Genome-wide identification and expression analysis of new cytokinin metabolic genes in bread wheat (*Triticum aestivum L.*)

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Cytokinins (CKs) are involved in determining the final grain yield in wheat. Multiple gene families are responsible for the controlled production of CKs in plants, including isopentenyl transferases for *de novo* synthesis, zeatin O-glucosyltransferases for reversible inactivation, β-glucosidases for reactivation, and CK oxidases/dehydrogenases for permanent degradation. Identifying and characterizing the genes of these families is an important step in furthering our understanding of CK metabolism. Using bioinformatics tools, we identified four new *TaIPT*, four new *TaZOG*, and 25 new *TaGLU* genes in common wheat. All of the genes harbored the characteristic conserved domains of their respective gene families. We renamed *TaCKX* genes on the basis of their true orthologs in rice and maize to remove inconsistencies in the nomenclature. Phylogenetic analysis revealed the early divergence of monocots from dicots, and the gene duplication event after speciation was obvious. Abscisic acid-, auxin-, salicylic acid-, sulfur-, drought- and light-responsive *cis*-regulatory elements were common to most of the genes under investigation. Expression profiling of CK metabolic gene families was carried out at the seedlings stage in AA genome donor of common wheat. Exogenous application of phytohormones (6-benzylaminopurine, salicylic acid, indole-3-acetic acid, gibberellic acid, and abscisic acid) for 3 h significantly upregulated the transcript levels of all four gene families, suggesting that plants tend to maintain CK stability. A 6- benzylaminopurine-specific maximum fold-change was observed for *TuCKX1* and *TuCKX3* in root and shoot tissues, respectively; however, the highest expression level was observed in the *TuGLU* gene family, indicating that the reactivation of the dormant CK isoform is the quickest way to counter external stress. The identification of new CK metabolic genes provides the foundation for their in-depth functional characterization and for elucidating their association with grain yield.

1 **Genome-wide identification and expression analysis of new cytokinin**

2 **metabolic genes in bread wheat (*Triticum aestivum* L.)**

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17   **Abstract**

18   Cytokinins (CKs) are involved in determining the final grain yield in wheat. Multiple gene

19   families are responsible for the controlled production of CKs in plants, including isopentenyl

20   transferases for *de novo* synthesis, zeatin O-glucosyltransferases for reversible inactivation, β-

21   glucosidases for reactivation, and CK oxidases/dehydrogenases for permanent degradation.

22   Identifying and characterizing the genes of these families is an important step in furthering our

23   understanding of CK metabolism. Using bioinformatics tools, we identified four new *TaIPT*,

24   four new *TaZOG*, and 25 new *TaGLU* genes in common wheat. All of the genes harbored the

25   characteristic conserved domains of their respective gene families. We renamed *TaCKX* genes on

26   the basis of their true orthologs in rice and maize to remove inconsistencies in the nomenclature.

27   Phylogenetic analysis revealed the early divergence of monocots from dicots, and the gene

28   duplication event after speciation was obvious. Abscisic acid-, auxin-, salicylic acid-, sulfur-,

29   drought- and light-responsive *cis*-regulatory elements were common to most of the genes under

30   investigation. Expression profiling of CK metabolic gene families was carried out at the

31   seedlings stage in AA genome donor of common wheat. Exogenous application of

32   phytohormones (6-benzylaminopurine, salicylic acid, indole-3-acetic acid, gibberellic acid, and

33   abscisic acid) for 3 h significantly upregulated the transcript levels of all four gene families,

34   suggesting that plants tend to maintain CK stability. A 6- benzylaminopurine-specific maximum

35   fold-change was observed for *TuCKX1* and *TuCKX3* in root and shoot tissues, respectively;

36   however, the highest expression level was observed in the *TuGLU* gene family, indicating that

37   the reactivation of the dormant CK isoform is the quickest way to counter external stress. The

38   identification of new CK metabolic genes provides the foundation for their in-depth functional

39   characterization and for elucidating their association with grain yield.

42

INTRODUCTION

44 Wheat (*Triticum aestivum* L.) is the predominant cereal crop, second only to rice as the most

45 important staple, with global production nearing 740 million tons of grain (*USDA, 2017*). The

46 projected increase in the human population will increase the production demand to 900 million

47 tons by 2050 (*FAO stat 2016*); thus, increasing the yield per unit area will be important for

48 meeting this mounting challenge (*Bartrina et al., 2011*).

49 Recent studies in model plants have revealed that cytokinin (CK) metabolic genes are

50 strongly associated with plant yield (*Ashikari et al., 2005; Bartrina et al., 2011*). CKs are

51 phytohormones that play key roles in regulating the vegetative and reproductive development of

52 plants (*Mok and Mok, 2001; Zalewski et al., 2010*). Most CKs are adenine derivatives and have

53 an isoprenoid or aromatic side chain attached to the N-6 of the purine ring (*Avalbaev et al.,*

54 *2012*). *trans*-Zeatin-, *cis*-zeatin-, and dihydrozeatin-type CKs have also been reported in plants,

55 but their abundance is species-specific (*Sakakibara, 2006*). Genetic manipulation of the genes

56 involved in CK homeostasis can be used for yield improvement, as a significant change in CK

57 content has been observed during grain development in crop plants, including wheat (*Jameson et*

58 *al., 1982*) and rice (*Ashikari et al., 2005*). CK homeostasis is carried out by several gene families

59 including isopentenyl transferases (IPTs) for biosynthesis, zeatin O-glucosyltransferases (ZOGs)

60 for reversible inactivation, β-glucosidases (GLUs) for reactivation, and cytokinin

61 oxidases/dehydrogenases (CKXs) for degradation (*Song et al., 2012*).

62 IPTs are the gene family responsible for CK synthesis. Two possible pathways of CK

63 synthesis have been proposed: (1) degradation of transfer RNA (tRNA) and (2) *de novo*

64 synthesis. The first pathway is catalyzed by tRNA IPTs (EC 2.5.1.8); However, it is not

65 considered as a major source of CK production (*Takei et al., 2001*). *De novo* biosynthesis of CKs

66 is carried out by adenylate IPTs (EC 2.5.1.27) by adding an isopentenyl group to the N6 terminal

67 domain of ATP (*Frébort et al., 2011*). To date, nine, eight, 11, and six IPT genes have been

68 reported in *Arabidopsis*, rice, maize, and wheat, respectively (*Chang et al., 2015; Song et al.,*

69 *2012*). Kakimoto (2001) examined the expression of *AtIPT* gene family and demonstrated that

70 *AtIPT3*, *AtIPT5* and *AtIPT7* were expressed in all the tissues, whereas *AtIPT6* and *AtIPT1* were

71 only expressed in the siliques . Moreover, in maize, the tRNA-IPT genes *ZmIPT1* and *ZmIPT10*

72 were reportedly highly expressed in all the organs, whereas the expression patterns of the

73   remaining *ZmIPT* genes were spatially and temporally specific (*Vyroubalová et al., 2009*). In

74   wheat, *TaIPT2*, *TaIPT5*, and *TaIPT8* are expressed during the reproductive stage, and *TaIPT2*

75   exhibits the highest expression level (*Song et al., 2012*). Controlled expression of IPT genes can

76   be used to improve plant growth and development (*Faiss et al., 1997*). Transgenic plants

77   harboring a high molecular weight gluten promoter fused with an IPT gene (*HMWipt*) exhibited

78   increased seed weight (*Daskalova et al., 2007*). IPT protein production under the control of the

79   P_{SARK} promoter in transgenic peanuts led to drought tolerance, delayed senescence and most

80   importantly, a 51–65% increase in seed yield compared with the wild type (*Qin et al., 2011*).

81   Zeatin, an active form of CK, was first identified in maize. Glycosylation of zeatin to O-

82   glucosyl-zeatin and O-xylosyl-zeatin is carried out by ZOG and O-xylosyl transferase (ZOX),

83   respectively (*Martin et al., 1999*). To date, three ZOG genes in *Arabidopsis*, three in wheat and

84   several ZOG genes in maize have been identified (*Song et al., 2012*). O-glucosylation of zeatin-

85   type CKs is reversible in nature. The deglycosylation of zeatin type CKs is catalyzed by GLU

86   (*Brzobohaty et al., 1993*). GLU genes are the members of the glycoside hydrolase 1 family and

87   are involved in the regulation of CK metabolism (*Song et al., 2012*). In *Arabidopsis* and rice, 47

88   (*Miyahara et al., 2011*) and 37 (*Sasaki et al., 2002*) GLUs have been annotated, respectively,

89   whereas in wheat only six *TaGLU* genes have been identified thus far (*Song et al., 2012*). The

90   substrate specificity of GLU was found to be conserved in ZOGs (*Falk and Rask, 1995*). As *de*

91   *novo* synthesis of CK is slow, it is likely that reversible degradation and activation of CKs play

92   important roles in maintaining the total CK pool in plants (*Frébort et al., 2011*).

