cs’ phosphatase catalytic domain harbored 11 conserved motifs. The result obtained from multiple alignments of the 86 BdPP2C domains indicated that not all of the BdPP2C members contained all of the 11 conserved motifs. For example, Bradi1g54110, Bradi3g03870, Bradi3g43440 and Bradi2g27977 were found to be partial deletions in the C-terminal of PP2C phosphatase catalytic domain, thus probably resulting in elimination of a few important motifs and loss of functions. Further analysis showed that scattered amino acid residues also existed among conserved motifs in several BdPP2C members, which suggests that the amino acid composition of plant PP2C domains is quite complex (Additional file 1: Figure S1). In addition, the 3-D structure prediction of 86 BdPP2C domains also showed the diversities of PP2C proteins (Additional file 2: Figure S2). However, as shown in Additional file 1: Figure S1, the residues contributing to Mg$^{2+}$/Mn$^{2+}$coordination in BdPP2Cs are absolutely conserved. The conserved amino acid residues [x[D], [DGxxG], [DG] and [GxxDN] (D, aspartic acid; G, glycine; N, asparagine) were found within motifs 1, 2, 8 and 11, respectively. The predicted protein tertiary structures further revealed that these 4 conserved residues were spatially clustered together in 84 BdPP2Cs (designated as canonical BdPP2Cs), except Bradi1g54080 and Bradi1g54110, which were designated as noncanonical members (Fig. 1, Additional file 3: Figure S3).

**Phylogenetic, gene structural and protein domain analyses of BdPP2C**

To investigate the phylogenetic relationships of PP2C genes between *B. distachyon* and other plants, we constructed a phylogenetic tree based on the alignments of PP2C domains in *B. distachyon*, *Arabidopsis* and rice using the maximum likelihood (ML) method. The phylogenetic analyses indicated that the 80 BdPP2C proteins were divided into 12 subfamilies (A–L), which is consistent with the PP2C groups found in *Arabidopsis* and rice. Each subfamily tree includes PP2C protein from *B. distachyon*, *Arabidopsis* and rice (Fig. 2). Not surprisingly, the BdPP2C clustered together with those from rice because both *B. distachyon* and rice are monocots, while AtPP2C tended to form independent branches. Furthermore, the distributions of PP2Cs in each subfamily were similar among *B. distachyon*, *Arabidopsis* and rice except for the subgroups I and K (Table 2), which suggests that the PP2C gene family has evolved from one common ancestor in the three plants. The other three members, i.e., Bradi1g04540, Bradi1g54110 and Bradi1g54080, grouped into a new clade, M. In addition, the remaining three members, i.e., Bradi2g04550, Bradi4g27880 and Bradi2g40950, did not cluster with any other group, and each of them formed a single branch (Fig. 2).

To determine the phylogenetic relationships among the BdPP2C proteins, we constructed a phylogenetic tree from the alignments of the 86 PP2C domains identified above. As shown in Fig. 2 and Fig. 3a, all of the BdPP2C proteins still remained in the same subfamily. Because the pattern diversity of exon/intron structure and protein domain often plays an important role in the evolution of gene families, the patterns of exon/intron structure of BdPP2C genes and conserved domains were examined according to their phylogenetic relationships. The investigation of exon/intron structures revealed that the majority of members in the same subfamily shared similar exon numbers and different exon and intron lengths. However, three members in the M group, consisting of 5, 13 and 17 exons, were largely distinct in exon/intron arrangements, although their phylogenetic relationship was supported by 100 % bootstrap value. More interestingly, almost all BdPP2Cs lacking introns were members of the K subgroup, and the number of PP2C members in the K subgroup in *B. distachyon* was four or two times more than that in *Arabidopsis* and rice, respectively (Fig. 3b, Table 2). Previous studies suggested that genes lacking introns would rapidly evolve via gene duplication events [29–31]. Therefore,
Table 1 List of 86 BdPP2C genes and their basic characterizations

