Genome-wide characterization of the C2H2 zinc finger transcription factors in *Pleurotus ostreatus* and expression analyses under abiotic stress

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The C2H2-type zinc finger proteins (C2H2-ZFPs) have been reported regulating various developmental processes or abiotic stresses response in eukaryotes. However, there is no comprehensive analysis of these transcription factors in *P. ostreatus*. Identification and characterization of these proteins in *P. ostreatus* could be desired to be found candidate genes to control the development or abiotic tolerance breeding. In this study, 18 *C2H2-ZFs* were identified in *P. ostreatus* genome. The result of phylogenetic analysis indicating these proteins have a low similarity. And, a wide range of variations also displayed among these genes, including the protein characteristics, gene intron-exon structure, and motif composition. Then, the expression patterns of *PoC2H2-ZFs* in mycelia, primordia, young and mature fruiting bodies were investigated by RT-qPCR. Besides, the expressions of some *PoC2H2-ZFs* can be regulated by auxin and cytokinin. And, under heat and cold stresses, members of *PoC2H2-ZFs* expression levels are changed dramatically, suggesting these genes may participate in different abiotic stress responses. The findings of this study will facilitate the study of the *C2H2-ZFs* role in *P. ostreatus* development and stress tolerance.

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Abstract

16 The C2H2-type zinc finger proteins (C2H2-ZFPs) have been reported regulating various
17 developmental processes or abiotic stresses response in eukaryotes. However, there is no
18 comprehensive analysis of these transcription factors in *P. ostreatus*. Identification and
19 characterization of these proteins in *P. ostreatus* could be desired to be found candidate genes to
20 control the development or abiotic tolerance breeding. In this study, 18 *C2H2-ZFs* were
21 identified in *P. ostreatus* genome. The result of phylogenetic analysis indicating these proteins
22 have a low similarity. And, a wide range of variations also displayed among these genes,
23 including the protein characteristics, gene intron-exon structure, and motif composition. Then,
24 the expression patterns of *PoC2H2-ZFs* in mycelia, primordia, young and mature fruiting bodies
25 were investigated by RT-qPCR. Besides, the expressions of some *PoC2H2-ZFs* can be regulated
26 by auxin and cytokinin. And, under heat and cold stresses, members of *PoC2H2-ZFs* expression
27 levels are changed dramatically, suggesting these genes may participate in different abiotic stress
28 responses. The findings of this study will facilitate the study of the *C2H2-ZFs* role in *P.*
29 *ostreatus* development and stress tolerance.

30

31 **Keywords:** *Pleurotus ostreatus*; C2H2-ZFPs; expression pattern; development; abiotic stress

32

Introduction

34 *Pleurotus ostreatus* is a widely cultivated mushroom for its nutritional value, relatively simple
35 cultivation techniques, and wide sources of cultivation materials(Chang & Miles, 2004; Khan &
36 Taina, 2012). The production of *P. ostreatus* relies on the precise control of fruiting body
37 development. The formation of fruiting bodies starts with two different mating types of hyphae
38 combined and form dikaryotic hyphae, which is called plasmogamy. These dikaryotic hyphae
39 can be aggregated to develop into primordia, which further differentiate into fruiting bodies.
40 Genome sequencing of model mushroom *Schizophyllum commune* indicating many predicted
41 transcription factors like zinc finger proteins (ZFPs), MYB, fungal specific transcription factor

42 (fst), and so on, are differentially expressed during sexual development (Ohm et al., 2010). In *P.*
43 *ostreatus*, the *Pofst3* was cloned as homolog gene of *S. commune fst3* and functional analysis has
44 characterized its role during primordia formation (Qi et al., 2019). These results indicating that
45 transcription factors may play an important role in mediating the development of *P. ostreatus*.
46 However, only a few transcription factors have been reported in this commercial mushroom.
47 Therefore, identification and characterization of more transcription factors in *P. ostreatus* can
48 provide the opportunity to identify the interesting proteins importantly in various development
49 processes, which can be desired for breeding to control mushroom development.
50   ZFPs are one of the largest transcription factor families in eukaryotic genomes (Laity, Lee
51 & Wright, 2001). The term "zinc finger" refers to these proteins harbor a conserved domain, in
52 which consisting of cysteine (C) and/or histidine (H) residues responsible for bonding with a zinc
53 ion comprising a two-stranded antiparallel beta-sheet and a helix (Takatsuji 1998). Based on the
54 number and location of the C and H residues, the ZFPs can be divided into different types:
55 C2H2, C2HC, C2HC5, C2C2, C3H, C3HC4, C4, C4HC3, C6, and C8 (Berg & Shi 1996).
56 Among them, C2H2-type zinc finger proteins (C2H2-ZFPs) are the ones most widely studied.
57 The zinc finger domain in these proteins containing two C and two H residues, which are
58 described as C-X(2-4)-C-X12-H-X(3-5)-H (where X represents any amino acid) (Pabo, Peisach
59 & Grant, 2001).
60   The C2H2-ZFPs have been widely studied in plants, functional analysis has shown that
61 *C2H2-ZFs* are involved in vegetative growth and reproductive development (An et al., 2012; Lu
62 et al., 2012; Sun et al., 2015). Besides, there also have been demonstrated the role of *C2H2-ZFs*
63 in mediating growth and development in fungi, including hyphal growth (Tian et al., 2017),
64 sexual development (Kim et al., 2009), oospores production (Wang et al., 2009), and so on.
65 Moreover, the *c2h2*-overexpression strains did not affect the normal development in *Agaricus*
66 *bisporus*, however, the yield per day of the strains peaked 1 day earlier, indicating the impact of
67 *C2H2-ZFs* in mushroom formation (Pelkmans et al., 2016), which makes a target gene for
68 breeding of this commercial mushroom. However, there are no findings conducted about *C2H2-*
69 *ZFs* roles in *P. ostreatus* development so far.
70   Apart from participating in regulating various development processes, *C2H2-ZFs* also have
71 been found to play crucial roles in defense to abiotic stress. In plants, they have been shown to
72 respond to heat (Mittler et al., 2006), and functional analysis has shown they are positively
73 improving drought (Yin et al., 2017), cold (Liu et al., 2017), and salt stress (Ciftci-Yilmaz et al.,
74 2007). In China, the traditional greenhouses are mainly used for cultivating *P. ostreatus*, which
75 poorly in environmental conditions control. Environmental stress, especially heat, always
76 threatening the development of mushrooms. The extreme and continuous high temperature can
77 inhibit mycelial growth (Yan et al., 2020), disruption of the cell wall integrity, and enhance the
78 ability of *Trichoderma asperellum* to infect mycelia (Qiu et al., 2018), then reduce the yield of
79 mushrooms. Therefore, identification of *C2H2-ZFs* in *P. ostreatus* also can be desired to find
80 candidate transcription factors for breeding to improve the ability to defend against
81 environmental stress.
82   Therefore, the *C2H2-ZFs* in *P. ostreatus* genome were identified and characterized by
83 bioinformatic analysis in this study. Then, the expression profiles of them in different tissues
84 were analyzed in order better understand their potential roles in regulating *P. ostreatus*

