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Comparative Expression and Phylogenetic Analysis of Maize Cytokinin Dehydrogenase/Oxidase (CKX) Gene Family

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Abstract Cytokinin dehydrogenase (CKX) degrades the cytokinin hormone in plants and plays an important role in cytokinin regulatory processes. CKX proteins are encoded by a multigene family with a varying number of members. In this study, 13 maize *CKX* sequences were collected in which ten transcripts were confirmed by RT-PCR. The tissue- and cytokinin-dependent expression studies indicated that *ZmCKX* genes exhibit a variety of expression patterns, suggesting diverse functions. Besides 13 maize CKXs, 7 *Arabidopsis*, 9 poplar, and 11 rice CKX proteins were further used to construct a phylogenetic tree. The CKX members were assigned to six groups, and the intron/exon structures, sequence motifs, and protein properties were conserved within groups. The genome distribution of *CKXs* supports that segmental duplication contributes to the expansion of the *CKX* gene family. By quantitative RT–PCR analysis of maize members and digital Northern analysis of *Arabidopsis*, poplar, and rice members for their tissue expression patterns, highly correlative expression profiles of *CKX* genes were found among some of the orthologs, whereas different expression manners were found between some of the paralogs. These results suggest functional conservation within each group of the CKX family and provide a clue for transfer of a gene function from one species to the other and further contribute to uncovering the role of *CKX* genes *in planta*.

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Introduction

Cytokinins (CKs) are a class of plant hormones that play a critical role in cell division and proliferation of many plant developmental and physiological processes, such as the delay of leaf senescence (Kim and others 2006), release of apical dominance (Tanaka and others 2006), control of shoot/root ratio (Werner and others 2001), signaling of nutrient status (Takei and others 2001), and reproductive competence (Ashikari and others 2005). Natural CKs are chemically N^6-substituted purine derivatives, which are either isopentenyladenin (iP)-type or zeatin-type derivatives (Mok and Mok 2001). Because cytokinins have central regulatory functions in plant development, the fine-tuned control of their catabolism plays an important role in ensuring the proper regulation of cytokinin functions. The homeostasis of the CK level in cells is regulated by the rate of biosynthesis, activation/inactivation, and degradation (Mok and Mok 2001). Cytokinin biosynthesis is catalyzed by the isopentenyltransferase (IPT) enzyme (Kakimoto 2001), and irreversible degradation of CKs is catalyzed by the enzyme cytokinin oxidase/dehydrogenase (CKX), which was encoded in plants by a small multigene family (Schmuelling and others 2003). Meanwhile, reversible inactivation and activation of CKs are catalyzed by zeatin O-glycosyltransferase (ZOG) and β-glucosidase (GLU), respectively (Brzobohaty and others 1993; Martin and others 1999).

The CKX enzyme degrades CKs by removing their N^6-substituted isoprene chain to produce an adenine and a corresponding unsaturated aldehyd-3-methyl-2-butenal

(Galuszka and others 2000). As a glycoprotein containing a covalently bound FAD domain, the CKX enzyme catalyzes the degradation of CKs with molecular oxygen as an electron acceptor or with other electron acceptors in a dehydrogenase reaction (Frebortova and others 2004). The CKX enzyme is thought to play an essential role in controlling the local CK level and subsequently contributing to the regulation of cytokinin-dependent processes *in planta*. The overexpression of *AtCKX* genes in transgenic tobacco plants reduces the endogenous cytokinin content and profoundly influences shoot and root development (Werner and others 2001). Furthermore, six *AtCKX* gene transformants in *Arabidopsis* displayed reduced cytokinin levels and showed distinct developmental alterations in the shoot and root (Werner and others 2003). Most importantly, changes in *OsCKX2* expression in rice are causally related to changes in grain yield, indicating that cytokinin metabolism is associated with crop productivity (Ashikari and others 2005).

Maize is one of the most important cereal crops used primarily for animal feed and also a significant source of biofuel production. Homology searches of the maize genome sequencing database revealed 13 maize *CKX* members (Vyroubalova and others 2009). Among them, *ZmCKX1* was first cloned and found to be expressed mainly in the kernel in a developmental manner (Morris and others 1999; Houba-Herin and others 1999). Further studies revealed that *ZmCKX1* expression was localized to the vasculature and was induced by cytokinins, abscisic acid, and abiotic stresses, suggesting a function in plant stress response (Brugiere and others 2003). *ZmCKX2* and *ZmCKX3* shared strong expression in leaves, whereas *ZmCKX4* and *ZmCKX5* were expressed mainly in immature tassels and in immature ears, respectively (Massonneau and others 2004). *ZmCKX10* showed a high constitutive expression pattern in plant tissues except embryo and endosperm (Smehilova and others 2009). Besides the different expression manner, ZmCKX proteins also differed in enzyme properties such as rates of CK oxidase activity and substrate specificity (Massonneau and others 2004; Smehilova and others 2009). ZmCKX1 can catalyze *trans*-zeatin, isopentenyladenosine, and isopentenyladenine but not dihydrozeatin, whereas ZmCKX10 shows a much wider substrate range. Distinct subcellular localization may also exist among ZmCKXs, because ZmCKX1 was found to localize in the apoplast, whereas ZmCKX10 localized in the cytosol. Their differences in tissue localization, subcellular compartmentation, and biochemical characteristics suggested distinct roles for maize CKX in cytokinin metabolism control and coordination of multiple cytokinin functions in plant growth and development. However, within the *ZmCKX* gene family, the physiological function of most members remains to be elucidated.