| Gene identifier | Gene name | Size (aa) | Mass (kDa) | pl   | Subcellular localization | Subgroup |
|-----------------|-----------|----------|-----------|------|--------------------------|----------|
| Bradi1g02920    | BdPP2C1   | 383      | 41.607    | 6.7  | cyto                     | D        |
| Bradi1g03690    | BdPP2C2   | 662      | 71.157    | 5.62 | chlo                     | C        |
| Bradi1g04520    | BdPP2C3   | 474      | 50.021    | 8.36 | chlo                     | K        |
| Bradi1g04540    | BdPP2C4   | 566      | 61.298    | 6.36 | chlo                     | M        |
| Bradi1g07870    | BdPP2C5   | 379      | 41.509    | 9.31 | chlo                     | D        |
| Bradi1g16630    | BdPP2C6   | 381      | 40.055    | 8.83 | chlo                     | B        |
| Bradi1g16810    | BdPP2C7   | 432      | 46.273    | 7.5  | chlo                     | H        |
| Bradi1g19620    | BdPP2C8   | 476      | 49.814    | 5.29 | cyto                     | L        |
| Bradi1g24400    | BdPP2C9   | 428      | 46.447    | 5.83 | nucl                     | E        |
| Bradi1g26690    | BdPP2C10  | 290      | 31.918    | 6.01 | cyto                     | F        |
| Bradi1g30200    | BdPP2C11  | 392      | 43.45     | 8.77 | mito                     | D        |
| Bradi1g31080    | BdPP2C12  | 366      | 40.344    | 4.94 | nucl                     | I        |
| Bradi1g33900    | BdPP2C13  | 281      | 30.172    | 5.01 | cyto                     | F        |
| Bradi1g36330    | BdPP2C14  | 309      | 33.654    | 6.25 | cyto                     | K        |
| Bradi1g36690    | BdPP2C15  | 309      | 33.423    | 5.51 | cyto                     | K        |
| Bradi1g36920    | BdPP2C16  | 359      | 38.823    | 4.85 | cyto                     | G        |
| Bradi1g37500    | BdPP2C17  | 312      | 33.409    | 6.33 | chlo                     | K        |
| Bradi1g37530    | BdPP2C18  | 312      | 33.409    | 6.33 | chlo                     | K        |
| Bradi1g38670    | BdPP2C19  | 362      | 38.975    | 8.9  | chlo                     | F        |
| Bradi1g47710    | BdPP2C20  | 353      | 38.882    | 5.01 | cyto                     | G        |
| Bradi1g54080    | BdPP2C21  | 263      | 29.067    | 7.75 | extr                     | M        |
| Bradi1g54110    | BdPP2C22  | 690      | 75.85     | 7.81 | chlo                     | M        |
| Bradi1g60520    | BdPP2C23  | 450      | 48.306    | 5.45 | chlo                     | H        |
| Bradi1g64780    | BdPP2C24  | 433      | 46.283    | 7.02 | chlo                     | H        |
| Bradi1g66520    | BdPP2C25  | 387      | 40.918    | 6.63 | chlo                     | B        |
| Bradi1g66650    | BdPP2C26  | 625      | 68.08     | 6.37 | nucl                     | C        |
| Bradi1g66920    | BdPP2C27  | 405      | 43.564    | 5.59 | nucl                     | A        |
| Bradi1g70680    | BdPP2C28  | 403      | 43.323    | 8.57 | chlo                     | D        |
| Bradi1g71690    | BdPP2C29  | 582      | 60.563    | 4.28 | chlo                     | K        |
| Bradi1g75940    | BdPP2C30  | 399      | 44.021    | 9.12 | nucl                     | D        |
| Bradi2g03970    | BdPP2C31  | 321      | 34.228    | 4.95 | chlo                     | K        |
| Bradi2g11350    | BdPP2C32  | 386      | 42.481    | 4.81 | cyto                     | G        |
| Bradi2g13820    | BdPP2C33  | 470      | 50.126    | 8.81 | nucl                     | H        |
| Bradi2g14420    | BdPP2C34  | 424      | 44.985    | 5.35 | nucl                     | A        |
| Bradi2g14740    | BdPP2C35  | 503      | 54.522    | 4.67 | nucl                     | F        |
| Bradi2g15840    | BdPP2C36  | 385      | 41.206    | 5.66 | nucl                     | A        |
| Bradi2g18510    | BdPP2C37  | 374      | 40.455    | 5.63 | chlo                     | A        |
| Bradi2g27977    | BdPP2C38  | 340      | 37.233    | 7.5  | chlo                     | E        |
| Bradi2g27977    | BdPP2C39  | 315      | 34.512    | 5.3  | chlo                     | E        |
| Bradi2g38640    | BdPP2C40  | 385      | 41.564    | 4.69 | cyto                     | G        |
| Bradi2g38770    | BdPP2C41  | 546      | 59.207    | 5.74 | chlo                     | C        |
| Bradi2g40550    | BdPP2C42  | 656      | 73.068    | 5.96 | cyto                     | C        |
| Bradi2g40950    | BdPP2C43  | 390      | 42.049    | 6.41 | chlo                     | M        |
### Table 1 List of 86 BdPP2C genes and their basic characterizations (Continued)