85 development. Besides, we also analyzed their expression pattern under environmental stress. The
86 results of the work would add useful information about the characterization of *C2H2-ZFs* in
87 mushrooms, and also providing with gene sources for control the development and abiotic
88 tolerance breeding in *P. ostreatus*.
89

Materials & Methods

Identification and characteristics of C2H2-ZFPs in *P. ostreatus*

92 First, the Hidden Markov Model (HMM) profile of the C2H2 domain sequences (PF00096) was
93 downloaded from the Pfam database and used as a query in the HMMER3.0 program against the
94 publicly available genome database of *P. ostreatus* which download from JGI
95 (http://genome.jgi.doe.gov/PleosPC15_2/) to search for C2H2-ZFPs with an *E*-value less than
96 $1e^{-4}$. Then, the candidate C2H2-ZFPs were submitted to SMART (http://smart.embl-
97 heidelberg.de) to confirm the presence of the C2H2 domain. Furthermore, the C2H2 domain
98 without the expression of "C-X$_{(2-4)}$-C-X$_{12}$-H-X$_{(3-5)}$-H" in protein were deleted by manual, and the
99 rest were regarded as PoC2H2-ZFPs. The subcellular localizations of PoC2H2-ZFPs were
100 predicted in WoLF PSORT (http://wolfpsort.org/), and the ExPasy site
101 (http://web.expasy.org/protparam/) was used to calculate the molecular weight (MW) and
102 isoelectric point (pI).
103

Phylogenetic analysis and multiple sequences alignment

105 A phylogenetic tree among C2H2-ZFPs in *P. ostreatus* was constructed using the MEGA-X
106 program (Sudhir et al., 2018) via the neighbor-joining (NJ) method based on the JTT model with
107 bootstrapping was performed 1000 times.
108   Multiple sequence alignment of the full C2H2-ZFPs and C2H2 domain were performed
109 using MEGA-X and loaded into JaLview software to visualize (Waterhouse et al., 2009).
110

Gene structure and motif analysis

112 The exon-intron organization of *C2H2-ZF* genes was obtained from genomic information and
113 drawn using Tbtools (Chen et al., 2020). Then, the proteins were submitted into online MEME
114 (http://meme-suite.org/tools/meme) to identify conserved motifs with 5 motif numbers, and the
115 optimum motif length was set with the default parameters (6-50 residues).
116

Strains, culture conditions, and sample collection

118 The strains *P. ostreatus 3125* provided by the Institute of Scientific Edible Fungal, Gaoyou,
119 China, was used for culture and sample collection. The fungi were grown in potato dextrose agar
120 (PDA) medium was transfer to sterility fully-boiled wheat grain medium and cultured at 25 °C in
121 the dark in a temperature-controlled incubator. Then, after 5 days later, put a little wheat grain
122 with mycelium into sterility growth bags, composed of 60% cottonseed hulls, 35% corncob, 10%
123 bran, 3% gypsum, 2% kalk, with 55% humidity, and continued cultured in the temperature-
124 controlled incubator. Waiting for mycelium growth fully in growth bags (Fig. 3a), the mycelium
125 was collected. To obtaining primordia, the growth bags were transferred to the culture room with

126 the condition was: 10-13 °C, 80% humidity. And the young and mature fruiting bodies were
127 collected while primordia beginning differentiates into fruiting bodies at the 6th and 12th days,
128 respectively (Fig. 3a). All the samples collected were frozen in liquid nitrogen immediately and
129 stored at -80 °C.
130
131 **Hormones and abiotic stress treatments**
132 The primordia were selected for treatment with hormones and environmental stress to analyze
133 the response of *PoC2H2-ZFs*. For hormones treatment, the primordia were covered with
134 absorbent cotton which was soaked with 200 ul 0.01 mM IAA, 0.01 mM zeatin, and H_2O,
135 respectively. Then, sampled after 1, 3 hours (h). For environmental stress treatment, the
136 primordia were cultured at 38 °C and 4 °C in a temperature-controlled incubator for 1, 3 h,
137 respectively, for heat and cold stress treatment, while cultures at the culture room with the
138 condition: 10-13 °C used as controls.
139
140 **Isolation of RNA, cDNA synthesis, and RT-qPCR analysis**
141 Total RNA from collected samples were isolated using Plant Total RNA Isolation Kit (Sangon
142 Biotech Co., Ltd, ShangHai). The cDNA was generated using MightyScript First Strand cDNA
143 Synthesis Master Mix (Sangon Biotech Co., Ltd, ShangHai) according to the manufacturer's
144 protocol. In addition, 2X SG Fast qPCR Master Mix (Sangon Biotech Co., Ltd, ShangHai) was
145 used to perform RT-qPCR. The *sar* gene was used as the reference (Castanera et al., 2015) and
146 primer sequences used for RT-qPCR were listed in Table S3. The relative expression level of
147 genes was analyzed using the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method. The heatmap of *PoC2H2-ZF* genes was
148 generated using Tbtools (Chen et al., 2020) .
149
150 # Results
151 **Identification, characterization, and phylogenetic analysis of C2H2-ZFPs in *P.***
152 ***ostreatus***
153 Based on the HMM results and manual correction, 18 C2H2-ZFPs were identified in the *P.*
154 *ostreatus* genome. All these proteins contained one to four conserved C2H2 domains where were
155 distributed in the N-terminus or C-terminus (Fig. S1). Detailed information about characteristics
156 of these proteins, like amino acid size, MW, isoelectric points, and so on, were also analyzed
157 (Table S1). The results showed PoC2H2-ZFPs had between 149 and 688 amino acids in
158 polypeptide length, with MW ranging from 16.4 (*PleosPC15_2|1089905*) to 74.8 kDa
159 (*PleosPC15_2|1054163*) and isoelectric points ranging from 4.66 (*PleosPC15_2|1079678*) to
160 10.8 (*PleosPC15_2|1089905*). According to the results of predicted subcellular localization, all
161 the PoC2H2-ZFPs were predicted as nuclear proteins (Table S1).
162   To analyze the phylogenetic relationships of these C2H2-ZFPs among *P. ostreatus*, a
163 phylogenetic tree was constructed based on their full protein sequences. As a result, the 18
164 PoC2H2-ZFPs were clustered into four groups (Fig. 1a). PleosPC15_2|1104202 form a single-
165 branch clade, indicating its independent origin. The bootstrap supports of some main branches
166 were very low. Besides that, most PoC2H2-ZFPs in the same group showed low bootstrap values
167 (<60%). Only PleosPC15_2|1111338 and PleosPC15_2|1095114 had strong bootstrap (100%),