In this study we collected 13 *ZmCKX* sequences from the maize genome sequencing database and confirmed the transcript of 10 members by RT-PCR. Tissue-specific expression and cytokinin-dependent regulation of *ZmCKXs* were also analyzed. In addition, we used phylogenetic and computational methods to compare the expression manner, protein properties, and gene structure of *ZmCKXs* with their orthologs in rice, poplar, and *Arabidopsis*. These analyses will be useful in exploring the functions of each *CKX* gene in maize and address the contribution of functional specification from model plants to crops.

Materials and Methods

Plant Materials

Maize inbred line *B73* was used for *ZmCKX*s cDNA cloning and quantitative PCR (qPCR) analysis. Young leaves and roots were harvested from five-leaf-stage seedlings (28-day-old plants after germination) grown in hydroponic solution. Mature leaves, mature tassels, and immature ears were obtained from maize plants grown in the field. For N^6-benzyladenine (BA) treatment, five-leaf-stage seedlings grown in a hydroponic system were treated by adding BA to a final concentration of 10 μM. Roots and shoots were collected from 1 to 72 h after treatment and frozen in liquid nitrogen.

Sequence Collection

Full-length sequences of *AtCKX*s were used as a query to search the poplar genome assembly v1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) or the TIGR rice database (http://rice.tigr.org). To obtain CKX sequences from lower plants, including green algae (*Chlamydomonas reinhardtii*, *Ostreococcus tauri*, and *Volvox carteri*), bryophytes (*Physcomitrella patens*), and lycophytes (*Selaginella moellendorfii*), the NCBI database (www.ncbi.nlm.nih.gov) was blasted using the sequences of ZmCK X1 or the bacterial CKXs (NfCKX1 or NsCKX1) (Crespi and others 1994; Schmuelling and others 2003). For maize *CKX* sequences, PlantGDB (http://www.plantgdb.org) and the maize sequence database (www.maizesequence.org) were blasted using the five ZmCKX protein sequences previously reported by Massonneau and others (2004) or protein sequences of OsCKX as queries. Together with maize expressed sequence tag (EST) sequences, the specific primers were designed to amplify *ZmCKX* cDNAs as follows: *ZmCKX1F*: 5′ TAATGGCGGTGGTTTATTACC TGCTG 3′, *ZmCKX1R*: 5′ GCTATCATCATCAGTTGAA GATGTCC 3′; *ZmCKX2F*: 5′ ACTCCCCTCCTTTTTGTT GGTTTGT 3′; *ZmCKX2R*: 5′ ACATACAGACCAACATG

ATTTCCTCG 3′; *ZmCKX3F*: 5′ CTTCTGTTTCCAGGCC GTCCCAAC 3′; *ZmCKX3R*: 5′ AGTAGCAGGAACTGT-CACAAAGGCA 3′; *ZmCKX4F*: 5′ CAATGACGCGGT GCCTCATGTTC 3′; *ZmCKX4R*: 5′ CAGCTGGATTACG TTACGAGTCGGC 3′; *ZmCKX4bF*: 5′ CAATGACGC GGTGCCTCATGTTCATG 3′, *ZmCKX4bR*: 5′ TCACGA GTCGGCGACGAGCGGCG 3′; *ZmCKX5F*: 5′ CGATGG CTAGAGCTACGACCTCCAC 3′, *ZmCKX5R*: 5′ GATCA TGAGGCAAGTAGCGGGGAC 3′; *ZmCKX6F*: 5′ TG AT GGAGGTTGCCATGGTCGTCAG 3′, *ZmCKX6R*: 5′ ATA AAATGCTAGCTCCCGTACGCAG 3′; *ZmCKX8F*: 5′ CT ATGGCAAGAAGGACTCGTTTCGTG 3′, *ZmCK X8R*: 5′ CTCAGGCAGAAGCAATGCCAGATG 3′; *Zm CKX10F*: 5′ ACATGATGCTCGCGTACATGGACCG 3′, *ZmCKX10 R*: 5′ ACTACACGGCGACGGACGCCGGGAC 3′; *ZmCK X12F*: 5′ ATAGCCAGCTAGCCATGGAGGG CA 3′, *Zm CKX12R*: 5′ GTCACATCGGGCTCGAGGAG GAGTC 3′.

These genes were amplified using *pfu* DNA polymerase (Invitrogen, Carlsbad, CA, USA) from a multiple-plant-tissues cDNA pool. The successful amplifications were subsequently cloned into the pGEM T-easy vector (Promega, Madison, WI, USA) for sequencing.

Gene structure (exon/intron structure) of *CKX*s was deduced from the comparison between its genomic sequence and the corresponding cDNAs or ESTs. Chromosomal locations of *CKX* genes were obtained using the BLAST server at the Genome Browser (http://www.arabidopsis.org, http://genome.jgi-psf.org/, http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice and http://www.maizesequence.org).