| Accession   | Description | Log2 Ratio | Fold Change | Location |
|-------------|-------------|------------|-------------|----------|
| Bradi2g41950| BdPP2C44    | 480        | 49.799      | cyto     |
| Bradi2g43700| BdPP2C45    | 289        | 31.763      | cyto     |
| Bradi2g45470| BdPP2C46    | 392        | 42.104      | chlo     |
| Bradi2g54810| BdPP2C47    | 403        | 42.912      | nucl     |
| Bradi2g62650| BdPP2C48    | 384        | 42.197      | mito     |
| Bradi3g03480| BdPP2C49    | 380        | 39.678      | chlo     |
| Bradi3g03870| BdPP2C50    | 289        | 30.628      | chlo     |
| Bradi3g05990| BdPP2C51    | 372        | 40.923      | nucl     |
| Bradi3g08390| BdPP2C52    | 366        | 38.917      | chlo     |
| Bradi3g09560| BdPP2C53    | 361        | 38.717      | chlo     |
| Bradi3g10350| BdPP2C54    | 1087       | 120.827     | chlo     |
| Bradi3g18000| BdPP2C55    | 318        | 34.579      | chlo     |
| Bradi3g25590| BdPP2C56    | 282        | 29.87       | cyto     |
| Bradi3g32240| BdPP2C57    | 402        | 43.492      | nucl     |
| Bradi3g32480| BdPP2C58    | 395        | 43.639      | chlo     |
| Bradi3g39540| BdPP2C59    | 525        | 56.595      | chlo     |
| Bradi3g43360| BdPP2C60    | 521        | 55.581      | chlo     |
| Bradi3g43430| BdPP2C61    | 418        | 45.232      | nucl     |
| Bradi3g43440| BdPP2C62    | 290        | 31.948      | nucl     |
| Bradi3g46160| BdPP2C63    | 268        | 28.242      | cyto     |
| Bradi3g46430| BdPP2C64    | 452        | 48.558      | chlo     |
| Bradi3g48280| BdPP2C65    | 378        | 41.453      | mito     |
| Bradi3g49540| BdPP2C66    | 365        | 38.711      | cyto     |
| Bradi3g49550| BdPP2C67    | 324        | 36.604      | cyto     |
| Bradi3g52110| BdPP2C68    | 596        | 64.92       | chlo     |
| Bradi3g54290| BdPP2C69    | 360        | 40.2        | cysk     |
| Bradi4g03520| BdPP2C70    | 390        | 42.584      | cyto     |
| Bradi4g11517| BdPP2C71    | 1022       | 116.521     | cyto     |
| Bradi4g19660| BdPP2C72    | 438        | 47.799      | cyto     |
| Bradi4g21150| BdPP2C73    | 378        | 40.097      | chlo     |
| Bradi4g27880| BdPP2C74    | 360        | 39.49       | nucl     |
| Bradi4g28100| BdPP2C75    | 363        | 38.537      | nucl     |
| Bradi4g32230| BdPP2C76    | 441        | 48.086      | chlo     |
| Bradi4g37710| BdPP2C77    | 362        | 39.39       | nucl     |
| Bradi4g40490| BdPP2C78    | 392        | 41.132      | chlo     |
| Bradi4g44750| BdPP2C79    | 436        | 47.474      | cyto     |
| Bradi5g08530| BdPP2C80    | 519        | 57.198      | chlo     |
| Bradi5g11780| BdPP2C81    | 444        | 47.535      | chlo     |
| Bradi5g11980| BdPP2C82    | 282        | 30.706      | cyto     |
| Bradi5g14730| BdPP2C83    | 431        | 47.974      | cyto     |
| Bradi5g19410| BdPP2C84    | 393        | 43.381      | cyto     |
| Bradi5g21140| BdPP2C85    | 316        | 34.51       | chlo     |
| Bradi5g24530| BdPP2C86    | 284        | 30.813      | nucl     |

cyto cytoplasm, chlo chloroplast, nucl nucleus, mito mitochondria, extr extracellular, cysk cytoskeleton
expansion of the BdPP2C K subfamily may be associated with gene duplication events.

When analyzing the phosphatase domains, we found that other domains were associated with the main PP2C domain, including S-TKc (ser/thr kinase catalytic domain), FHA (forkhead associated domain) and CNB (cyclic nucleotide-binding domain). As shown in Fig. 3c, S-TKc was found in Bradi3g10350, Bradi4g15117 and Bradi2g40550; FHA was identified in Bradi1g54110 and Bradi1g04540; CNB was only present in Bradi3g10350. However, the transmembrane region identified in AtPP2Cs and OsPP2Cs was not found in any BdPP2Cs. The S-TKc or FHA domains have been found in several Arabidopsis and rice PP2C genes, including AT2G40860, Os01g36080 and Os11g37540 or At5G19280, Os03g59530 and Os07g11010. PP2Cs with FHA domain known as the KAPP (kinase-associated protein phosphatase) were reported to play important roles in interacting with RLK and have been characterized as the first downstream negative regulator of RLK in plant development in Arabidopsis [18]. Hence, it would be interesting to investigate the different biological functions of BdPP2Cs harboring these special domains.

**Chromosomal location and duplications of BdPP2C genes**

To analyze the genomic distribution of BdPP2C genes, we marked their approximate position on each chromosome based on the information obtained from the Brachypodium genome database. The results showed that all of the 86 BdPP2C genes were localized on five chromosomes and their distribution appeared to be uneven. Further analysis indicated that the number of BdPP2C genes on each chromosome was proportional to the chromosome length. 30, 18, 21, 10 and 7 BdPP2C genes were mapped on chromosome 1, 2, 3, 4 and 5, respectively (Fig. 4). We also marked the densities of CpG islands on each chromosome. As shown in Fig. 4, the deeper color region on chromosomes represents the higher density of CpG islands; the gray or white region on chromosomes represents the lower density of CpG islands. We found that most of the BdPP2C genes were located on gray or white regions, indicating that they might be transcriptionally active.