168 which regard as one sister pairs. The results of sequence alignment indicating the PoC2H2-ZFPs
169 showed low sequence similarity (Fig. S1).

170

171 **Gene structure and conserved motif analysis of *PoC2H2-ZFs***
172 To gain insights into the genetic structure of *PoC2H2-ZFs*, the exon-intron organization of them
173 was analyzed. The results showed the diverse gene structures were exhibited in *PoC2H2-ZFs* and
174 also appeared to be more variable in the same group (Fig. 1b). The number of introns varied
175 from 0 to 4 in group Ⅰ, 1 to 2 in group Ⅱ, *PleosPC15_2|1104202* showed 5 exons and 4
176 introns, and in group Ⅳ had 1 to 7 introns (Fig. 1b).
177   To further investigate the diversity of the PoC2H2-ZFPs, the motif composition was
178 analyzed using MEME online server (Fig. 1c). The results identified five putative conserved
179 motifs (while the number was set beyond five, the *E*-value of the motif (X >5) was greater than
180 one. Data was not shown). Motif 1 was predicted encoding the conserved region (C-X_2-C-X_{12}-H-
181 X_3-H) that corresponds to the characteristic motif of C2H2 domain (Table S2), were detected in
182 N-terminus or C-terminus of all PoC2H2-ZFPs (Fig. 1c). And, the motif 3 also represented one
183 type of C2H2 domain (C-X_4-C-X_{12}-H-X_3-H) (Table S2), and the PleosPC15 2|1046937,
184 PleosPC15 2|1079678, and PleosPC15 2|1091415 in group Ⅰ, PleosPC15 2|1077016,
185 PleosPC15_2|1051309, PleosPC15_2|1054163, and PleosPC15_2|157229 in group Ⅳ contained
186 it in sequences (Fig. 1c). Motif 2 was predicted encoding a false motif of the C2H2 domain that
187 lacks a conserved H residue in the C-terminus (Table S2), also showed distributed in 17
188 PoC2H2-ZFPs (Fig. 1c). From the data, the PoC2H2-ZFPs were showed diverse motif
189 compositions. In group Ⅰ, PleosPC15_2|1107157, PleosPC15_2|1088285, and
190 PleosPC15 2|1089005 containing the motifs 1 and 3, while motifs 1, 2, and 3 were contained in
191 others (Fig. 1c). For group Ⅱ, the PleosPC15_2|160383 only had one motif 1 (Fig. 1c). Among
192 them, the PleosPC15 2|1111338 and PleosPC15 2|1095114 also containing motifs 4 and 5 in N-
193 terminus and C-terminus, respectively (Fig. 1c). In group Ⅳ, the PleosPC15_2|1102393,
194 PleosPC15_2|1095325, and PleosPC15_2|1053338 share the same motif composition that
195 containing motifs 1 and 2 in the N-terminal region, and others also containing one motif 3 in the
196 C-terminal region (Fig. 1c).

197

198 **Conserved domain analysis of the PoC2H2-ZFPs**
199 To investigate the characteristics of C2H2 domains in *P. ostreatus*, multiple sequence alignment
200 was performed to analyze conservative amino acids in the C2H2 domain. The result revealed
201 there were 23-26 amino acids in 29 predicted C2H2 domains (Fig. 2a). The variation of sequence
202 length was due to the amino acid numbers changed in C-$X_{(2-4)}$-C and H-$X_{(3-5)}$-H region. Among
203 them, 15 C2H domains contained two amino acids in the C-$X_{(2-4)}$-C region while others
204 contained four amino acids (Fig. 2a). 24 C2H2 domains contained three amino acids, 3 contained
205 four amino acids, and 2 contained five amino acids in the H-$X_{(3-5)}$-H region (Fig. 2a). Two C and
206 H residues were conserved in domains. Besides that, the F and L residues also showed the most
207 conservative (Fig. 2a). The signature sequences of C-$X_{(2-4)}$-CX_3FX_5LX_2H$_{(3-5)}$H in C2H2 domains
208 was corresponding to the conserved amino acid analysis of motif 1 (Fig. 2b), suggesting this
209 feature motif was conserved in PoC2H2-ZFPs.