93   CKXs (EC: 1.5.99.12) are the only enzymes that permanently degrade CKs by cleaving the

94   N6-unsaturated side chain of the CK to adenine and adenosine in a single step (*Ma et al., 2011*).

95   Two conserved domains involved in the catalytic activity of CKXs have been reported, a FAD

96   binding domain at the N terminus and a CK binding domain at the C terminus of the protein

97   (*Avalbaev et al., 2012*). Pačes et al. (1971) first reported CKX activity in tobacco, whereas the

98   first CKX gene (*ZmCKX1*) was isolated from maize (*Houba-Hérin et al., 1999*). Since then,

99   many CKX genes have been identified in multiple plant species (*Galuszka et al., 2000*). To date,

100   seven CKX genes from *Arabidopsis*, 11 from rice, 13 from maize and 13 from wheat have been

101   partially or completely identified (*Lu et al., 2015; Song et al., 2012*). As CKX is a multi-gene

102   family, every member of the family is expected to have specific biochemical properties (*Yeh et*

103   *al., 2015*), i.e., organ localization, subcellular localization, and substrate specificity. Using gain-

104   or loss-of-function methods, all of the *AtCKX* genes have been functionally studied (*Zalabák et*
105   *al., 2013*). Detailed expression analysis of *HvCKX* genes has suggested that *HvCKX1*, *HvCKX4*,
106   *HvCKX9* and *HvCKX11* are more highly expressed in developing kernels, and by using RNA
107   interference technology, *HvCKX-1-* and *HvCKX9*-silenced plants were found to produce more
108   spikes and a greater number of seeds (*Zalewski et al., 2014*).

109   In rice, the production of more CK as a result of reduced *OsCKX2* expression increased the
110   total yield by increasing the number of reproductive organs (*Ashikari et al., 2005*). Yeh et al.
111   (2015) used short hairpin RNA-mediated silencing technology to hinder the expression of
112   *OsCKX2* in rice, resulting in an increased number of tillers and increased grain weight. Based on
113   quantitative expression analysis, 12 bread wheat varieties varying in the numbers of grains per
114   spike were found, and the variation was positively correlated with *TaCKX2.1* and *TaCKX2.2*
115   genes (*Zhang et al., 2011*). *TaCKX6a02-D1a*, an allelic isoform of *TaCKX6a02-D1*, was
116   correlated with grain size, grain weight and grain filling rate. These results were also confirmed
117   in 169 recombinant inbred lines (Jing 411 × Hongmangchun 21) and 102 wheat varieties under
118   different environmental conditions. A 29-bp insertion-deletion mutation in the 3′ untranslated
119   region was thought to be responsible for this variation. In another experiment, copy number
120   variation in the *TaCKX4* gene linked to *Xwmc169* on chromosome 3AL was associated with
121   grain weight (*Chang et al., 2015; Lu et al., 2015*).

122   In summary, all members of the aforementioned CK metabolic gene families have been
123   identified in model plants, and in-depth functional studies have been carried out. Nevertheless,
124   the gene family members have not yet been completely identified in wheat. The hexaploidy
125   (AABBDD = 42), large genome size (~17 GB) and complexity of interactions between the three
126   genomes are among the reasons for this lack of information. In this study, we explored new
127   genes belonging to the major CK metabolic families in wheat, laying a foundation for their
128   detailed characterization.

129

130   **MATERIALS AND METHODS**
131   **Plant material**
132   *Triticum urartu* seeds treated with 1% H_2O_2 were grown in petri dishes. After 5 days, the
133   seedlings were transferred to hydroponic tanks and grown in controlled conditions (25°C, 16:8 h
134   photoperiod). Half-strength Hoagland solution (*Hoagland, D. R., & Arnon, D. I. 1938*) modified

135   for solution culture was provided, and the nutrient solution was changed twice a week during the

136   course of the experiment. Fifteen days after germination, seedlings were treated with plant

137   hormones: 5 μM 6-benzylaminopurine (6-BA), 0.5 mM salicylic acid (SA), 10 μM indole-3-

138   acetic acid (IAA), 30 μM gibberellic acid (GA$_3$), and 10 μM abscisic acid (ABA) for 3 h, along

139   with the control treatment. A total of 20 seedlings per biological replicate and three biological

140   replicates per treatment were used. Immediately after 3 h treatment, root and shoot tissues were

141   collected and frozen in liquid nitrogen for RNA extraction.

142

143   **RNA extraction and cDNA synthesis**

144   Conventional RNA extraction was performed using TRIzol reagent (TIANGEN Biotech Co.,

145   Ltd., Beijing, China) (*Chomczynski & Sacchi, 2006*). The purity and quality of the RNA samples

146   were verified using 1% agarose gel electrophoresis. For cDNA synthesis, 1.5 μg of the RNA

147   template was used in a reaction mixture of 20 μL. A FastQuant RT kit (with gDNase)

148   (TIANGEN Biotech Co., Ltd.) was used according to the manufacturer's instructions, with the

149   final incubation time extended to 30 min at 42°C.

150

151   **Isolation of CK metabolic genes**

152   To retrieve new members of the gene families involved in CK metabolism, the homology search

153   approach was used. cDNA sequences and the conserved domains of all previously annotated

154   genes involved in CK metabolism, i.e., IPTs, CKXs, GLUs, and ZOGs from *Arabidopsis*, maize,

155   and   rice,   were   used   to   query   the   wheat   database

156   (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php). Matched sequences having E-values $\leq 2e^{-7}$

157   were downloaded. A separate preliminary sequence alignment and a phylogenetic tree for each

158   gene family were constructed to clean the duplicate sequences. Using a BLASTx search of the

159   NCBI database (https://www.ncbi.nlm.nih.gov/), protein structures and conserved motifs specific

160   to each protein family were confirmed. The theoretical isoelectric points (PIs), molecular weights

161   (MWs)   (http://web.expasy.org/compute_pi/),   and   N-glycosylation   sites

162   (http://www.cbs.dtu.dk/services/NetNGlyc/) of CK metabolic proteins were also determined.

163

Gene structure and phylogenetic analysis

164

165 The structures of CK metabolic gene families and the number of introns and exons were

166 determined using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) (*Hu et al.,*

167 *2015*). For phylogenetic analysis, translated amino acid sequences were used, as protein

168 sequences are more conserved among species. Separate ClustalW multiple alignments

169 (*Thompson, Higgins & Gibson, 1994*) of the protein sequences for each gene family were carried

170 out using Bioedit software (*Hall, 1999*). Based on the conserved domains and full-length protein

171 sequences, an unrooted neighbor-joining phylogenetic tree (bootstrap 1,000) was developed

172 using Geneious software (*Kearse et al., 2012*).

173

174 *In silico* **promoter analysis**

175 To identify *cis*-regulatory elements in the promoter regions of gene families involved in CK

176 metabolism, 2-kb upstream regions of the translation sites of the respective genes were extracted

177 from the local wheat genomic database. *In silico* promoter analysis was carried out for all the

178 reported genes of the respective multi-gene families. *Cis*-regulatory elements responsive to light,

179 phytohormones, abiotic stress, heat shock and low temperature were considered. MatInspector

180 software (*Cartharius et al., 2005*) based on the PLACE library

181 (http://www.dna.affrc.go.jp/PLACE/) (*Higo et al., 1999*) was used to explore the *cis*-regulatory

182 elements.

183

184 **Quantitative expression analysis**

185 As there was significant sequence similarity in the exonic regions of wheat sub-genomes, gene-

186 specific homoeologous quantitative polymerase chain reaction (qPCR) primers for all members

187 of the *TaCKX, TaIPT*, and *TaZOG* families were developed. As *TaGLU* is a large family, qPCR

188 primers were designed from selected family members (seven new and seven old genes). The

189 *Ta4045* primer was used as an internal control (*Paolacci et al., 2009*), and SYBR Green I Master

190 Mix (Roche Diagnostics, Indianapolis, IN, USA) was used in the reaction mixture according to

191 the manufacturer's instructions. qPCR was conducted using the LightCycler 480 system (Roche

192 Diagnostics), with an initial denaturation step at 94°C for 5 min, followed by 45 cycles of

193 denaturation at 94°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 20 s. Three

194 biological and two technical replicates were used to reduce the error. Genes with reliably

195 detectable expression are presented here.