During evolutionary processes, large segmental duplications and small-scale tandem duplications are two major mechanisms to generate new genes, which contribute to the genome complexities in the plant kingdom [32]. Indeed, previous studies have reported that PP2C gene families mainly expanded through whole-genome and chromosomal segment duplications, both in rice and Arabidopsis [23]. In B. distachyon, paralogous relationship analysis showed that six major chromosomal duplication events happened among the B. distachyon chromosomes, covering 92.1% of the whole genome [25]. Closely related genes located within a distance of less than 200 kb on the same chromosome are defined as tandem duplications, otherwise they are segmental duplications [33]. According to this principle, 22 pairs of paralogous BdPP2C genes were found to be involved in segmental duplication events and no tandem duplication gene pairs were found in the BdPP2C family. Analysis of these 22 pairs of segmental duplication BdPP2C genes showed that 16 were located in 6 major duplicated chromosomal blocks (Fig. 5a). Amino acid alignment analysis indicated that two counterparts of each gene pair were from the same subgroup (Table 3). To determine the selection pressures for these duplicated BdPP2C genes, we calculated the substitution ratio of non-synonymous (Ka) to synonymous (Ks) mutations for these 22 pairs of duplicated BdPP2C genes. The ratio Ka/Ks can be used to measure the selection acting among the duplicated gene pairs. Commonly, if the value of Ka/Ks is less than 1, the duplicated gene pairs may evolve from purifying selection (also called negative
selection); Ka/Ks = 1 means neutral selection; while Ka/
Ks > 1 means positive selection [34]. The result showed
that Ka/Ks for 22 pairs of duplicated BdPP2C genes was
less than 1, suggesting that all duplicated BdPP2C genes
have evolved mainly from purifying selection (Table 3).
We also calculated the divergence time (as T = Ks/2λ)
among 22 pairs of duplicated BdPP2C genes based on a
clock-like rate of $6.5 \times 10^{-9}$ mutations per synonymous
site per year, as proposed previously [35]. The result in
Table 3 showed that divergence events of duplicated
BdPP2C genes were estimated to have occurred around
50.8–131.5 Mya (million years ago), and the majority of
gene pairs diverged long before the divergence time of
grass species (56–73 Mya) [25, 36]. This finding is
consistent with a previous report that genes involved in
conserved signal transduction regulation pathways are
preferentially retained [37], and this could explain why
many BdPP2C genes were retained during the long evo-
lutionary history. In addition, we examined orthologous
PP2C gene pairs between B. distachyon and Arabidopsis

**Fig. 2** Phylogenetic analysis of PP2C proteins among B. distachyon, Arabidopsis and rice. Alignments of 256 PP2C domains from B. distachyon, Arabidopsis and rice were performed with ClustalW2, and the phylogenetic tree was constructed using the ML method with MEGA 6.0
BdPP2C genes were highly expressed preferentially in stems; and two (BdPP2C73 and BdPP2C84) were highly expressed in leaves. However, none of the BdPP2C genes was highly preferentially expressed in roots (Fig. 6). The expression profiles data of BdPP2C genes in various tissues are listed in Additional file 4: Table S3. We also compared the 22 duplicated BdPP2C gene pairs and found that the expression pattern was very similar for 6 gene pairs, i.e., BdPP2C11/30, BdPP2C19/83, BdPP2C20/69, BdPP2C30/58, BdPP2C35/45 and BdPP2C55/85. On the other hand, the remaining 15 pairs showed differential expression patterns. Recent studies suggested that there was a positive correlation of duplicated gene pairs between the degree of differential expression and their divergence time, which meant that the longer the duplication, the more obvious the differentiation of the expression [39–41]. In our analysis, we found that the divergence times for these seven duplicated gene pairs with similar expression pattern were actually more recent (50.8–86.9Mya). These results were also consistent with the expression of AtPP2C and OsPP2C paralogous gene pairs, indicating the diversification of expression between duplicated PP2C gene pairs.

It has been demonstrated that subgroup A PP2Cs in Arabidopsis, foxtail millet and rice are transcriptionally up-regulated upon exogenous ABA treatment or stress conditions that stimulate ABA biosynthesis [23, 24, 42, 43]. Seven members of subgroup A AtPP2Cs have been characterized as negative regulators of ABA responses in ABA-mediated physiological processes [44–49]. In B. distachyon, subgroup A BdPP2Cs includes eight members (BdPP2C27, BdPP2C34, BdPP2C36, BdPP2C37, BdPP2C44, BdPP2C46, BdPP2C47 and BdPP2C75). The qRT-PCR result suggested that six of these genes were highly induced by exogenous ABA treatment, the exceptions being BdPP2C27 and BdPP2C34. Consistently, BdPP2Cs from subgroup A were also differentially up-regulated by drought, salt, cold, heat or H2O2 treatment. Among them, BdPP2C36, BdPP2C37 and BdPP2C44 were continuously up-regulated and remained at a high level upon all of these treatments. Both BdPP2C46 and BdPP2C47 were up-regulated by drought, salt, cold, or H2O2, whereas they were down-regulated by heat treatment. For BdPP2C75, the expression level increased under drought or salt treatment; however, cold, heat and H2O2 repressed its expression (Fig. 7). In addition, BdPP2C70 from subgroup D, BdPP2C13 from subgroup F and BdPP2C32 from subgroup G also exhibited strongly increased expression levels in response to ABA and abiotic treatments (Fig. 7), suggesting that they may participate in the regulation of ABA signaling pathways. Similarly, the expression profiles of BdPP2Cs in other subgroups under exogenous hormone or abiotic treatments for 3 h or 6 h were analyzed as well using qRT-PCR. The other two exogenous hormones, i.e., MeJA and SA, were also tested. The heatmaps derived from expression profiles revealed that about 50–80 % of the