Quantitative RT-PCR (qPCR)

The expression profiles of *ZmCKX* genes were examined using the qPCR method. Total RNA of different maize samples was extracted using Trizol reagent (Invitrogen). DNA contamination in RNA samples was removed by treatment with RNase-Free DNase set (Invitrogen) and was further verified by PCR amplification using the *Actin1* intron primers (GenBank accession No. J01238; the primers were *ZmACT1F*: CAGACATAGACCCAAACCCGAT, and *ZmACT1R*: ACAGTTGCCCATTGTCAAAGAA). After reverse transcription by M-MLV reverse transcriptase (Invitrogen), the PCR reactions were performed using SYBR® Green dye (Applied Biosystems, Foster City, CA, USA) in a final volume of 25 µl containing 13 µl of Power SYBR Green PCR Master Mix, 20 pmol of forward- and reverse-specific primers, and a 1:10 dilution of cDNA template. Using a 7500 real-time PCR system (Applied Biosystems), amplification was carried out in a two-step PCR procedure with 40 cycles of 15 s at 95°C for denaturation, 15 s at 60°C for annealing, and 60 s at 72°C for extension. The primers used in the qPCR analyses are listed in Table 1. Data were analyzed using 7500 SDS v1.3 software (Applied Biosystems). Dissociation curves obtained by heating the amplicon from 60 to 98°C were analyzed to verify the reaction specificity. The expression of the *GAPDH* gene (GenBank accession No. NM_0011 11943.1; the primers were *ZmGAPDHF*: 5′ CTGGTTTC TACCGACTTCCTTG 3′ and *ZmGAPDHR*: 5′ CGGCATACACAAGCAGCAAC 3′) or the *Alpha tubulin4* gene (GenBank accession No. AJ420856.1; the primers were *ZmTUB4F*: 5′ GCTATCC TGTGATCTGCCCTGA 3′ and *ZmTUB4R*: 5′ CGCCAAAC TTAATAACCCAGTA 3′) served as the internal control. Three biological replicates of qPCR were performed for each sample.

CKX Activity Assay

Lyophilized plant samples were powdered and extracted with twofold excess (v/w) of 0.2 M Tris HCl buffer (pH 8.0) containing 1 mM phenylmethylsulphonyl fluoride and 0.3% Triton X-100. Cell debris was removed by centrifugation at 12,000 *g* for 10 min. The assay was performed according to the method described by Frebort and others (2002). Root and shoot samples were incubated in a reaction mixture at 37°C containing of 100 mM McIlvaine buffer (pH 6.5), 0.5 mM electron acceptor DCPIP, and 0.25 mM substrate iP for 4 and 12 h, respectively. The reaction was then stopped by adding 40% trichloroacetic acid and centrifuged at 12,000 *g* for 5 min. After the addition of 2% 4-aminophenol (solution in 6% trichloroacetic acid) into the supernant, the absorbance was measured at 352-nm wavelength using a UV spectrophotometer (Beckman Coulter, Brea, CA, USA). The protein concentration of a sample was assayed according to the Bradford method with bovine serum albumin as the standard (Bradford 1976).

Phylogenetic Analysis

Phylogenetic analyses were conducted using the MEGA program (Tamura and others 2007). Multiple sequence alignment of *Arabidopsis*, poplar, rice, and maize CKX proteins was performed using ClustalW. The neighbor-joining method was used to construct the phylogenetic tree. To estimate evolutionary distance, the proportions of amino acid differences were computed using Poisson correction distance. The pairwise deletion option was used to circumvent the gaps and missing data. The reliability of different phylogenetic clusters was evaluated by the bootstrap test (1000 bootstrap replications).

Prediction of Isoelectric Point and Subcellular Localization of CKXs Protein

The isoelectric point (pI) of CKXs was predicted according to the Compute pI/Mw software available at http://www.expasy.ch/tools/pi_tool.html, and the subcellular localization of