196

197 **Statistical analysis**

198 The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels for each treatment (*Livak*

199 *& Schmittgen, 2001*). Student's *t*-test was used to determine the significant differences in the

200 expression levels between the control and treated samples. All statistical analyses were carried

201 out using Microsoft Excel software.

202

203 **RESULTS**

204 **Bioinformatics analysis of CK metabolic genes**

205 Following in-depth mining of the wheat genomic database, 13 *TaCKX*, seven *TaZOG*, nine

206 *TaIPT*, and 32 *TaGLU* genes were identified (Fig. 1A–D). With few exceptions, most of the

207 identified genes had homoeologues in the A, B, and D sub-genomes; whereas the CKX gene

208 family members *TaCKX12* and *TaCKX13*, the IPT gene family member *TaIPT4*, and the GLU

209 family member *TaGLU21* did not have homoeologues. In wheat, CK metabolic genes were not

210 uniformly distributed among and along the lengths of chromosomes (Fig. 2). Most of the genes

211 resided away from the centromere towards the distal parts and formed CK metabolic gene-rich

212 regions. The maximum number of CK metabolic genes were found on chromosome groups three

213 and two, whereas only one gene (*TaCKX7*) was found on chromosome group six. None of the

214 members of the *TaCKX* or *TaIPT* gene families were found on chromosome group four, nor were

215 any *TaIPT* genes found on chromosome group six. As *TaZOG* is a small gene family, its

216 members resided only on chromosome groups two, three, five and seven. *TaGLU* genes were

217 distributed on all the chromosomes except chromosome group six. Based on wheat reference

218 sequence 1.0, homoeologues of *TaCKX11* and *TaZOG2* were predicted on an unknown

219 chromosome. cDNA sequences of these homoeologues were BLASTed against *Aegilops tauschii*

220 in the Ensemble database and more than 90% homology was found for the respective genes.

221 Based on this, we predicted that these homoeologues belong to the D genome.

222   Phylogenetic analyses were conducted for each gene family using previously reported

223 *Arabidopsis*, rice, maize, and wheat genes. The results showed that most of the identified genes

224 from wheat had orthologs in other monocot species, that is why CK metabolic genes in wheat

225 were given names according to their homology in related species (Fig. 3A–D).

226   We were unable to identify new putative genes in the *TaCKX* gene family, as all of the

227 sequences were identical to previously identified *TaCKX1-11* genes (*Feng et al., 2008; Ma et al.,*

228 *2011; Song et al., 2012; Zhang et al., 2007*). However, we report the full-length *in silico*

229 extraction of the *TaCKX* gene family and rectification of its nomenclature. In the literature,

230 multiple names for a single *TaCKX* gene sequence were observed (Table 1). We have renamed

231 the *TaCKX* gene sequences according to their homology with rice and maize (Fig. 3A).

232 According to this systematic renaming, the *TaCKX* gene family consists of 13 members, with

233 gene structures varying from 0 to 4 introns (Fig. 4A), predicted protein lengths of 516–555

234 amino acids (aa), PIs of 5.62–8.68, 0–5 glycosylation sites, and MWs ranging from 55.7 to 59.8

235 kDa (Table 2A). Phylogenetic analysis also revealed a D genome-specific duplication of the

236 *TaCKX2* gene, forming a cluster (Fig. 3A) with more than 85% sequence similarity.

237   While mining the database for *TaZOG*, *TaIPT* and *TaGLU* gene families, four new

238 *TaZOG*s (*TaZOG1, TaZOG2, TacisZOG3* and *TacisZOG4*), four new *TaIPT*s (*TaIPT1, TaIPT4,*

239 *TaIPT9* and *TaIPT10*) and 25 new *TaGLU* genes were identified (Fig. 1B–D).

240   In the *TaZOG* gene family, with the exception of *TaZOG2*, remainder of the *TaZOG* genes

241 consisted of open reading frames (ORFs) with no introns (Fig. 4B). Exploring their predicted

242 proteins revealed lengths of 467–551 aa, PIs of 5–6.93, and MWs of 50.6–59.3 kDa. Most of the

243 *TaZOG* proteins were predicted to be localized to the plasma membrane, whereas the *cis*-type

244 ZOG proteins were anticipated to be secretory in nature (Table 2B). Genes in the *TaIPT* gene

245 family were also ORFs with no introns, except *TaIPT9* (Fig. 4C). The predicted protein lengths

246 of *TaIPT* genes ranged from 292 to 499 aa, expected MWs from 31.6 to 52.1 kDa, and PIs from

247 5.05 to 9.24, and the N-glycosylation sites of *TaIPT*s varied from 0 to 1 (Table 2C). Other than

248 the tRNA IPT genes (*TaIPT9* and *TaIPT10*), the rest were predicted to localize to the chloroplast.

249   In contrast to the IPT family, the GLUs constitute a large gene family. The 25 predicted

250 *TaGLU* genes contained a minimum of 10 introns (Fig. 4D), and the predicted protein size for all

251 *TaGLU* genes varied from 406 to 585 aa. Glycosyl hydrolase family 1/β-glucosidase appeared to

252 be the characteristic conserved domain of this family, and the MW was predicted to range from

253 46.1 to 64.6 kDa. Via *in silico* localization, most of the newly identified *TaGLU*s appeared to be

254 chloroplastic in nature (Table 2D). As wheat is a monocot, newly predicted *TaGLU* genes were

255 homologous to rice rather than the dicot *Arabidopsis*, and this characteristic was clearly mirrored

256 in the phylogenetic analysis of the GLU family (Fig. 3D).

257

258 ***In silico* promoter analysis**

259 Promoter analysis of the CK metabolic gene families revealed that drought-responsive *cis-*

260 elements were common to the promoter regions of all members of the *TaIPT*, *TaZOG*, *TaGLU*

261 and *TaCKX* gene families (Table 3A, 3B, 3C and 3D). ABA- and sulfur-responsive *cis*-elements

262 were common to members of the *TaCKX* and *TaZOG* families only (Table 3A and 3B)*,* and cold-

263 responsive *cis*-elements were only found in the promoter regions of *TaGLU* genes (Table 3D).

264

265 **Expression analysis**

266 To determine which of the CK biosynthetic and degrading genes were highly expressed or

267 responsive to phytohormones and abiotic stress, the expression patterns of the treated samples

268 were recorded. Genes with reliably detectable expression are presented here. The experiment was

269 conducted on AA genome donor (*Triticum urartu*) of hexaploid wheat. As a basic genome, it has

270 played a central role in wheat evolution and the domestication process (*Ling et al., 2013*).

271 In general, with the application of phytohormones, the transcript levels of all the genes

272 under study were upregulated as compared to the control treatment, except for *TuGLU4*,

273 *TuGLU9*, and *TuGLU12*, as in roots; their expression levels were drastically lower than that of

274 control treatment (Fig. 5A-D).

275 As all the gene families under study are involved in CK metabolism, majority of the genes,

276 with few exceptions, showed significant maximal changes in their transcript levels following

277 exogenous CK treatment. Following CK treatment, most of the genes were responsive to GA_3

278 treatment, as GA_3 is also a major plant growth regulator; however, their transcript levels varied.

279 While exploring the response of CK metabolic gene families to ABA treatment, we observed that

280 the mRNA contents of *TuGLU3* and *TuGLU13* in leaf tissues only were significantly higher than

281 those in control plants (Fig. 5B & 5D).

282 For the *TuCKX* gene family, the highest expression level in leaf tissue was recorded for

283 *TuCKX9* (Fig.5A). In shoots and roots, *TuCKX3* and *TuCKX1*, respectively, showed 6BA-

284 specific maximum fold-changes in their expression patterns (Fig.5A and 5B). In the *TuIPT* gene

285 family, all detectable *TuIPT* genes were upregulated by exogenously applied phytohormones, but

286   the newly identified *TuIPT10* exhibited maximum transcript abundance and was more highly

287   expressed in shoot tissues than in root tissues (Fig.5A and 5B). In the *TuZOG* family, *TuZOG3*

288   had the highest expression levels and a significant 6BA-specific response in *T. urartu* roots

289   (Fig.5A).