210

Expression analysis of *PoC2H2-ZFs* during different developmental stages

To investigate the expression profiles of the *PoC2H2-ZFs* during different developmental stages, a heatmap was displayed based on the RT-qPCR result by analyzing their expression patterns at four different tissues (mycelia, primordia, young fruiting body, and mature fruiting body) (Fig. 3a). The result showed *PoC2H2-ZFs* had distinct spatial and temporal expression patterns. All of the *PoC2H2-ZFs* had a continuous expression in four different tissues (Fig. 3b). The *PeosPC15_2|1046937*, *PeosPC15_2|1107157*, *PeosPC15_2|1102653*, *PeosPC15_2|1077016,* and *PeosPC15_2|1053338* were showed lowest expression level in four tissues (Fig. 3b). Besides that, most *PoC2H2-ZFs* expression is relatively higher in mycelia, primordia, and young fruiting body, but lower in the mature fruiting body (Fig. 3b). The *PeosPC15_2|1077016*, *PeosPC15_2|112393*, and *PeosPC15_2|1088285* had high expression in mycelia. Five genes (*PeosPC15_2|1079678*, *PeosPC15_2|1089905*, *PeosPC15_2|1111338*, *PeosPC15_2|1095114*, and *PeosPC15_2|1095325*) exhibited the increased expression from the mycelia to primordia (Fig. 3b). The expression level of all *PoC2H2-ZFs* was increased when the primordia differentiate into the fruiting body (Fig. 3b). While the fruiting body turning to ripen, most of the *PoC2H2-ZFs* decrease the expression, only the expression of three genes (*PeosPC15_2|1088285*, *PeosPC15_2|1089905*, and *PeosPC15_2|1102653*) increased continuously (Fig. 3b).

Expression analysis of *PoC2H2-ZFs* response to auxin and cytokinin

To study the response of *PoC2H2-ZFs* under hormones, the primordia was treatment with IAA and zeatin for 1 h and 3 h, respectively. Twelve genes were selected to study their expression profiles, and in our results, only the expression of *PeosPC15 2|1046937* was not affected treatment with auxin or cytokinin, other members were showed regulated by the hormones (Fig. 4). Like *PeosPC15_2|1091415* and *PeosPC15_2|1102653*, their expression was downregulated treatment with zeatin for 1 h, while *PeosPC15_2|1079678* and *PeosPC15_2|1089905* was increased (Fig. 4). In addition, four genes (*PeosPC15_2|1079678*, *PeosPC15_2|1095114*, *PeosPC15 2|1051309*, and *PeosPC15 2|157229*) was upregulated treatment with zeatin after 3 h (Fig. 4). In auxin treatments, the expression of *PeosPC15_2|1079678* was increased after 1 h. Other members, like *PeosPC15_2|1079678*, *PeosPC15_2|1089905*, and *PeosPC15_2|1095114*, were increased after 3 h, while *PeosPC15_2|1102653* was downregulated (Fig. 4). From our data, we can see that *PoC2H2-ZFs* have different expression responses to auxin and cytokinin.

Expression analysis of *PoC2H2-ZFs* under different abiotic stresses

Then, to investigate the potential roles of *PoC2H2-ZFs* in abiotic stress, their expression profiles were analyzed under heat and cold stress. The results showed that the expression of fiv genes (*PeosPC15_2|1079678*, *PeosPC15_2|1089905*, *PeosPC15_2|1095114*, *PeosPC15_2|1051309*, and *PeosPC15_2|157229*) was upregulated after heat stress treatment with 1 h (Fig. 5). The *PeosPC15_2|1102653* showed downregulated with heat treatment after 3 h, while the expression of *PeosPC15_2|1102653* and *PeosPC15_2|1051309* was increased (Fig. 5). For cold treatments with 3 h, the expression of *PeosPC15_2|1046937* and *PeosPC15_2|1102653* were significantly suppressed, whereas *PeosPC15_2|1111338*, *PeosPC15_2|1051309*, *PeosPC15_2|1089905*, *PeosPC15_2|157229*, and *PeosPC15_2|1104202* was increased (Fig. 5). There are two *PoC2H2-*

254 *ZFs* (*PeosPC15_2|1091415* and *PeosPC15_2|1053338*) that showed the expression was not
255 affected after 1, 3 h with cold and heat treatment (Fig. 5). These data suggesting that *PoC2H2-*
256 *ZFs* have different expression responses to abiotic stresses.

257

Discussion

259 The C2H2-ZF proteins as one largest transcription factor that widely exited in the eukaryotic
260 kingdom have been reported to play important roles in mediating plant growth and stress
261 response (An et al., 2012; Ciftci-Yilmaz et al., 2007; Liu et al., 2017; Lu et al., 2012; Sun et al.,
262 2015; Yin et al., 2017). Moreover, there also have been demonstrated the role of *C2H2-ZFs* in
263 fungi growth and development, like hyphal growth (Tian et al., 2017), microsclerotia formation
264 (Tian et al., 2017), sexual development (Kim et al., 2009), and so on. In particular, its effect on
265 changing the yield of *Agaricus bisporus* (Pelkmans et al., 2016), one of the widely cultivated
266 commercial mushrooms, making the *C2H2-ZFs* be important candidate genes for breeding edible
267 mushrooms. *P. ostreatus* is one of the widely cultivated mushrooms in China, however, the study
268 about this important transcription factor is limited in it. In this paper, a systematic analysis of
269 *C2H2-ZFs* was performing in *P. ostreatus*, including phylogenetic relationships, gene structures,
270 conserved motifs, expression patterns during growth and development, and under various
271 hormones and abiotic stress treatments.

272   In this study, 18 *C2H2-ZFs* were identified in *P. ostreatus* through genome-wide analysis.
273 The proteins were further divided into four subfamilies based on the phylogenetic relationship
274 analysis. However, the low bootstrap values were displayed among C2H2-ZFPs in each
275 subfamily (Fig. 1a), which we thought may due to the low sequence similarity of non-C2H2
276 domain sequences (Fig. S1). Our results showed the characteristics of *PoC2H2-ZFs*, including
277 molecular weight, PI values, protein length, exon and intron number, and motif composition
278 (Table S1, Fig. 1b and 1c), have a wide range of variations among proteins, suggesting the high
279 diversity in structural and physicochemical properties maybe existed in *PoC2H2-ZFs*. These data
280 suggesting that *PoC2H2-ZFs* may have distinct origins and showed diversity in function.