### Table 2 The distribution of PP2C genes in Arabidopsis, rice and B. distachyon

| Subgroup of PP2C genes | Numbers of AtPP2Cs | Numbers of OsPP2Cs | Numbers of BdPP2Cs |
|------------------------|--------------------|--------------------|--------------------|
| A                      | 10                 | 10                 | 8                  |
| B                      | 6                  | 3                  | 4                  |
| C                      | 7                  | 6                  | 5                  |
| D                      | 9                  | 11                 | 9                  |
| E                      | 12                 | 12                 | 8                  |
| F                      | 13                 | 11                 | 11                 |
| G                      | 6                  | 8                  | 6                  |
| H                      | 3                  | 7                  | 7                  |
| I                      | 2                  | 12                 | 7                  |
| J                      | 2                  | 1                  | 1                  |
| K                      | 3                  | 6                  | 12                 |
| L                      | 2                  | 0                  | 2                  |
| M                      | 0                  | 0                  | 3                  |
| Single branch 1        | 1                  | 1                  | 1                  |
| Single branch 2        | 1                  | 1                  | 1                  |
| Single branch 3        | 2                  | 1                  | 1                  |
BdPP2C genes were induced by various abiotic conditions or ABA treatment. On the other hand, most of the BdPP2C genes were up-regulated within 3 h of treatment by MeJA or SA, whereas only 15 and 26 BdPP2C genes maintained the increase until 6 h, respectively. In our analysis, the expression profiles of BdPP2C genes under abiotic stresses, including cold, heat, drought and high salinity, showed differential and overlapping expression patterns. Previous studies have suggested that different stresses can activate the same genes in distinct signaling pathways [50, 51], which might be the result of the production of some common signaling component such as ABA or calcium triggered by different stress stimuli. Increasing these signaling components leads to activation of protein kinases, including MAPK, CDPK, CCaMK and SnRK2, and then regulates the activities of transcription factors, leading to downstream responses. Moreover, such overlapping expression patterns might be the basis of crosstalk between different pathways, promoting the complex signal pathways for plants under abiotic stress conditions. Furthermore, we treated the B. distachyon seedlings with CdCl2 and ZnCl2 solutions, two heavy-metal stresses, and analyzed the changes in transcript abundances of BdPP2C genes. The expression analysis revealed that 60% of BdPP2C genes shared a similar response pattern between CdCl2 and ZnCl2 treatments. For instance, the expression levels of BdPP2C2, BdPP2C13, BdPP2C30, BdPP2C32, BdPP2C46 and BdPP2C47 were up-regulated after 3 h and retained relatively high levels for 6 h when treated with CdCl2 and ZnCl2.
However, for BdPP2C37, BdPP2C39, BdPP2C40, BdPP2C43, BdPP2C55, BdPP2C69, BdPP2C77, BdPP2C82 and BdPP2C84, their expression was down-regulated after 3 h and then up-regulated or returned to basal levels after 6 h.

The previous study has reported that AtPP2CG1 is induced by drought, salt or exogenous ABA treatment and thus positively regulates salt tolerance in Arabidopsis [52]. In this study, BdPP2C32, a homolog of AtPP2CG1, was also found to be strongly responsive to ABA and abiotic treatments (Fig. 7). Interestingly, CdCl₂ and ZnCl₂ treatments resulted in an obvious increase in its expression, which indicates that PP2C members in the G group may be related to heavy-metal-resistant pathways in plants. In addition, we also investigated the expression levels of BdPP2Cs after phytopathogens’ infection. Bd21 seedling was infected with F. graminearum (F0968) and two M. grisea strains (Guy11, avirulent ACE1 genotype; PH14, virulent ACE1 genotype). As shown in Fig. 7, many BdPP2Cs were significantly down-regulated at 4 h and 12 h after infection with all three phytopathogens. Only 20 BdPP2Cs were up-regulated by a single phytopathogen within 4–12 h, such as BdPP2C13, BdPP2C14, BdPP2C25, BdPP2C42 and BdPP2C62. It has been demonstrated that the PP2C WIN2 (At4g31750), a homolog in Arabidopsis with BdPP2C13, can enhance the resistance to phytopathogen P. syringae [18]. Consistent with this, the expression of BdPP2C13 was remarkably increased after exogenous salicylic treatment and phytopathogen PH14 treatment. The expression profiles of BdPP2C genes under different treatments are listed in Additional file 4: Tables S4 and S5. These results indicate that PP2Cs may be involved not only in the regulation of abiotic stresses but also in biotic stresses.