290   *TuGLU*s are responsible for the reactivation of reversibly inactivated CKs, and this gene

291   family appeared to be more highly expressed than *TuCKX*s, *TuIPT*s, or *TuZOG*s. Among the

292   *TuGLU*s, *TuGLU7* had the highest transcript level in shoot and root tissues; however, it was

293   significantly responsive to phytohormones only in shoots (Fig. 5B, 5D and Fig. 6B). In contrast,

294   *TuGLU1* appeared to have a root-specific expression and 6BA-specific response (Fig.5D).

295

296   **DISCUSSION**

297   CKs are phytohormones that play important role in the regulation of plant growth. Their role in

298   cell differentiation, nutrient signaling, and leaf senescence have been well established (*Yeh et al.,*

299   *2015*). Multigene families are reported to maintain CK homeostasis for normal plant growth. In

300   model plants, the genes responsible for CK metabolism have already been identified and well

301   characterized. Using a comparative genomics approach, the conserved domains and full-length

302   coding sequences of CK anabolic (IPT and GLU) and catabolic (ZOG and CKX) genes from

303   *Arabidopsis*, rice, and maize were used as queries to search the wheat local genomic database.

304   We were unable to identify new genes in the wheat *TaCKX* family. However, for the *TaZOG*,

305   *TaIPT*, and *TaGLU* gene families, we report four, four, and 25 new genes, respectively.

306   Naming newly identified genes on the basis of their orthologs in closely related species is a

307   systematic way forward (*Lee, Redfern & Orengo, 2007*), as inconsistencies in nomenclature can

308   be misleading (*Goyal et al., 2018*). When reviewing the literature, some irregularities were found

309   in the nomenclature of the *TaCKX* gene family, i.e., multiple naming of homoeologues or single

310   naming of different paralogs (Table 1). The polyploid nature of common wheat and the

311   unavailability of its reference sequence until recently may have led to this discrepancy. In this

312   work, a systematic approach was followed and *TaCKX* gene family members were renamed

313   according to their true orthologs in rice and maize.

314   Phylogenetic analysis on the basis of sequence similarity is a powerful tool to predict

315   orthologous genes of interest and their functions in important crop species (*Song et al., 2012*).

316   *AtIPT2* from *Arabidopsis*, *ZmIPT1* from maize, and *OsIPT9* from rice are actually tRNA IPT

317   genes responsible for the synthesis of zeatin-type CKs in their respective species (*Brugiere et al.,*
318   *2008; Miyawaki et al., 2006; Sakamoto et al., 2006*). Based on the sequence and gene structure
319   similarities, newly identified *TaIPT9* in wheat may have a similar function. Phylogenetic
320   analysis also revealed that newly identified *TaGLU* genes from wheat are more similar to rice
321   than *Arabidopsis*, depicting the early divergence of monocots from dicot species.

322   Softberry and NetNGlyc servers were used to predict subcellular localization and
323   glycosylation sites, respectively. Variable subcellular localization and the presence or absence of
324   glycosylation sites within members of each family predicts their variable functions and substrate
325   specificities (*Köllmer et al., 2014*), which will later be confirmed practically. For example,
326   *TaIPT9*, which produces zeatin-type CKs, is predicted to localize to the cytoplasm, in contrast
327   with the remainder of the *TaIPT* genes, which are predicted to localize to chloroplasts.

328   By controlling the efficiency of gene promoters, *cis*-regulatory elements contribute
329   significantly to the regulation of gene expression. Identifying the targeted *cis*-elements can aid in
330   devising detailed functional studies. Among the putative regulatory elements, ABA-, auxin-, SA-
331   , sulfur-, drought- and light-responsive *cis*-regulatory elements were predicted in most of the
332   promoters of *TaCKX*, *TaIPT*, *TaGLU* and *TaZOG* genes. The broad range of regulatory elements
333   predicts their expression in multiple plant tissues, which may help these gene families stabilize
334   CK content under different environmental stresses.

335   Before moving forward and carrying out detailed studies of the newly predicted genes, it is
336   necessary to characterize them practically based on expression levels and responsiveness to
337   different stimuli. *T. urartu* seedlings grown under exogenous application of 6-BA, SA, GA$_3$, IAA
338   and ABA hormones were used to develop expression profiles of the above-mentioned gene
339   families. In general, after 3 h of treatment, the transcript levels of all CK metabolic genes were
340   upregulated compared to the control treatment. With the application of external stimuli, CKX
341   genes readily began to degrade active CK. To maintain homeostasis of the CK pool, by feedback
342   mechanism, genes for biosynthetic activity were also triggered. As *de novo* synthesis of CK is
343   relatively slow (*Frébort et al., 2011*), de-glycosylation of O-glycosylated CKs plays a major role
344   in stabilizing CK level (*Vyroubalová et al., 2009*). This can be explained by the higher
345   expression level of *TuGLU* genes compared to those of *TuIPT* genes (Fig. 5A-D). In contrast to
346   the high expression levels of *TuGLU* genes in leaves and roots under external stimuli, the
347   transcript levels of *TuGLU4*, *TuGLU9*, and *TuGLU12* were antagonistic in both tissues (Fig. 5A-

348 D). This can be explained by the tissue-specific expression/function of CK metabolic genes

349 (*Vyroubalová et al., 2009*).

350 In conclusion, we predicted four new *TaZOG*, four new *TaIPT*, and 25 new *TaGLU* genes

351 in wheat and evaluated their sensitivity towards phytohormones. Future studies will be able to

352 mine their biochemical and functional characteristics and their associations with target traits in

353 crop plants.

354

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360

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514

Table legends

516   Table 1 Previously assigned nomenclature of wheat cytokinin oxidase/dehydrogenase (*TaCKX*)
517   gene family.

518

519   Table 2 Characteristic features of wheat *TaCKX* (a), *TaZOG* (b), *TaIPT* (c) and *TaGLU* (d) gene
520   families.

521

522   Table 3 *cis-* regulatory elements in the promoter region of wheat *TaCKX* (a), *TaZOG* (b), *TaIPT*
523   (c) and *TaGLU* (d) gene families.

524

526

527 **Figure legends**

528

529 **Figure 1 The unrooted phylogenetic tree of 13 *TaCKX* (A), seven *TaZOG* (B), nine *TaIPT* (C)**
530 **and 32 *TaGLU* (D) genes from wheat.**
531 The tree was produced at amino acid level using neighbor joining method and bootstrapped at
532 1000 replications. Newly identified genes are in red color.

533

534 **Figure 2 Chromosome locations of *TaCKX*, *TaZOG*, *TaIPT* and *TaGLU* genes in wheat.**
535 Wheat reference sequence 1.0 was used to develop the physical map of the wheat CK metabolic
536 genes.

537

538 **Figure 3 The unrooted phylogenetic tree of *CKX* (A), *ZOG* (B), *IPT* (C) and *GLU* (D) genes**
539 **from *Arabidopsis (At)*, rice (*Os*), maize (*Zm*) and wheat (*Ta*).**
540 The tree was produced at amino acid level using neighbor joining method and bootstrapped at
541 1000 replications. Newly identified genes are in red color.

542

543 **Figure 4 Predicted gene structures of wheat *TaCKX* (A), *TaZOG* (B), *TaIPT* (C) gene**
544 **families and of newly identified *TaGLU* (D) genes.**
545 Exons and introns are illustrated by filled boxes and single lines respectively. UTRs are shown in
546 blue color lines. Gene structures are developed using Gene Structure Display server
547 (http://gsds.cbi.pku.edu.cn).

548

549 **Figure 5 Quantitative expression profiles of selected putative cytokinin regulatory genes**
550 ***TaCKX , TaZOG , TaIPT , TaGLU* in leaf (A & B) and root (C &D) tissue of *T. urartu***
551 **exposed to exogenously applied phyto-hormones treatment.**
552 A & C: Selected CK regulatory genes with relatively lower expression. B & D: Selected CK
553 regulatory genes with relatively higher expression. *Ta4045* gene primer was used as internal
554 control. Two technical and three biological replicates were used to reduce the error. Error bars
555 represent Standard Deviation (n=3).