Table 1 *CKX* genes and their expression profiles in maize

Gene name	Accession No.[a]	Locus/Bac[b]	qPCR primers (5′–3′)	Expression level[c]					Reference
				Root	Shoot	Mature leaf	Immature ears	Tassel	
ZmCKX1	NM_00111 2121	AC225703	TGGAGATGAAGAACAAGTACGACC, ATGACCTTGACATACATCAGTGCAG	9.59 ± 2.13	6.31 ± 1.03	5.78 ± 1.80	1.17 ± 0.42	20.81 ± 4.42	Houba-Herin and others (1999), Morris and others (1999)
ZmCKX2	NM_0011 12056	AC190976.3	GTGGCCAAAGATACCTCAAGCAGT, ACGGAAAATACATACAGACCAACATGA	3.52 ± 0.27	24.65 ± 2.53	46.00 ± 1.75	4.29 ± 0.93	16.83 ± 1.74	Massonneau and others (2004)
ZmCKX3	AJ606944	AC191624.3	CACGGGCACTCCCTCATGG, CGACAGACGGTGGCGACAC	0.78 ± 0.11	2.66 ± 0.69	1.06 ± 0.29	0.88 ± 0.11	1.65 ± 0.03	Massonneau and others (2004)
ZmCKX4	GU160398	AC203958.3	GCCGACTCGTAACGTAATCCAG, GCGATTAACAACCCCACATTTG	4.09 ± 0.14	3.18 ± 0.24	1.96 ± 0.35	8.84 ± 1.35	11.78 ± 1.51	This work
ZmCKX4b	GU160399	AC197031.3	CTCGTCGCCGACTCGTGATCGT, CTGTACGGTTACCGCTGTAGCTACTAC	2.16 ± 0.19	15.82 ± 2.13	7.79 ± 1.12	48.91 ± 3.05	16.80 ± 0.69	This work
ZmCKX5	GU160400	AC213523.3	CGCCGCAAGAGGAAATACGA, GCCAGCAACAGCAACACACGA	0.22 ± 0.16	<0.01	0.30 ± 0.22	1.94 ± 1.81	1.17 ± 1.47	This work
ZmCKX6	GU160401	AC186422.3	GTCGCTGGCTTCAGGGATCTGC, CGACGTGTTGGTGTCCCACTTGTCT	23.33 ± 3.26	31.74 ± 4.05	11.56 ± 1.32	28.58 ± 3.62	48.57 ± 3.08	This work
ZmCKX7	GRMZM2G11 4427_T01	AC208724.3	[d]TACAGCGTGGAGGTGTCCTACTTGG, [d]GGAGTGGGTAGATGAGGATGAGGC	9.83 ± 2.82[e]	7.93 ± 1.43[e]	2.05 ± 0.26[e]	1.80 ± 0.64[e]	6.93 ± 0.89[e]	This work
ZmCKX8	GU160402	AC210850.4							This work
ZmCKX9	GRMZM2G004 225_T01	AC194337.2	GCATTTCGGCGAGAAATGGAG, GAGAATCAGAGCCCGTTTGCTG	<0.01	<0.01	<0.01	<0.01	<0.01	This work
ZmCKX10	FJ269181	AC197220.4	CAGAAGATCTTCCCTCGGGTCC, CCATCAGCCTCCCCTCCTCCT	3.04 ± 0.13	2.86 ± 0.47	2.02 ± 0.25	6.67 ± 0.86	9.60 ± 1.26	Smehilova and others (2009)
ZmCKX11	GRMZM2G122 340_T01	AC199218.3	CAGGTCGACTTCAGCCCGGACT, TGCTGTTGCTGGTAGGAGTGGTATG	<0.01	<0.01	<0.01	<0.01	<0.01	This work
ZmCKX12	GU160403	AC183306.2	GCACCAACAAGAAGGTCTGCTACTAC, CCACAACATGGTCAGCAGCGGAG	13.50 ± 3.21	68.08 ± 7.29	30.08 ± 0.11	19.47 ± 1.48	73.73 ± 0.77	This work

[a] cDNA accession number of *ZmCKX*7, 9, and 11 obtained from www.maizesequence.org; others from www.ncbi.nlm.nih.org

[b] Locus represented by the genome BACs in www.maizesequence.org

[c] Expression level of maize *CKXs* obtained from qPCR analyses in this work. The value was the relative level to 0.01 × *ZmGAPDH* expression level. Values less than 0.0001 were not listed due to the possibility of being unreliable and were marked as <0.01

[d] The primers shared by *ZmCKX7* and *ZmCKX8*

[e] Additive expression level of *ZmCKX7* and *ZmCKX8*

CKXs by TargetP software in the CBS database (http://www.cbs.dtu.dk/services/TargetP).

Motif Identification of CKX Protein

The motifs of the CKX protein were identified using MEME (http://meme.sdsc.edu/meme/meme. html), with motif length set at 6–200 and motif sites set at 4-200. The matched motifs with low quality were manually removed based on an e-value of less than 1×10^{-15}. The identified motifs were further characterized using the Conserved Domain Search Service (CD Search) (Marchler-Bauer and Bryant 2004).

In silico Tissue Expression Analysis of *Arabidopsis*, Poplar, and Rice *CKX* Genes

The tissue expression of *AtCKX*s and *OsCKX*s were obtained from GeneInvestigator (Zimmermann and others 2004). The expression profiles of poplar *CKX* genes were obtained from a poplar eFP browser database (http://www.bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi). Using TMEV software, relative gene expression data were genewise normalized and subsequently displayed in relation to the phylogenetic tree.

Results

Cloning of Putative *CKX* Genes from Maize

Five *ZmCKX* gene sequences had been identified in previous work (Houba-Herin and others 1999; Morris and others 1999; Massonneau and others 2004). We obtained an additional eight *ZmCKX* sequences through BLAST searching in the maize sequence database (Table 1). RT–PCR using gene-specific primers was conducted to confirm the transcripts of individual *ZmCKX*s. In a total of ten open reading frames (ORFs) of *ZmCKX*s, all but *ZmCKX7*, *9*, and *11* were cloned and subsequently sequenced. Six novel *ZmCKX* sequences were uploaded into GenBank with accession numbers from GU160398 to GU160403 (Table 1).