As mentioned above, 22 pairs of paralogous BdPP2C genes involved in segmental duplication events have been found, which suggested that chromosome gene duplication may play an important role in the expansion and evolution of the BdPP2C gene family. It has been observed that segmentally duplicated genes displayed a greater degree of functional divergence, and they exhibited pseudo-functionalization, neo-functionalization and retention of gene functions [53]. We analyzed the expression pattern of 22 segmentally duplicated gene pairs under various stresses, which showed that 17 pairs of genes had very similar expression pattern, indicating the retention of
Fig. 5 (See legend on next page.)
their functions (Additional file 5: Figure S5). Most of the duplicated gene pairs were found to have a high amino acid sequence homology and share similar exon/intron structures. Although the diversification of expression occurred between duplicated PP2C gene pairs, their functions still were retained. This indicated that diversification of expression may occur after a very short period of time, while function diversification may need more time.

The relevance analysis between cis-acting element and stress-induced BdPP2C gene expression

Cis-acting elements in promoter regions of genes often play key roles in stress responses in plants. For example, ABA-responsive elements (ABREs) are responsive to ABA, drought or salt signals [54]. LTR is involved in low-temperature response and regulation [55]. TCA-element and CGTCA-motif have good correlation with the expression levels after MeJA and SA treatment, respectively [56]. Analyses of cis-acting elements in the promoter regions of the 86 BdPP2C genes showed that every B. distachyon PP2C gene carried one or more ABRE, HSE, LTR, TCA-element, and CGTCA-motif in their promoter regions, suggesting the significant relationship between BdPP2Cs and stress responses (Additional file 6: Figure S4). Among them, BdPP2C genes from subgroup A contained the greatest number of ABRE, which is consistent with Arabidopsis, foxtail millet and rice [23, 43]. Further analyses demonstrated that a good correlation existed between the number of the ABREs and the expression of BdPP2C in the majority of subgroups after 3 h or 6 h ABA treatment according to the statistical analysis. Similarly, this correlation was also found to exist between the number of ABREs and the expression levels after MeJA and SA treatment, respectively [56].

Table 3: BdPP2Cs present in segmental duplication in B. distachyon genome

| Locus ID   | Subgroup | Ka    | Ks    | Ka/Ks | Divergence time (Myr) |
|------------|----------|-------|-------|-------|------------------------|
| BdPP2C2/26 | C        | 0.52  | 1.00  | 0.52  | 76.9                   |
| BdPP2C3/56 | K        | 0.68  | 1.55  | 0.438709677 | 119.2               |
| BdPP2C7/24 | H        | 0.10  | 0.76  | 0.131578947 | 58.5                |
| BdPP2C11/30| D        | 0.2   | 0.78  | 0.25641026 | 60                   |
| BdPP2C13/50| F        | 0.11  | 1.34  | 0.082089552 | 103.1               |
| BdPP2C19/53| F        | 0.28  | 0.75  | 0.373333333 | 57.7                |
| BdPP2C20/69| G        | 0.14  | 1.13  | 0.123893805 | 86.9                |
| BdPP2C25/6 | B        | 0.38  | 1.51  | 0.251655629 | 116.2               |
| BdPP2C27/47| A        | 0.35  | 0.76  | 0.460526326 | 58.5                |
| BdPP2C29/56| K        | 0.25  | 1.00  | 0.25   | 76.9                   |
| BdPP2C30/58| D        | 0.15  | 0.88  | 0.170454545 | 67.7                |
| BdPP2C32/40| G        | 0.22  | 1.12  | 0.1964285  | 86.2                |
| BdPP2C34/44| A        | 0.25  | 1.41  | 0.177304965 | 108.5               |
| BdPP2C35/45| F        | 0.15  | 0.66  | 0.227272727 | 50.8                |
| BdPP2C36/46| A        | 0.31  | 0.74  | 0.418918919 | 56.9                |
| BdPP2C37/44| A        | 0.32  | 1.65  | 0.193939394 | 126.9               |
| BdPP2C55/85| F        | 0.12  | 0.84  | 0.142857143 | 64.6                |
| BdPP2C60/72| E        | 0.46  | 1.71  | 0.269005848 | 131.5               |
| BdPP2C64/76| H        | 0.28  | 1.03  | 0.27184466 | 79.2                 |
| BdPP2C69/32| G        | 0.61  | 1.00  | 0.61   | 76.9                   |
| BdPP2C73/78| B        | 0.41  | 0.82  | 0.5    | 63.1                 |
| BdPP2C76/81| H        | 0.34  | 1.30  | 0.2615384 | 100                 |
levels after salt or drought treatment (Additional file 7: Figure S6A–F). This result indicates that BdPP2C genes may be involved in drought or salt stress through the ABA-dependent pathway.