556

557

558 **Figure 6 Comparison among expression profiles of selected cytokinin regulatory genes**

559 ***TaCKX, TaZOG, TaIPT*** and ***TaGLU,*** **in leaf and root tissue of** ***T. urartu*** **exposed to**

560 **exogenously applied phyto-hormones treatment.**

561 A: Selected CK regulatory genes with relatively lower expression. B: Selected CK regulatory

562 genes with relatively higher expression. *Ta4045* gene primer was used as internal control. Two

563 technical and three biological replicates were used to reduce the error. Error bars represent

564 Standard Deviation (n=3).

565

566

Previously assigned nomenclature of wheat cytokinin oxidase/dehydrogenase (*TaCKX*) gene family.

1   **Table 1: Previously assigned nomenclature of wheat cytokinin oxidase/dehydrogenase**
2   (***TaCKX***) **gene family**

Sr#	Gene name[a]	Previously assigned nomenclature
1	*TaCKX1*	***TaCKX1*** (*Feng et al., 2008; Song, Jiang & Jameson, 2012*)
2	*TaCKX2*	***TaCKX2*** (JN381556.1 GenBank), *TaCKX2.5* (*Mameaux et al., 2012*)
3	*TaCKX3*	*TaCKX6* (*Song, Jiang & Jameson, 2012*), *TaCKX8* (JQ925405.1 GenBank)
4	*TaCKX4*	***TaCKX4*** (*Song, Jiang & Jameson, 2012*)
5	*TaCKX5*	***TaCKX5*** (*Lei, Baoshi & Ronghua, 2008*)
6	*TaCKX7*	*TaCKX8* (*Song, Jiang & Jameson, 2012*)
7	*TaCKX8*	*TaCKX11* (*Song, Jiang & Jameson, 2012*)
8	*TaCKX9*	*TaCKX10* (*Song, Jiang & Jameson, 2012*)
9	*TaCKX10*	*TaCKX9* (*Song, Jiang & Jameson, 2012*)
10	*TaCKX11*	*TaCKX2* (*Lei, Baoshi & Ronghua, 2007*), *TaCKX3* (*Ma et al., 2010; Song, Jiang & Jameson, 2012*)
11	*TaCKX12*	*TaCKX2.1* (*Zhang et al., 2011*), *TaCKX6D* (*Zhang et al., 2012*)
12	*TaCKX13*	*TaCKX2.2* (*Zhang et al., 2011*)
13	*TaCKX14*	*TaCKX2.4* (*Mameaux et al., 2012*)

3   [a] *TaCKX* genes were renamed on the basis of their true orthologs in rice and maize to remove
4   inconsistencies in the nomenclature.
5   Gene names which matched the new nomenclature are in bold letters.

Characteristic features of wheat *TaCKX* (a), *TaZOG* (b), *TaIPT* (c) and *TaGLU* (d) gene families.

1   **Table 2 Characteristic features of wheat *TaCKX* (a), *TaZOG* (b), *TaIPT* (c) and *TaGLU* (d)**

2   **gene families.**

(a) Cytokinin oxidase/dehydrogenase (*TaCKX*)

	Genes	Length (aa)	PI	MW (kDa)	Subcell location	Glyco. sites
1	*TaCKX1*	524	8.68	56.9	ER & Vacuole	5
2	*TaCKX2*	555	6.18	59.8	ER & Vacuole	2
3	*TaCKX3*	523	6.28	57.7	ER & Vacuole	0
4	*TaCKX4*	527	6.53	57.8	ER & Vacuole	3
5	*TaCKX5*	531	6.03	57.8	ER & Vacuole	2
6	*TaCKX7*	535	8.49	58.5	ER & Vacuole	4
7	*TaCKX8*	528	5.62	57.2	ER & Vacuole	0
8	*TaCKX9*	521	6.86	58.3	ER & Vacuole	5
9	*TaCKX10*	532	6.1	58.0	ER & Vacuole	3
10	*TaCKX11*	516	5.93	55.7	ER & Vacuole	0
11	*TaCKX12*	547	5.57	59.2	ER & Vacuole	1
12	*TaCKX13*	545	6.05	58.8	ER & Vacuole	1
13	*TaCKX14*	552	5.56	59.4	ER & Vacuole	1

(b) Zeatin O-glucosyltransferases (*TaZOG*)

1	*TaZOG1*	491	5.99	53.4	Plasma membrane	2
2	*TaZOG2*	481	5.74	53.1	Secreted	0
3	*TaZOG3*	551	5	59.3	Plasma membrane	2
4	*TaZOG4*	529	5.43	56.3	Plasma membrane	1
5	*TacisZOG1*	467	5.87	50.8	Secreted	0
6	*TacisZOG3*	466	6.23	50.6	Secreted	0
7	*TacisZOG4*	528	6.93	57.5	Secreted	0

(c) isopentenyl transferases (*TaIPT*)

1	*TaIPT1*	292	5.23	31.6	chloroplast	0
2	*TaIPT2*	355	5.05	38.0	chloroplast	0
3	*TaIPT3*	369	9.24	39.2	chloroplast	0
4	*TaIPT5*	351	8.16	37.8	chloroplast	1

5	*TaIPT6*	351	8.47	37.8	chloroplast	1
6	*TaIPT7*	346	6.68	37.1	chloroplast	0
7	*TaIPT8*	392	9.09	41.2	chloroplast	0
8	*TaIPT9*	466	6.68	52.1	Cytoplasm	0
9	*TaIPT10*	499	6.77	50.5	Cytoplasm	1

(d) wheat β-glucosidases (*TaGLU*)

	Genes	Length (aa)	PI	MW (kDa)	subcell location	Glyco. sites
1	*TaGLU5*	475	5.46	53.1	vacuole	4
2	*TaGLU6*	427	7.15	49	chloroplast	2
3	*TaGLU7*	508	9	56.6	chloroplast	1
4	*TaGLU8*	585	6.72	64.6	chloroplast	2
5	*TaGLU9*	532	6.9	59.5	vacuole	2
6	*TaGLU11*	508	4.91	56.6	vacuole	3
7	*TaGLU12*	519	6.93	58.6	chloroplast	4
8	*TaGLU13*	508	5.7	57.5	chloroplast	1
9	*TaGLU14*	519	6.79	59.5	chloroplast	2
10	*TaGLU15*	430	5.35	48.4	chloroplast	1
11	*TaGLU16*	511	6.12	57.8	vacuole	1
12	*TaGLU17*	504	9.55	55.8	chloroplast	1
13	*TaGLU19*	506	5.36	56.7	vacuole	2
14	*TaGLU21*	473	5.4	52.4	vacuole	2
15	*TaGLU22*	485	5.2	53.8	vacuole	2
16	*TaGLU23*	477	5.6	53.4	vacuole	2
17	*TaGLU24*	502	8.37	57.5	chloroplast	3
18	*TaGLU26*	448	6.67	51.5	chloroplast	4
19	*TaGLU28*	525	8.72	59.4	chloroplast	4
20	*TaGLU30*	517	9.26	58	vacuole	3
21	*TaGLU31*	503	6.05	56.5	vacuole	6
22	*TaGLU32*	522	7.28	58.5	vacuole	1
23	*TaGLU34*	515	6.92	58.4	chloroplast	4
24	*TaGLU35*	406	6	46.1	chloroplast	2
25	*TaGLU38*	502	7.29	58.4	chloroplast	5

4 PI = Isoelectric point,   MW = Molecular weight,   Glyco. Sites = Glycosylation sites ER=   Endoplasmic
5 reticulum
6 For *TaGLU* family, characteristic features of only newly identified genes are presented here.
7 PI & MW predicted by ExPASy (http://web.expasy.org/compute_pi/)

8   Subcell location predicted by Softberry (http://www.softberry.com/)

9   Glyco. Sites predicted by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/)

cis- regulatory elements in the promoter region of wheat *TaCKX* (a), *TaZOG* (b), *TaIPT* (c) and *TaGLU* (d) gene families.