Tissue Expression Analysis of *ZmCKX* Genes

The maize *CKX* transcript abundance was determined by qPCR analyses (Table 1). The level of maize *CKX* expression in the tested tissue was relatively low, about 1/100 to 1/10 of the expression level of the *ZmGAPDH* gene. The additive level of *ZmCKX7* and *ZmCKX8* were indicated as *ZmCKX7 + 8* because their transcripts could not be distinguished by the qPCR method. In addition, expression of *ZmCKX9* and *ZmCKX11* was not listed because of their low expression levels (less than 1/10,000 of *ZmGAPDH* expression), which was also in agreement with the finding of failed ORF amplification by RT–PCR in this study and of their very low expression levels as described by Vyroubalova and others (2009). *ZmCKX6*, *ZmCKX10*, and *ZmCKX12* were abundant and constitutively expressed in all tissue tested, whereas other *ZmCKX*s showed visible spatial patterns of expression (Table 1). For example, *ZmCKX2* and *ZmCKX3* were preferentially expressed in young leaves and in mature leaves, respectively. The transcripts of *ZmCKX4* and *ZmCKX4b* were more abundant in reproductive organs.

Cytokinin-dependent Expression of *ZmCKX* Genes

To investigate cytokinin-dependent expression of individual *ZmCKX* genes, their transcript abundance was analyzed by qPCR in maize seedlings treated with an exogenous cytokinin (Fig. 1). After supplying 10 μM of chemical cytokinin (BA) in nutrient solution from 1 h to 3 days, the transcripts of *ZmCKX1*, *ZmCKX4*, *ZmCKX4b*, and *ZmCKX5* were subsequently induced in both root and shoot (Fig. 1a, b). Interestingly, *ZmCKX2* expression was induced in shoots but not in root. By contrast, the expression level of some other *ZmCKX*s did not show a significant response in shoot, and even decreased in root. The corresponding CKX enzyme activities were significantly increased in both root and shoot after 12 h of BA treatment (Fig. 1c). However, such responses appeared slower than those of *ZmCKX* gene expression. Thus, as a result of enhanced levels of *ZmCKX*s gene expression by its substrate, CKX activities were subsequently increased for accelerating CK degradation, aiming to control CK homeostasis in maize.

Phylogenetic Analysis of Plant *CKX* Genes

To construct the phylogeny of the *CKX* gene family, a subset of plant CKX protein sequence from representative species was collected. It consisted of 13 *CKX* genes from maize and their orthologs, including 7 *CKX* genes from *Arabidopsis*, 11 *CKX* genes from rice, and 9 putative *CKX* genes from poplar (Table 2). To gain more insight into the evolution of the CKX family, two previously reported bacteria *CKX* genes (*NsCKX1* and *RfCKX1*) (Schmuelling and others 2003), six putative *CKX* genes from a bryophyte (*Physcomitrella patens*) and two *CKX* genes from a lycophyte (*Selaginella moellendorfii*) were also included in the phylogenetic analysis (Fig. 2). Plant CKX protein contains two highly conserved domains, an oxidase FAD binding domain (domain ID: PF01565) and a cytokinin dehydrogenase 1, FAD/cytokinin binding domain (PF09265) (http://www.ebi.ac.uk/interpro). We then constructed the phylogenetic tree by alignment of either the full-length protein sequences or two conserved domain sequences and similar phylogenetic relationships