281   Conserved "QALGGH" sequences have been reported unique to the plant C2H2 domain
282 (Takatsuji, 1999). In many plants, it was detected in most C2H2-ZFPs (Cao et al., 2016; Hu et
283 al., 2019). In *P. ostreatus*, there was not found such conserved sequences in C2H2-ZFPs (Fig.
284 2a). The result of sequence alignment indicating the C2H2 domain in *P. ostreatus* can describe
285 as C-$X_{(2-4)}$-$CX_3FX_5LX_2H_{(3-5)}$H, that F and L residues also showed the most conservative (Fig.
286 2a). Motif 1 encoding the characteristic motif of the C2H2 domain was detected distributed in all
287 PoC2H2-ZFPs (Table S2, Fig. 1c). The conserved amino acid analysis of motif 1 indicating the
288 signature sequences it encoded was corresponding to the result of sequence alignment (Fig. 2).
289 These results suggesting the C-$X_{(2-4)}$-$CX_3FX_5LX_2H_{(3-5)}$H sequences were conserved in PoC2H2-
290 ZFPs.

291   The *C2H2-ZFs* have been reported to participate in multiple growth and development
292 processes in fungi. Investigate the expression profiles of *PoC2H2-ZFs* can help us understand the
293 potential role of their involvement in growth and development in *P. ostreatus*. In *Verticillium*
294 *dahlia*, the loss function of the C2H2 transcription factor VdMsn2 leads to a significant
295 reduction in hyphal growth (Tian et al., 2017), indicating the impact of *C2H2-ZFPs* on hyphal

growth. In *P. ostreatus*, the *PeosPC15_2|1077016*, *PeosPC15_2|112393*, and *PeosPC15_2|1088285* were observed expression highly in mycelia (Fig. 3b), suggesting they may play an important role in mycelia growth. Previous studies have reported the *C2H2-ZFs* regulating the formation of primordia. Inactivation of *c2h2* in *S. commune* resulted in the aggregates formed, but couldn't further differentiate into primordia (Ohm et al., 2011). From our data, five genes (*PeosPC15_2|1079678*, *PeosPC15_2|1089905*, *PeosPC15_2|1111338*, *PeosPC15_2|1095114*, and *PeosPC15_2|1095325*) exhibited the increased expression from the mycelia to primordia (Fig. 3b), indicating they may be involved in the formation of primordia in *P. ostreatus*. Besides, *C2H2-ZFs* also have been reported involved in the development of the fruiting body. In *Aspergillus nidulans*, deletion of *nsdC*, a gene that encoded one C2H2 transcription factor, resulted in the loss of fruiting body formation (Kim et al., 2009). In our data, the expression of all *PoC2H2-ZFs* was increased from primordia to the young fruiting body (Fig. 3b), suggesting the *C2H2-ZFs* may play an important role in the development of the fruiting body in *P. ostreatus*. There are three genes (*PeosPC15_2|1088285*, *PeosPC15_2|1089905*, and *PeosPC15_2|1102653*) that showed the increased expression at the mature fruiting body (Fig. 3b), which implies that might be involved in the *P. ostreatus* ripening process. The *PsCZF1* gene encoding a *C2H2-ZFs* in *Phytophthora sojae* has been demonstrated involved in the production of oospores and swimming zoospores (Wang et al., 2009). Thus, the *PeosPC15_2|1088285*, *PeosPC15_2|1089905*, and *PeosPC15_2|1102653* seem to participate in the development of the spore (Fig. 3b).

In *Arabidopsis thaliana*, the *ZINC FINGER PROTEIN 5* (*ZFP5*) was reported can mediate cytokinin and ethylene effects on the root hairs formation and growth (An et al., 2012). *ZFP6* has been identified as essential regulators of trichome initiation by integrating gibberellin and cytokinin (Zhou et al., 2013). In addition, *GhWIP2* encoded one C2H2-ZFP that mediating cell expansion during organ growth by modulating crosstalk between auxin, gibberellins, and abscisic acid in *Gerbera hybrida* (Ren et al., 2018). These results indicating *C2H2-ZFs* can integrate various hormones that regulating the multiple aspects of development processes. Auxin can be produced by *P. ostreatus* (Bose, Shah & Keharia., 2013), and auxin and cytokinin have been reported to affect mycelia growth by exogenous treatment (Ramachela & Sihlangu, 2016). In this study, members of *PoC2H2-ZFs* expression were showed a significant response to auxin and cytokinin in primordia (Fig. 4). Therefore, our data indicating that auxin and cytokinin may be involved in *PoC2H2-ZFs* function in growth and development processes.

Previous studies have revealed that *C2H2-ZFs* increased resistance to abiotic stress in plants. In *Arabidopsis*, root growth in transgenic plants with constitutive expression of *Zat10* showed more tolerance to heat stresses (Mittler et al., 2006). Overexpression of *ZAT18* in *Arabidopsis* increased the ability to tolerate drought in transgenic plants, while mutation of this gene resulted in decreased drought tolerance (Yin et al., 2017). In soybean, the expression of the *C2H2-ZF* gene *GmSCOF-1* was induced by low temperature, and the overexpression lines were showed increased cold tolerance that increases expression levels of cold-responsive genes (Kim et al., 2001). For *P. ostreatus* cultivation, bad environmental conditions always influence the growth and development processes. Like high temperature, always inhibit mycelial growth, disruption of the cell wall integrity and increased the risk of fungal contamination, reduce the yield of mushrooms (Qiu et al., 2018). From our data, members of *PoC2H2-ZFs* could be

339 induced by heat and cold stress (Fig. 5), indicating this important transcription factor may have a
340 conserved function that responds to heat and cold in fungi. Notably, some genes like
341 *PeosPC15_2|1089905* and *PeosPC15_2|1102653* were found induced by heat or cold stress (Fig.
342 5), suggesting these genes may play a variety of roles in the response to various stresses.
343

Conclusions

345 In this study, we identified 18 *C2H2-ZFs* in *P. ostreatus* genome. However, a wide range of
346 variations was observed in their phylogenetic relationship, gene structure, motif composition,
347 and so on. The different expression profiles of *PoC2H2-ZFs* suggest they play diverse roles in
348 different tissue growth and development. In addition, hormones and abiotic stress induced
349 expression suggested that *PoC2H2-ZFs* may involve multiple processes that hormones regulated
350 as well as abiotic stress responses.
351