2 **Table 3** *cis*- **regulatory elements in the promoter region of wheat *TaCKX* (a), *TaZOG* (b),**

3 ***TaIPT* (c) and *TaGLU* (d) gene families.**

5 **(a) Cytokinin oxidase/dehydrogenase (*TaCKX*)**

	Auxin	SA	ABA	Sulphur	Drought	Cold	Light	GA₃
TaCKX1	4	4	5	1	9	2	1	1
TaCKX2	0	0	7	4	8	4	0	1
TaCKX3	1	1	1	1	9	2	3	0
TaCKX4	5	5	20	9	22	10	2	1
TaCKX5	1	1	4	4	10	0	2	0
TaCKX7	6	5	7	6	5	6	2	3
TaCKX8	6	6	9	5	6	3	2	1
TaCKX9	0	0	2	3	6	0	4	4
TaCKX10	2	2	7	1	6	2	0	2
TaCKX11	1	1	8	3	8	4	2	0
TaCKX12	5	5	7	4	10	4	0	0
TaCKX13	8	8	12	4	18	9	2	0
TaCKX14	0	0	6	4	11	4	0	1
(b) Zeatin O-glucosyltransferases (*TaZOG*)								
TaZOG1	2	2	10	2	34	5	0	0
TaZOG2	2	2	5	2	9	0	2	0
TaZOG3	0	0	8	6	10	4	2	2
TaZOG4	10	10	15	6	20	6	1	0
TacisZOG1	7	7	3	3	7	9	0	1
TacisZOG3	4	4	2	2	6	0	2	0
TacisZOG4	1	1	4	3	8	2	1	2
(c) isopentenyl transferases (*TaIPT*)								
TaIPT1	2	2	6	2	6	2	1	2
TaIPT2	0	0	1	0	5	0	2	0
TaIPT3	0	0	0	1	3	5	0	1
TaIPT4	2	2	4	2	6	0	0	0

TaIPT6	3	3	2	5	8	6	3	0
TaIPT7	6	6	9	6	12	3	3	1
TaIPT8	2	1	6	3	16	9	2	0
TaIPT9	3	3	5	1	9	4	1	0
TaIPT10	3	3	0	6	8	3	1	1

(d) wheat β-glucosidases (*TaGLU*)

	Auxin	SA	ABA	Sulphur	Drought	Cold	Light	GA$_3$
TaGLU5	2	2	2	1	6	1	5	1
TaGLU6	4	4	7	4	6	8	1	0
TaGLU7	7	7	9	2	28	7	0	0
TaGLU8	5	5	4	2	8	6	0	0
TaGLU9	6	5	2	4	4	11	0	0
TaGLU11	1	1	3	3	11	3	3	0
TaGLU12	5	4	5	2	5	5	2	0
TaGLU13	1	1	8	2	22	1	1	0
TaGLU14	4	4	0	3	2	6	1	1
TaGLU15	3	2	5	2	12	2	0	3
TaGLU16	0	0	0	4	9	4	0	0
TaGLU17	5	5	7	1	11	13	0	1
TaGLU19	6	6	8	1	12	3	2	0
TaGLU21	2	2	1	3	5	1	3	2
TaGLU22	2	2	3	4	4	6	1	2
TaGLU23	3	3	20	0	12	16	1	1
TaGLU24	1	0	4	1	6	6	0	1
TaGLU26	2	2	7	1	17	2	2	0
TaGLU28	1	0	5	4	11	3	3	0
TaGLU30	2	2	8	1	11	8	0	0
TaGLU31	1	1	7	3	9	1	5	0
TaGLU32	4	4	7	4	6	4	0	0
TaGLU34	0	0	1	5	5	1	1	0
TaGLU35	2	2	2	2	6	2	1	1
TaGLU38	4	4	6	6	10	3	1	2

6   Arabic numerals represent the number of repeats of *cis*-regulatory elements in the promoter region of cytokinin
7   metabolic gene families; whereas, 0 represents absence of specific *cis*-element.
8   For *TaGLU* family, *cis*-regulatory elements of only newly identified genes are presented here.

9   PLACE library was used to predict the *cis*-elements, and Auxin, Salicylic acid (SA), Abscisic acid (ABA),

10   Sulphur, Drought, Cold, light and Gibberellic acid (GA_3) responsive *cis*-elements were given consideration.

Figure 1

The unrooted phylogenetic tree of 13 *TaCKX* (A), seven *TaZOG* (B), nine *TaIPT* (C) and 32 *TaGLU* (D) genes from wheat.

The tree was produced at amino acid level using neighbor joining method and bootstrapped at 1000 replications. Newly identified genes are in red color.

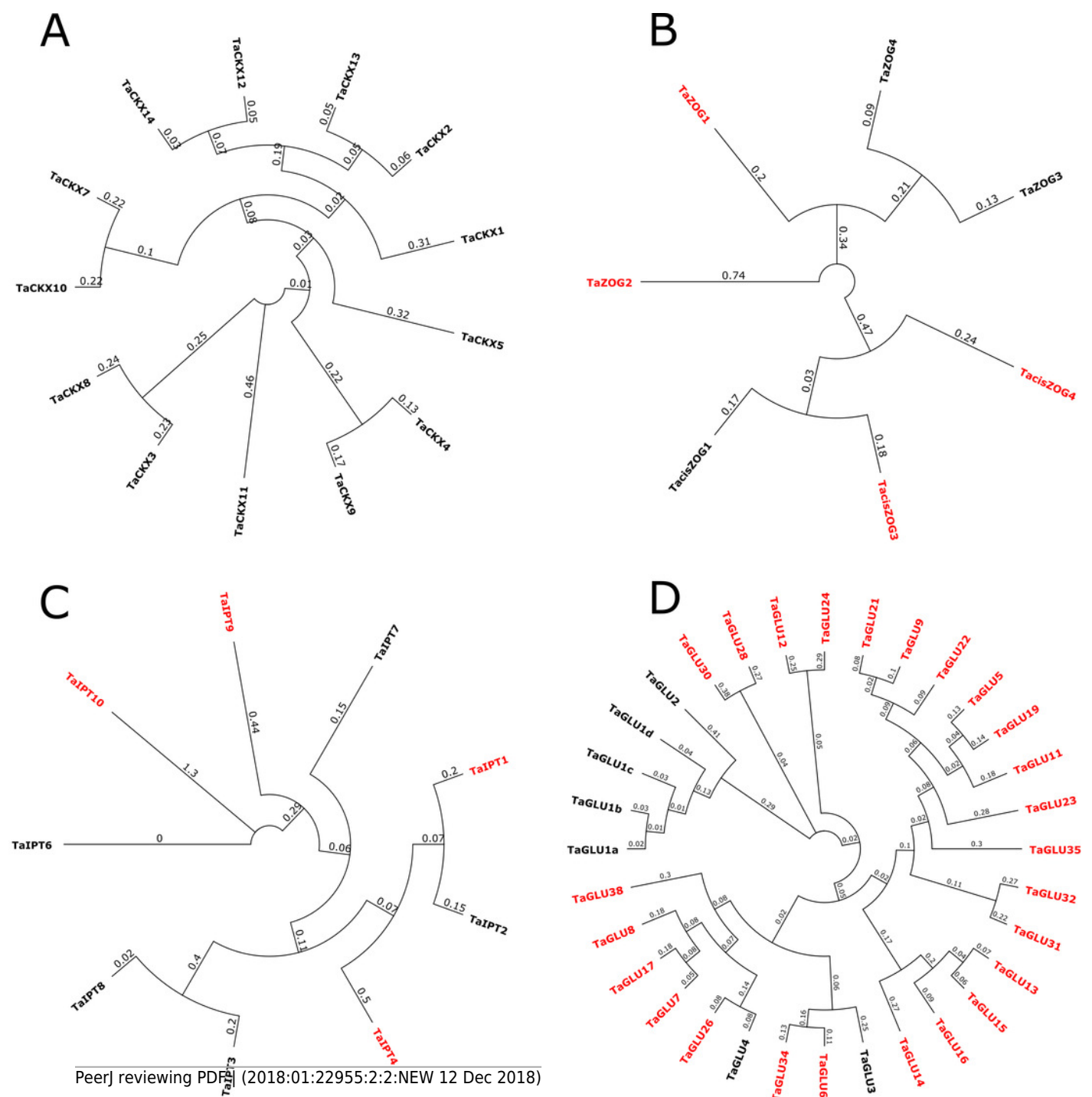



Figure 2

Chromosome locations of *TaCKX*, *TaZOG*, *TaIPT* and *TaGLU* genes in wheat.

Wheat reference sequence 1.0 was used to develop the physical map of the wheat CK metabolic genes.