Conclusions
In the present study, beyond a comprehensive analysis of phylogenetic relationships, gene structures, conserved domains, chromosomal locations, gene duplications, cis-elements and expression patterns of the PP2C gene family was carried out for the first time in a new model monocot i.e., B. distachyon, one of the key features of our results was the multiple level approaches to identify BdPP2C members from sequence alignment, motifs and domain analysis to 3D protein structure confirmation (Fig. 1). Another key feature of our result was the expression patterns of the BdPP2C genes indicated that almost all members displayed up-regulation in response to abiotic stresses such as cold, heat, PEG and NaCl treatments, but down-regulation to biotic stresses such as Ph14, Guy11 and F0968 infection (Fig. 7).

Methods
Database searching and sequence retrieval
To identify PP2C candidates in B. distachyon, the InterPro domain “IPR001932” was used to search the Plaza2.5 database (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v2_5/). The resulting protein sequences were manually examined with Pfam (http://pfam.sanger.ac.uk/search) and SMART (http://smart.embl-heidelberg.de/) to confirm the presence of the PP2C domains [57]. Arabidopsis PP2C protein sequences were downloaded from the TAIR database (https://www.arabidopsis.org). PP2C protein sequences of rice were downloaded from the Rice Genome Annotation Project Database (https://rice.plantbiology.msu.edu/). The molecular weight (MW) and theoretical isoelectric point (pI) of the PP2C candidates were calculated using the Compute pI/MW tool in the ExPASy server (http://web.expasy.org/compute). The WoLF PSORT program (http://expasy.org/resources/search/keywords:subcellular%20location) was used to predict protein subcellular localization. The prediction of protein tertiary structure was done with the Phyre2 tool (http://www.sbg.bio.ic.ac.uk/phyre2/) [58].

Phylogenetic analysis, exon/intron analysis and domain analysis
For phylogenetic tree construction, amino acid sequences of PP2C domains in B. distachyon, Arabidopsis and rice were aligned using the Clustal W2 program [59], and then phylogenetic trees were constructed using the ML method based on the JTT matrix-based model with MEGA 6.0 [60]. The data for the phylogenetic tree were deposited in Treebase Web (Accession URL: http://purl.org/phylo/treebase/phyloows/study/TB2:517847/YOU). The exon/intron structures of the PP2C gene candidates were examined using the online Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.ch) based on their corresponding genomic sequences [61]. The conserved
domains were predicted using the InterPro database (http://www.ebi.ac.uk/interpro/).

Chromosomal locations and synteny analysis
The chromosomal locations of PP2C genes were derived from the Plaza2.5 database. For synteny analysis, we investigated gene duplication events of PP2C genes in the B. distachyon genome and among B. distachyon, Arabidopsis and rice genomes from the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/index/locus) [62]. Then the synteny blocks were illustrated with CIRCOS software (http://circos.ca/software/download/circos) [63].

Plant materials and hormone, abiotic-and biotic-stress treatments
B. distachyon Bd21 was cultured in 1/4 Hogland’s solution at 25 °C/22 °C (day/night) with supplemental lighting for two weeks. Young roots, stems and leaves were harvested for tissue-specific expression analysis. Before the stress treatments, the 2-week-old seedlings were soaked in deionized water for two hours. For abiotic stress treatment, the seedlings were incubated in a solution containing 20 % PEG, 200 mM NaCl, 10 mM H2O2 or 100 μM ZnCl2/CdCl2. The cold or heat treatment was applied by incubating seedlings in a beaker containing water pretreated to 4 °C or 42 °C, respectively. For phytohormone analysis, the seedlings were cultured in deionized water containing 100 μM MeJA, 100 μM ABA or 1 mM SA. Whole seedlings were collected from each stress treatment after 0 h, 3 h and 6 h exposure. Phytopathogen treatment was conducted by spraying 2-week-old seedlings with Fusarium graminearum (F0968) or two Magnaporthe grisea strains (Guy11, avirulent ACE1 genotype; PH14, virulent ACE1 genotype) for 4 h and 12 h according to the previous report [55]. These tissues and seedlings were immediately frozen in liquid nitrogen and stored at −80 °C until used.

Quantitative reverse transcription PCR (qRT-PCR) and cluster analysis
The total RNA was extracted from plant tissues and seedlings using RNAiso Plus (Takara, Dalian, China)
according to the manufacturer’s instructions. About 1–2 μg total RNA was used to perform reverse transcription using the PrimeScript™ RT reagent kit with gDNA Eraser to remove genomic DNA contamination (Takara, Dalian, China). The resulting cDNA was diluted 20-fold with sterile distilled water. Quantitative PCR (qPCR) was performed in an ABI StepOne Real-Time Cycler (Applied Biosystems, USA) using 10 μL reaction volume containing 1 μL cDNA, 0.4 μL forward primer (10 μM), 0.4 μL reverse primer (10 μM), 0.2 μL ROX and 5 μL SYBR Premix Ex Taq™ II (Takara, Dalian, China). The following condition was used: pre-denaturation at 95 °C for 30 s, 40 cycles of amplification at 95 °C for 5 s and 60 °C for 30 s. Gene-specific primers was designed using the Primer5 program and their specificity was checked by blastN. The primers used for qPCR are listed in Additional file 4: Table S2 and actin was used as an internal control [56, 64, 65]. The data reported in this study were calculated based on three biological replicates. In addition, the expression profiles were calculated using the \( \Delta\Delta CT \) values \( \Delta\Delta CT = (\text{CT}_{\text{Control gene}} – \text{CT}_{\text{Control actin}}) – (\text{CT}_{\text{Treated gene}} – \text{CT}_{\text{Treated actin}}) \), and obtained by PermutMatrixEN version 1.9.3 software, and then shown by a green-red gradient. The data were statistically analyzed using OriginPro 7.5. The differentially expressed genes were defined as those showing up-or down-regulation greater than twofold with \( p \)-value less than 0.05. All qPCR data were submitted to the NCBI GEO dataset with accession number GSE70366.