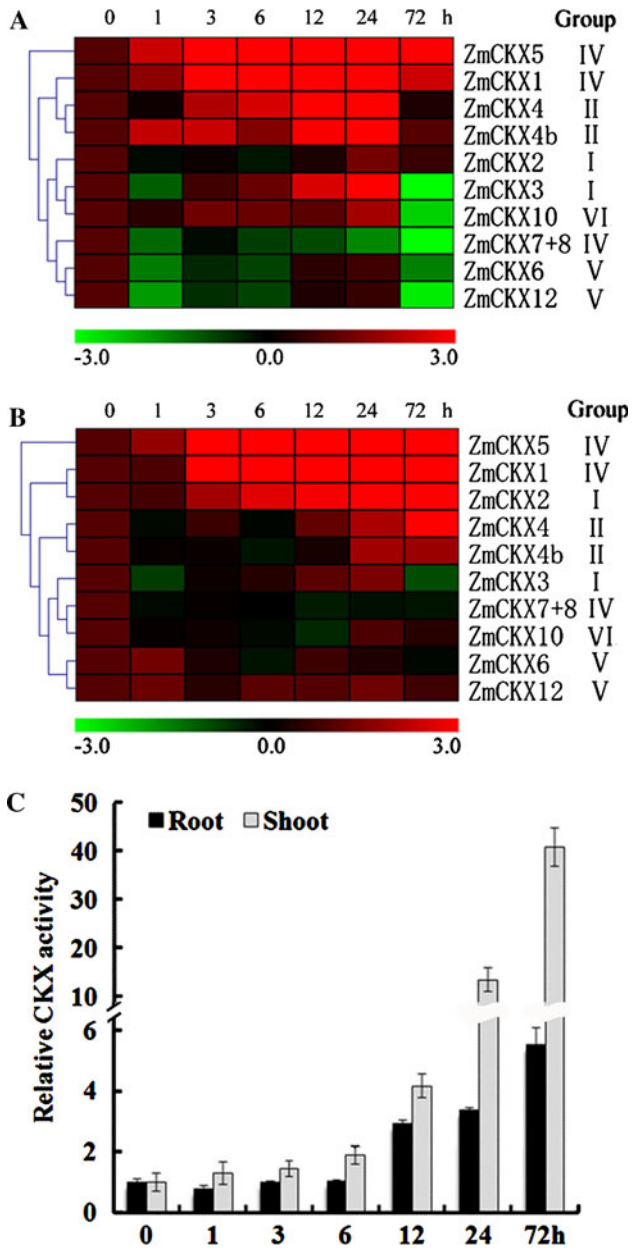

Fig. 1 *ZmCKX*s expression in root (**a**) and shoot (**b**) and their corresponding CKX activities (**c**) of maize exposed to BA for 1-72 h. *ZmCKX*s expression levels were determined by qPCR analyses, and the relative gene expression data were genewise normalized to the control plant, in which expression level was fixed to 1. *ZmTub4* gene expression served as control. Color scale represented the signal $\log_2$ value. All values of CKX activities were derived by iP substrate from at least three biological replicates with at least two independent measurements, and bars indicated standard deviation. CKX activity in controls (0 h) of root and shoot were determined as 14.21 and 0.33 pkat mg^{-1}, respectively

were observed (data not shown). Based on the phylogeny derived from full-length protein sequences, all CKXs from higher plants were assigned into six groups (I-VI) with a well-supported bootstrap value (Fig. 2). By contrast, the CKXs of the bryophyte and lycophyte, representing lower plant species, were clustered into one group. Moreover, two bacteria CKXs showed significant distances to plant members due to their low identities (<30%) with plant CKX proteins (Fig. 2). Within the different groups of CKXs in higher plants, group I and group VI each contained ten members represented as the larger groups, whereas other small groups consisted of four to six members (Fig. 2). Groups I, II, and VI contained both dicot and monocot genes, indicating that gene expansion in these groups occurred before the monocot/dicot split. By contrast, groups IV and V consisted of only monocot genes and group III of dicot genes.

Gene Structures and Chromosome Distribution of *CKX* Genes

CKX genes were found to be widely distributed in higher-plant genomes (Fig. 3). In rice, 11 *CKX*s are distributed in seven chromosomes, with two sets of segmental duplication (*OsCKX4* and *OsCKX9*, *OsCKX3* and *OsCKX8*) and a pair of tandem duplication (*OsCKX6* and *OsCKX7*). In maize, 13 *CKX*s are distributed in six chromosomes, with three pairs of segmental duplication (*ZmCKX11* and *ZmCKX12*, *ZmCKX4* and *ZmCKX4b*, *ZmCKX2* and *ZmCKX3*). Although localization of *ZmCKX8* in the genome was not available, it also might be a tandem duplication of *ZmCKX7*, which is closely related to a pair of tandem duplication in rice (*OsCKX6* and *OsCKX7*). *Arabidopsis CKX*s are located in all chromosomes, including a pair of segmental duplication, *AtCKX2* and *AtCKX4*. Members of the poplar *CKX* genes were found to be present in one plastid chromosome besides the genome chromosomes. Three segmental duplication events were also found in poplar *CKX* genes (*PtCKX4* and *PtCKX5*, *PtCKX6* and *PtCKX9*, *PtCKX1* and *PtCKX8*). Interestingly, most of the maize duplicated genes in one pair were found to be closely related to a rice ortholog, suggesting that recent duplication events of the maize gene occurred after maize/rice speciation. Similar duplication events were also observed between poplar and *Arabidopsis* genes (Fig. 2). These results suggested that recent gene duplication events could explain the gene expansion within the *CKX* gene family, particularly in maize and poplar.

The intron/exon structure of *CKX* genes was determined by comparative analysis of their genome sequences and transcript sequences. As shown in Fig. 2, the bacterial *CKX* genes are presented as one exon without any intron, whereas the plant *CKX* genes consist of three to six exons at conserved positions. This suggests that an RNA splicing event occurred at an early evolutionary stage of plant CKX proteins. In contrast to the varied gene structures in the lower plant CKX group, they are relatively conserved in

Table 2 *CKX* Genes and their expression profiles in rice, *Arabidopsis*, and poplar