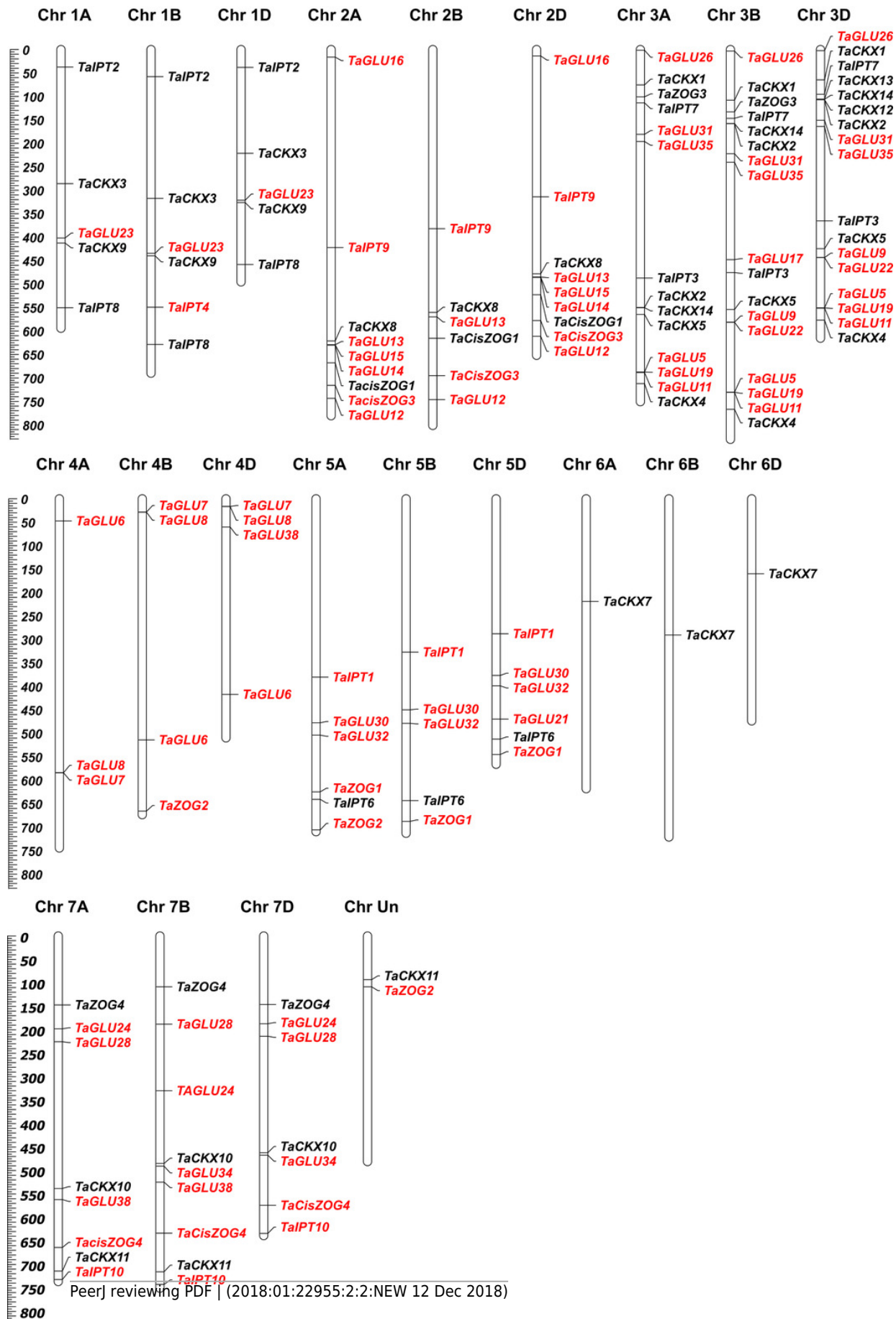



Figure 3

The unrooted phylogenetic tree of *CKX* (A), *ZOG* (B), *IPT* (C) and *GLU* (D) genes from *Arabidopsis (At)*, rice (*Os*), maize (*Zm*) and wheat (*Ta*).

The tree was produced at amino acid level using neighbor joining method and bootstrapped at 1000 replications. Newly identified genes are in red color.

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

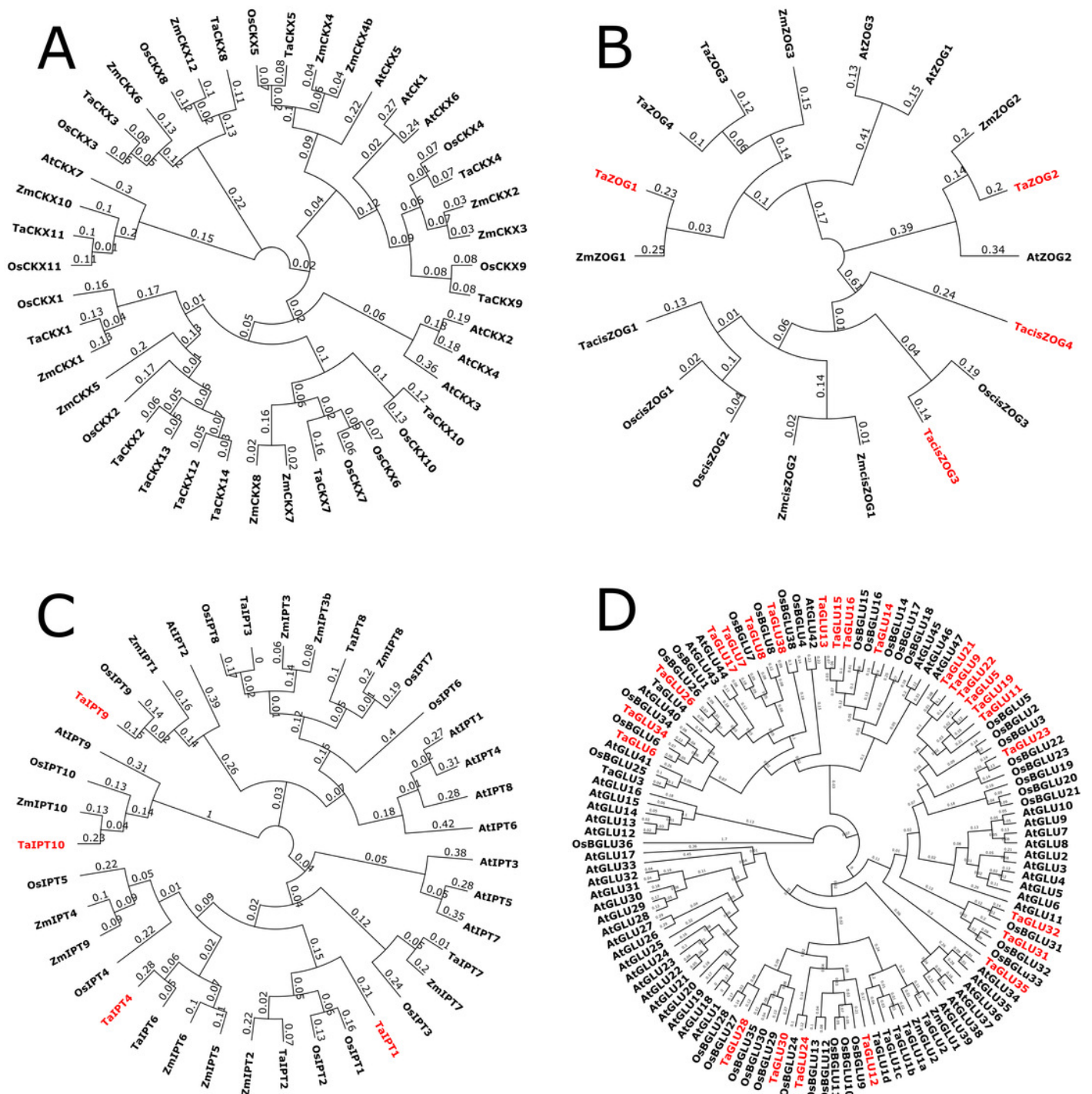



Figure 4

Predicted gene structures of wheat *TaCKX* (A), *TaZOG* (B), *TaIPT* (C) gene families and of newly identified *TaGLU* (D) genes.