### Promoter analysis

The 1500 bp promoter sequences of BdPP2C genes were obtained from the Plaza2.5 database. The cis-acting regulatory elements in these promoter sequences were analyzed using the PLANT CARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [66].

### Availability of supporting data

The qRT-PCR data supporting the genes expression results of this study are available in the NCBI GEO database with accession number GSE70366. The supporting tables for phylogenetic tree were deposited in Treebase Web (URL: http://purl.org/phylo/treebase/phylows/study/TB2:SI7847You).

### Additional files

- **Additional file 1: Figure S1.** The amino acid alignment of 86 BdPP2C domains in B. distachyon. (TIF 9808 kb)
- **Additional file 2: Figure S2.** The predicted 3-D structure of 86 BdPP2C domains in B. distachyon. (TIF 8875 kb)
- **Additional file 3: Figure S3.** The predicted tertiary structure of 86 BdPP2C domains in B. distachyon. (TIF 7796 kb)

### Additional file 4: Table S1.

The amino acid sequences of PP2C genes in B. distachyon, Arabidopsis and rice. Table S2. The primers used for real-time qPCR in this study. Table S3. The expression profiles of BdPP2C genes in various tissues. Table S4. The expression profiles of BdPP2C genes under abiotic or hormone treatment. Table S5. The expression profiles of BdPP2C genes under biotic stress. Table S6. The orthologous PP2C gene pairs between B. distachyon and Arabidopsis or B. distachyon and rice. (DOCX 308 kb)

### Additional file 5: Figure S5.

Expression pattern of duplication BdPP2C genes under biotic/abiotic stresses and phytohormone treatments. (TIF 9276 kb)

### Additional file 6: Figure S4.

cis-acting elements analysis in promoter sequences of 86 BdPP2C genes. (TIF 9862 kb)

### Additional file 7: Figure S6.

The correlation analysis between cis-acting elements and stress-induced expression of BdPP2C genes. (A) The correlation analysis between ABRE and ABA-induced expression of BdPP2C genes. The samples were treated with ABA for 3 h. (B) The correlation analysis between ABRE and ABA-induced expression of BdPP2C genes. The samples were treated with ABA for 6 h. (C) The correlation analysis between ABRE and NaCl-induced expression of BdPP2C genes. The samples were treated with NaCl for 3 h. (D) The correlation analysis between ABRE and NaCl-induced expression of BdPP2C genes. The samples were treated with NaCl for 6 h. (E) The correlation analysis between ABRE and PEG-induced expression of BdPP2C genes. The samples were treated with PEG for 3 h. (F) The correlation analysis between ABRE and PEG-induced expression of BdPP2C genes. The samples were treated with PEG for 6 h. (TIF 6349 kb)

### Abbreviations

ABRE: ABA-responsive element; AMPK: AMP-activated protein kinase; CAT: Ca2+/calmodulins-dependent protein kinases; CDPK: calcium dependent protein kinases; CNB: cyclic nucleotide-binding domain; DSPTPs: dual-specificity phosphatases; FHA: forkhead associated domain; KAPP: kinase associated protein phosphatase; MAPK: mitogen-activated protein kinases; MW: molecular weight; Mya: million years ago; PP1: protein phosphatase 1; PP4: protein phosphatase 4; PPs: protein phosphatase 5; PP6: protein phosphatase 6; PP7: protein phosphatase 7; PP2Aa: protein phosphatases 2A; PP2Bs: protein phosphatases 2B; PP2Cs: protein phosphatases 2C; Pf: isoelectric point; PM: phosphophosphatase; PP: phospho-protein phosphatase; PTPs: protein tyrosine phosphatases; qRT-PCR: quantitative reverse transcription-PCR; RLK: receptor like protein kinase; SnRK2: Nonfermenting1-related protein kinases2; S-TKc: Ser/thr kinase catalytic domain.

### Competing interests

The authors declare that they have no competing interests.

### Authors’ contributions

JC and ZC conceived of the study. JC, ZC, MJ and PL performed the bioinformatic analysis and carried out the experiments. JC and ZC wrote the manuscript. All authors read and approved the final manuscript.

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