Gene name	Locus/Bac[a]	Probe ID[b]	Expression level[c]					Reference
			Root	Shoot/young leaf[d]	Mature leaf	Female tissue[e]	Male tissue[f]	
OsCKX1	Os01g09260	OsAffx.9698.1.S1_x_at	629 ± 158	108 ± 21	122 ± 11	4 ± 0	147	Ashikari and others (2005)
OsCKX2	Os01g10110	Os.43596.1.S1_at	409 ± 212	214 ± 12	242 ± 52	208 ± 80	83	Ashikari and others (2005)
OsCKX3	Os10g34230	Os.46895.1.S1_at	318 ± 25	354 ± 27	175 ± 38	217 ± 42	166	Ashikari and others (2005)
OsCKX4	Os01g71310	Os.50470.1.S1_at	371 ± 76	2616 ± 242	3398 ± 595	25 ± 9	819	Ashikari and others (2005)
OsCKX5	Os01g56810	Os.33309.1.S1_at	1028 ± 243	510 ± 176	638 ± 83	783 ± 127	646	Ashikari and others (2005)
OsCKX6	Os02g12770	OsAffx.24214.1.S1_at	30 ± 5	13 ± 7	13 ± 2	2 ± 0	77	Ashikari and others (2005)
OsCKX7	Os02g12780	OsAffx.24214.1.S1_at						Ashikari and others (2005)
OsCKX8	Os04g44230	OsAffx.26455.1.S1_at	44 ± 7	86 ± 20	89 ± 11	71 ± 15	42	Ashikari and others (2005)
OsCKX9	Os05g31040	OsAffx.27093.1.S1_at	24 ± 4	494 ± 41	612 ± 105	10 ± 1	777	Ashikari and others (2005)
OsCKX10	Os06g37500	OsAffx.15797.1.S1_at	30 ± 24	23 ± 3	25 ± 4	15 ± 3	21	Ashikari and others (2005)
OsCKX11	Os08g35860	OsAffx.29543.1.S1_x_at	1420 ± 225	737 ± 120	814 ± 146	4385 ± 450	1697	Ashikari and others (2005)
AtCKX1	AT2G41510	AT2G41510	160 ± 86	86 ± 5	178 ± 54	58 ± 8	101 ± 13	Werner and others (2003)
AtCKX2	AT2G19500	AT2G19500	189 ± 5	237 ± 9	220 ± 6	171 ± 27	602 ± 46	Werner and others (2003)
AtCKX3	AT5G56970	AT5G56970	211 ± 6	147 ± 7	194 ± 20	141 ± 7	637 ± 75	Werner and others (2003)
AtCKX4	AT4G29740	AT4G29740	1008 ± 49	775 ± 39	1919 ± 130	89 ± 9	175 ± 14	Werner and others (2003)
AtCKX5	AT1G75450	AT1G75450	554 ± 9	464 ± 21	770 ± 33	864 ± 22	928 ± 95	Werner and others (2003)
AtCKX6	AT3G63440	AT3G63440	198 ± 11	308 ± 29	240 ± 10	267 ± 20	42 ± 4	Werner and others (2003)
AtCKX7	AT5G21482	AT5G21482	No data	No data	No data	No data	No data	Werner and others (2003)
PtCKX1	pt03g1575	PtpAffx.203528.1.S1_at	55.09	72.89	117.5	84.7	174.23	This work
PtCKX2	pt06g1009	PtpAffx.206397.1.S1_at	12.16	25.39	28.2	581.16	498.2	This work
PtCKX3	pt07g0777	PtpAffx.207235.1.S1_at	515.16	39.9	55.73	210.69	342.93	This work
PtCKX4	pt05g1410	PtpAffx.205795.1.S1_at	55.26	35.06	57.7	6345.86	2021.86	This work
PtCKX5	pt02g0291	PtpAffx.201634.1.S1_at	126.8	310.39	165.53	132.06	152.83	This work
PtCKX6	pt06g1495	PtpAffx.206106.1.S1_at	1.86	27.33	6.3	8.86	50.63	This work
PtCKX7	pt06g0402	Ptp.938.1.A1_s_at	400.66	177.6	568.1	4615.86	1403.93	This work
PtCKX8	pt00220g0009	PtpAffx.218925.1.S1_at	223.83	103.8	46.23	916.29	755.43	This work
PtCKX9	pt16g0357	PtpAffx.213290.1.S1_at	48.03	44.16	12.63	112.46	31.3	This work

[a] Locus represented by the AGI locus in *Arabidopsis*, the TIGR locus in rice, and the JGI locus in poplar

[b] Probe ID of *Arabidopsis CKX*s obtained from database www.arabidopsis.org, rice and poplar from http://www.affymetrix.com/analysis/index.affx

[c] Expression level of *Arabidopsis* and rice *CKX*s were from GENEINVESTIGATOR (www.genevestigator.ethz.ch), and poplar from http://www.bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi

[d] Expression level of *Arabidopsis CKX*s in juvenile leaves, poplar in young leaves, and rice in shoot

[e] Expression level of *Arabidopsis CKX*s in carpel, poplar in female catkins, and rice in pistil

[f] Expression level of *Arabidopsis CKX*s in stamen, poplar in male catkins, and rice in stamen