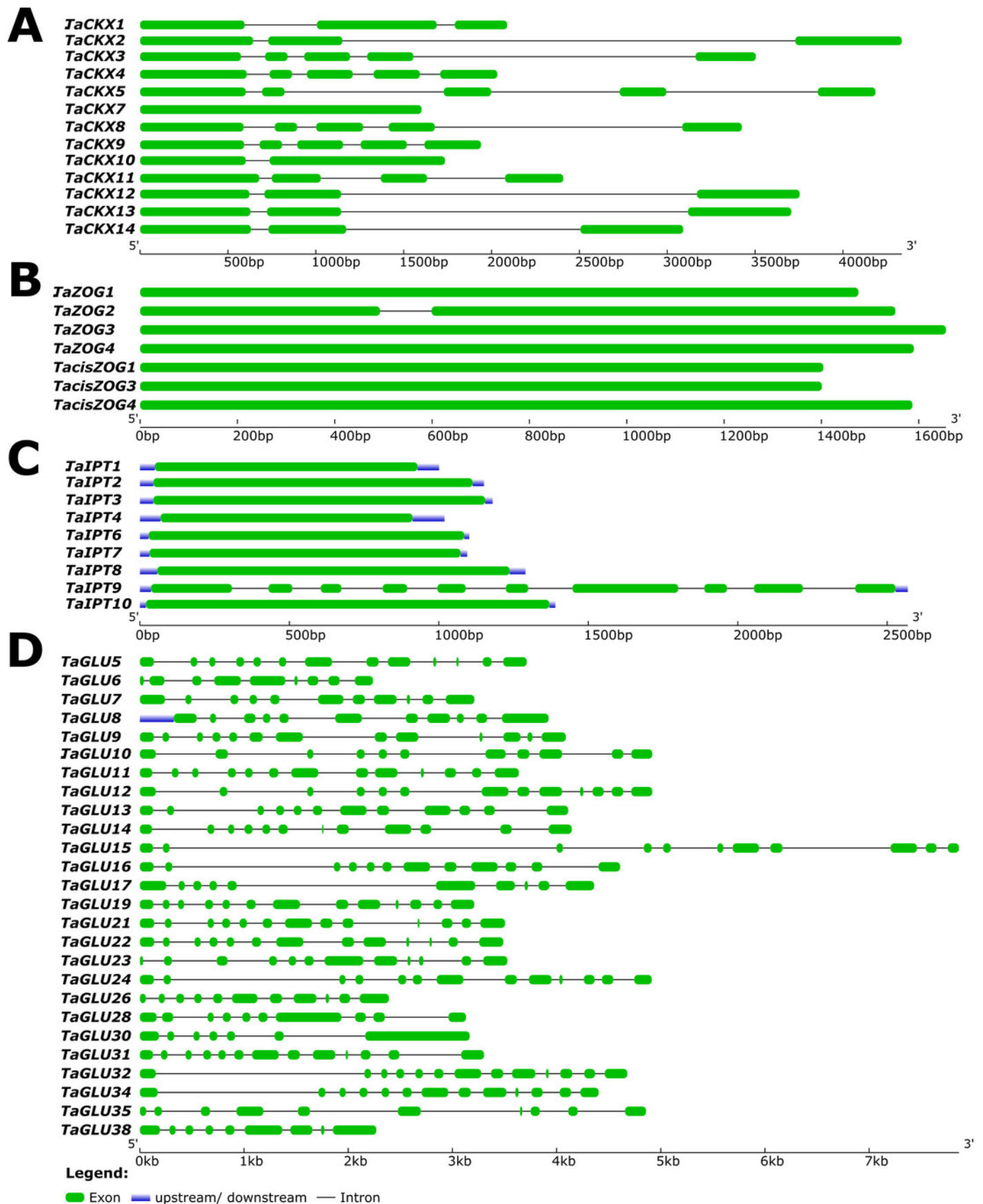
Exons and introns are illustrated by filled boxes and single lines respectively. UTRs are shown in blue color lines. Gene structures are developed using Gene Structure Display server (http://gsds.cbi.pku.edu.cn).

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Figure 5

Quantitative expression profiles of selected putative cytokinin regulatory genes *TaCKX* , *TaZOG* , *TaIPT* , *TaGLU* in leaf (A) and root (B) tissue of *T. urartu* exposed to exogenously applied phyto-hormones treatment.

A & C: Selected CK regulatory genes with relatively lower expression. B & D: Selected CK regulatory genes with relatively higher expression. *Ta4045* gene primer was used as internal control. Two technical and three biological replicates were used to reduce the error. Error bars represent Standard Deviation (n=3).
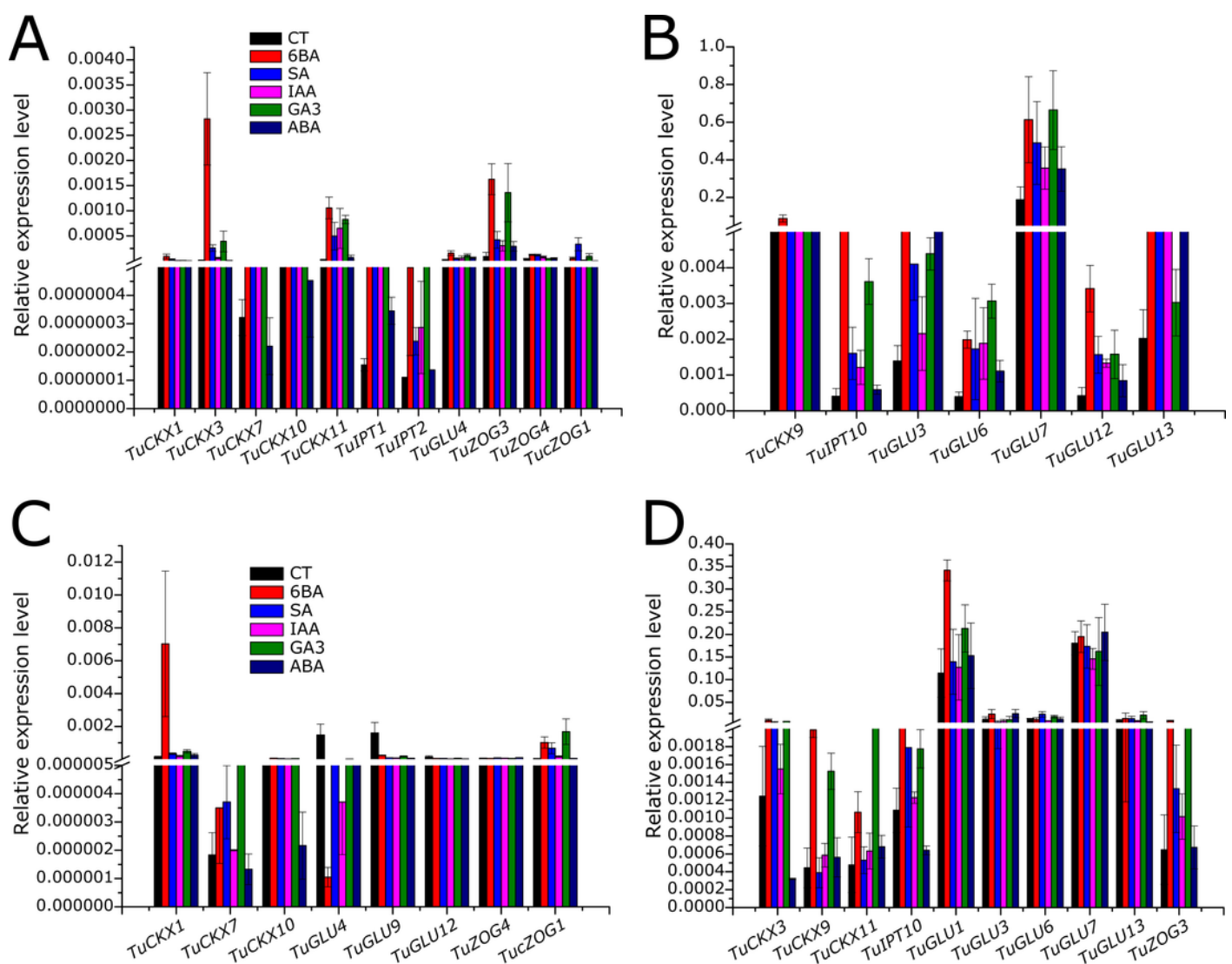
Figure 6

Comparison among expression profiles of selected cytokinin regulatory genes *TaCKX*, *TaZOG*, *TaIPT* and *TaGLU,* in leaf and root tissue of *T. urartu* exposed to exogenously applied phyto-hormones treatment.

A: Selected CK regulatory genes with relatively lower expression. B: Selected CK regulatory genes with relatively higher expression. *Ta4045* gene primer was used as internal control. Two technical and three biological replicates were used to reduce the error. Error bars represent Standard Deviation (n=3).