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355

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465

Figure 1

Phylogenetic relationships, gene structures, and conserved motifs analysis of *C2H2-ZFs* in *P. ostreatus.*

(a) The Phylogenetic tree of PoC2H2-ZFPs. The four major subfamilies are marked with different colored backgrounds and indicated by Roman numerals on the left. (b) Gene structures of *PoC2H2-ZF*. Exons are represented by green boxes, and introns by black lines. (c) Conserved motifs in PoC2H2-ZFPs. Five colored boxes represent the various putative motifs. The sequences of each putative motif encoded are shown in Table S2.
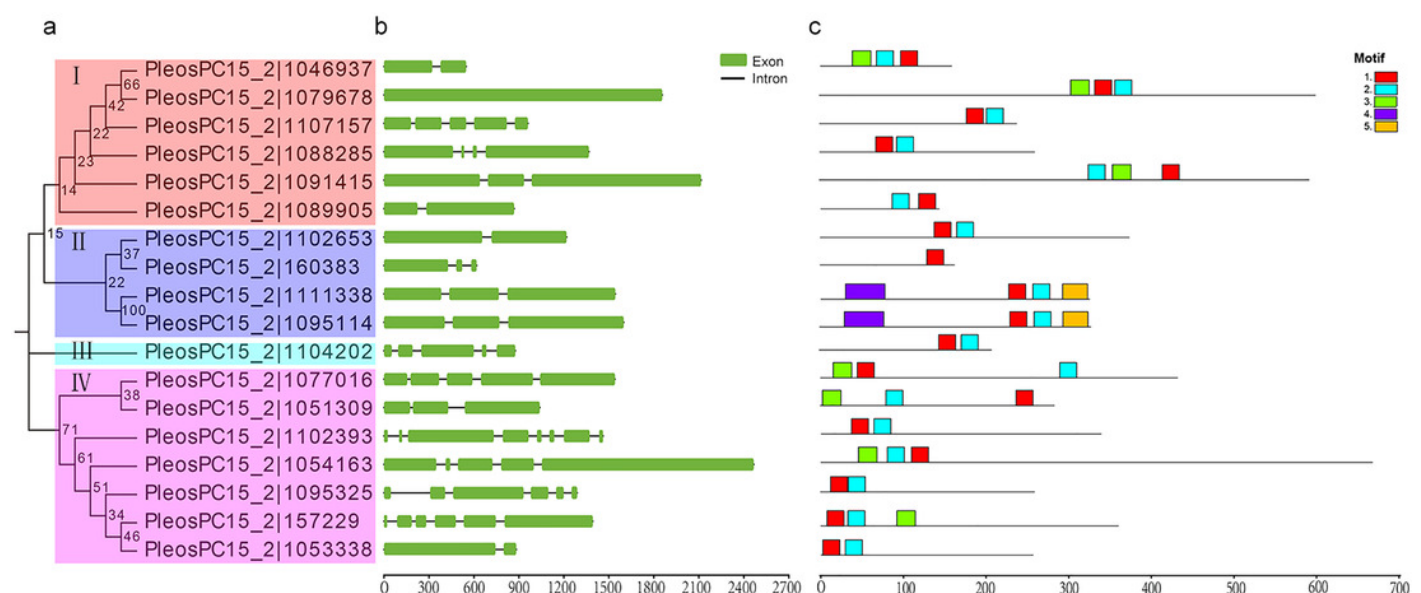
Figure 2

Multiple alignment and conserved amino acids analysis of the C2H2 domains in PoC2H2-ZFPs.

(a) Multiple sequence alignments of the C2H2 domains in PoC2H2-ZFPs. The C2H2 domains in PoC2H2-ZFPs were predicted on SMART (http://smart.embl-heidelberg.de)(with E-value <

$1e^{-2}$). The conserved amino acid sequence with 100% identity was marked with an asterisk.

(b) Conserved amino acid analysis of motif 1. The height of amino acids indicates the conservation ratio.

a

				*			*										*			*

PleosPC15_2|1046937   F V C P V P G C G S T F T R S F N L K G H I R - - S H
PleosPC15_2|1079678-1   Y P C I V P G C Q K T F A R L F S L R A H Q R - - I H
PleosPC15_2|1079678-2   F R C S H - - C P A S F V R N H D L K R H T K - - L H
PleosPC15_2|1107157   F F C Q Y - - C D R G F T A R H N Y T R H L G - - A H
PleosPC15_2|1088285   H G C W M - - C H K S F D R P S T L R K H L L - - V H
PleosPC15_2|1091415-1   H I C N Y E D C S K T F T R R S D L A R H Q R - - I H
PleosPC15_2|1091415-2   F I C S F D G C G K T F I Q R S A L H V H S R - - V H
PleosPC15_2|1091415-3   H C C E Y P G C G R T F G D S S L A R H R R - - T H
PleosPC15_2|1091415-4   Y K C D N A G C E K T F T R R T T L T Q H M R - - I H
PleosPC15_2|1089905-1   F V C E V S E C N K R F V R G E H L K R H V R S - I H
PleosPC15_2|1089905-2   F I C P Q K G C G K T F S R R D N L G Q H A R - - V H
PleosPC15_2|1102653-1   H L C P V - - C G S R F N R P S S L R I H I N - - T H
PleosPC15_2|1102653-2   F K C P W P D C G R E F N V N S N M R R H Y R - - N H
PleosPC15_2|160383   H V C P I - - C S K P F E R S S S L Q T H M H - - I H
PleosPC15_2|1111338   Y K C N V - - C S R A F A R A F N L K T H M S - - T H
PleosPC15_2|1095114   F K C T V - - C C H A F D R R W N L S Q H M L - - T H
PleosPC15_2|1104202-1   Y E C N V - - C M K R F Y R P S G L R I H L A - - S H
PleosPC15_2|1104202-2   F I C P V E G C G R S F G V L S N M R R H A R - - L H
PleosPC15_2|1077016-1   Y K C T F E G C E K A Y T K P S R L E E H E R - - S H
PleosPC15_2|1077016-2   Y Q C S H A D C S K S F S T S Q K L R A H A K - - T H
PleosPC15_2|1051309-1   F E C P Q - - C E R Q F R S S M A L G D H C R S K A H
PleosPC15_2|1051309-2   Y D C Y L - - C T R K F K T L S G L N T H L N S P A H
PleosPC15_2|1102393   F A C P Q - - C D A A F T R M D A L R R H Q R S R - H
PleosPC15_2|1054163   H V C E I - - C K K S F K R P Q D L K K H E K - - I H
PleosPC15_2|1095325   F K C G M - - C P R R L N T A G G L A V H I Q Q - V H
PleosPC15_2|15729-1   F D C P R - - C K R P F K R K G D L N R H I Q - - L H
PleosPC15_2|15729-2   F V C P I D G C T S S I K R R S A F A K H I K - - S H
PleosPC15_2|105338-1   H E C E I - - C H K L F P R P S G L Q T H M N - - T H
PleosPC15_2|105338-2   F P C T V Q G C N K R F A V R S N A K R H L R - - T H

b
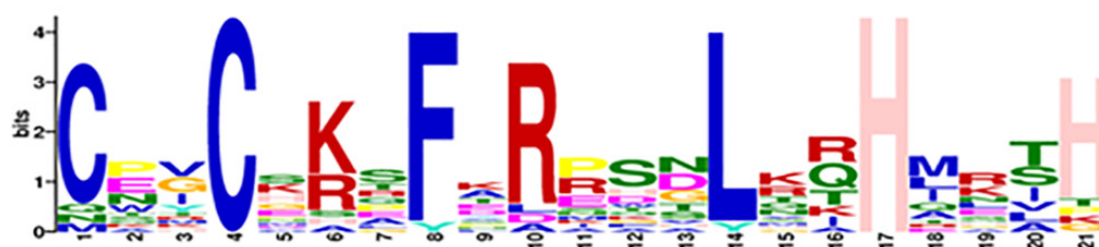
Figure 3

Expression analysis of *PoC2H2-ZFs* in different tissues.

(a) Mycelia (M), primordia (P), young fruiting body (Y F B), and mature fruiting body (M F B) were sampled to analyze the expression profiles of *PoC2H2-ZFs*. (b) The result of RT-qPCR was analyzed using the $2^{-\Delta CT}$ method and then transformed in log2 level to visualize in a heatmap.
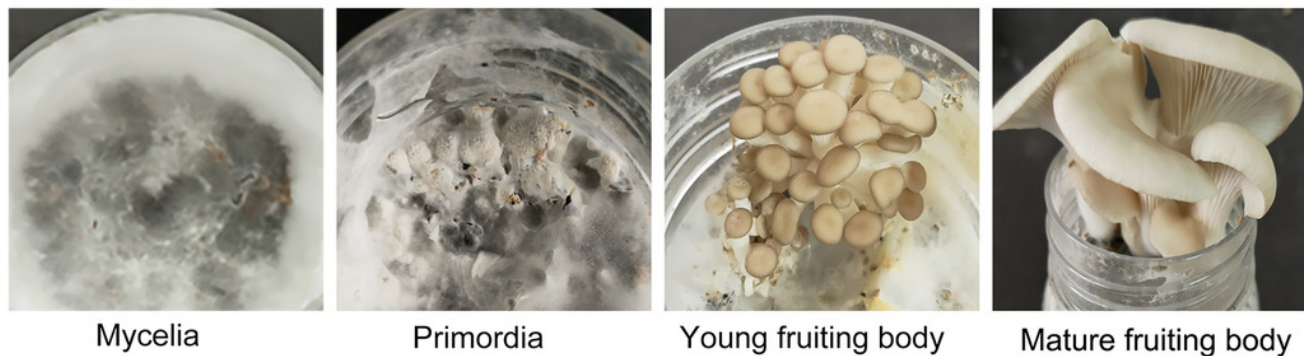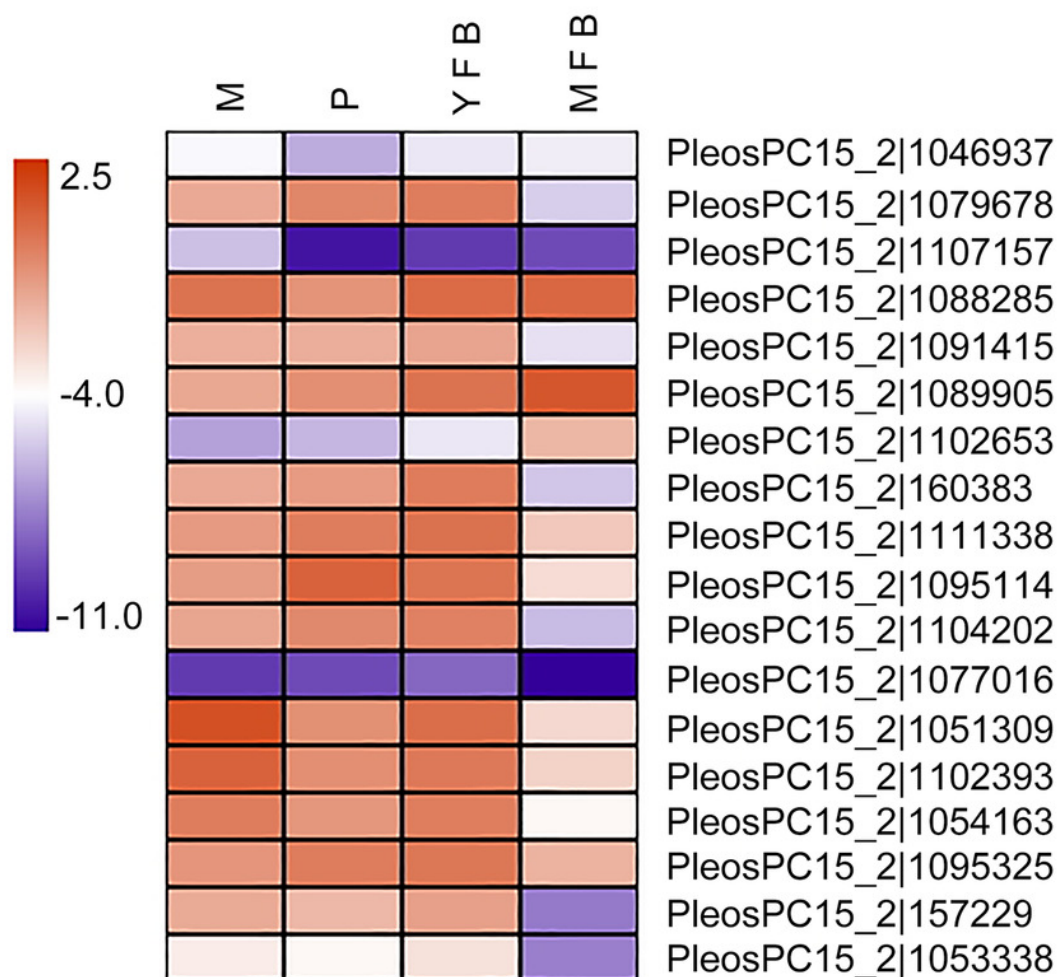
Figure 4

Expression pattern of *PoC2H2-ZFs* under auxin and cytokinin treatment.

Twelve selected *PoC2H2-ZFs* were used to analyze their expression patterns at 1, 3 h after IAA and zeatin treatment, respectively. Primordia treatment with H_2O was analyzed as controls. The result was analyzed using the $2^{-\Delta\Delta CT}$ method. The data were compared using the student's t-test, and the asterisk indicates a significant difference at $P < 0.05$ (n = 3).

PleosPC15_2|1046937 *PleosPC15_2|1079678* *PleosPC15_2|1107157*

PleosPC15_2|1091415 *PleosPC15_2|1089905* *PleosPC15_2|1102653*

PleosPC15_2|1111338 *PleosPC15_2|1095114* *PleosPC15_2|1104202*

PleosPC15_2|1051309 *PleosPC15_2|157229* *PleosPC15_2|1053338*

control   auxin   cytokinin
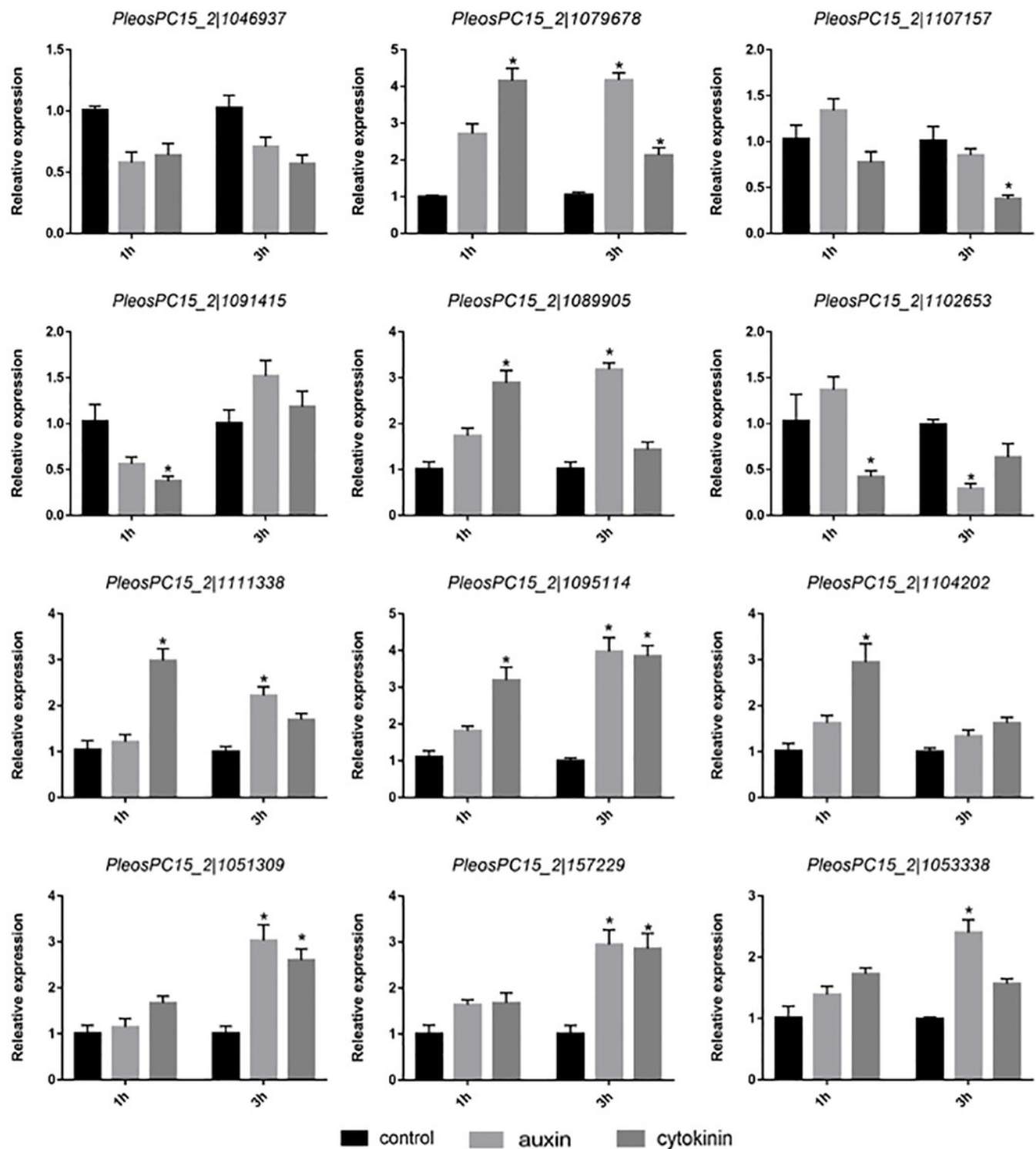
Figure 5

Expression pattern of *PoC2H2-ZFs* response to cold and heat stress.

The *PoC2H2-ZFs* were used to analyze their expression patterns at 1, 3 h after 4 °C and 38 °C treatment, respectively. Primordia cultured at room temperature (10-13 °C) were analyzed as controls. The result was analyzed using the $2^{-\Delta\Delta CT}$ method. The data were compared using the student's t-test, and the asterisk indicates a significant difference at $P < 0.05$ ($n = 3$).