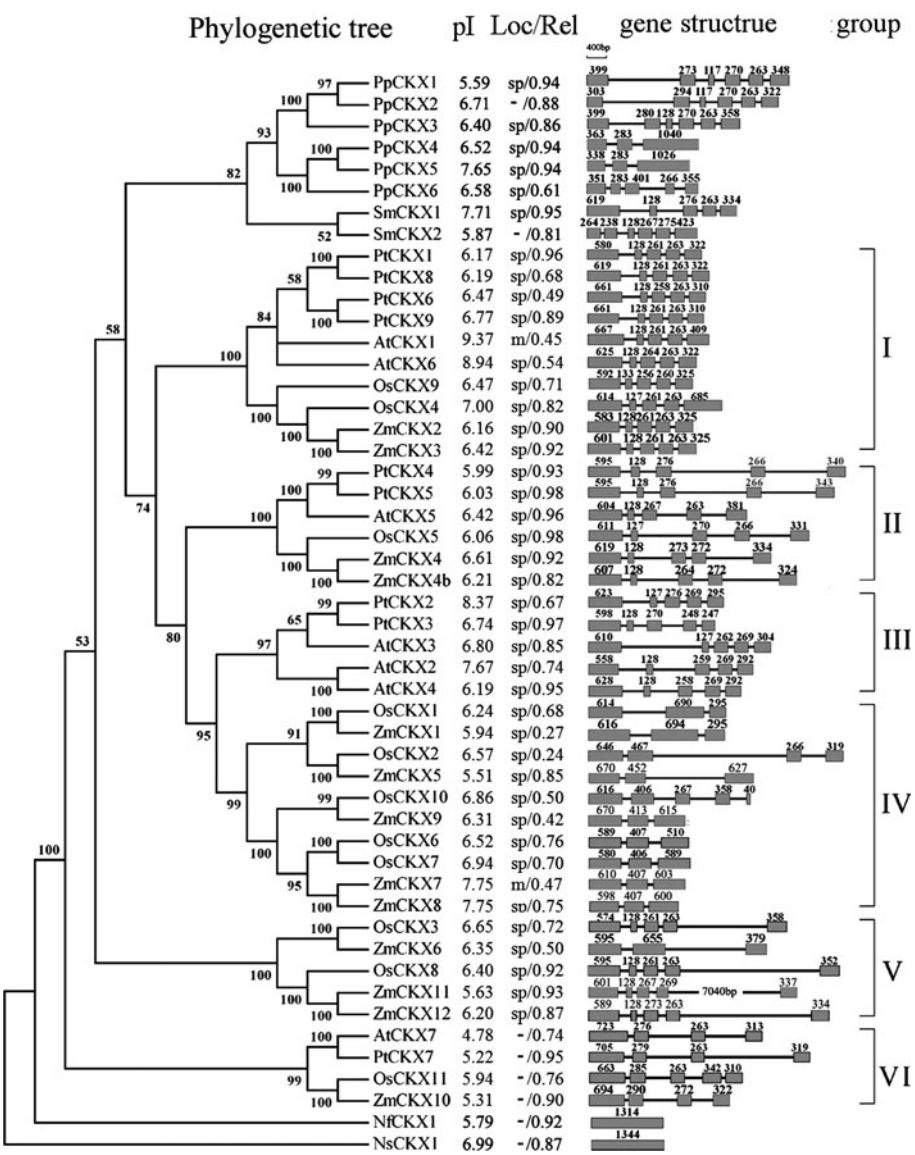

Fig. 2 Phylogenetic tree of the *CKX* gene family and their corresponding intron/exon organization, isoelectric point (pI), and predicted subcellular localization. The tree was generated by protein sequence alignment with ClustalW using the neighbor-joining method. Bootstrap support values over 50 are shown. Higher-plant CKX proteins were assigned into six groups, and the corresponding clades of each group are indicated at the right. Introns and exons are represented by black lines and black boxes, respectively. The length of each exon in base pairs is indicated. The pI was predicted by pI/Mw solfware in http://www.expasy.ch/tools/pi_tool.html. The subcellular localization/reliability (Loc/Rel) was calculated by TargetP (http://www.cbs.dtu.dk/services/TargetP) and assigned to three types: secretory pathway (sp), mitochondria (m), and no predication (−)

each group of higher plant CKX members. Members in groups I, II, and III had five exons, and the exon lengths were highly conserved, with the second, third, and fourth exons having lengths of 127-129, 256-276, 260-272 bp, respectively. However, group I members contain short introns and groups II and III contain long introns. Interestingly, most *CKX* genes in the monocot-specific groups IV and V reduced their number of exons from five down to four or even three, which would be due to the combination of the second, third, fourth, and fifth exons of their ancestors during early evolutionary separation. Remarkably, group VI *CKX*s had a special exon/intron structure with a relatively longer first exon (663-723 bp) compared to members in all other groups (558-670 bp) and also with a second exon of distinct length (276-290 bp).

Protein Properties and Conservative Domains of Plant CKX Proteins

Using MEME tools a total of 16 distinct motifs were identified in the CKX family (Fig. 4). Many motifs are conserved within all plant CKX members. Among them, motifs 1 and 2 are representative of the oxidase FAD binding domain and the cytokinin dehydrogenase 1, FAD/cytokinin binding domain, respectively. In addition, motifs 12, 13/14, 15, and 16 were uniquely presented in groups IV, V, II, and VI, respectively. The lower-plant CKX proteins also contain motif 16 which is unique in group VI. As a result, the motif identification further confirmed the divergence of CKXs between the phylogenetic groups.

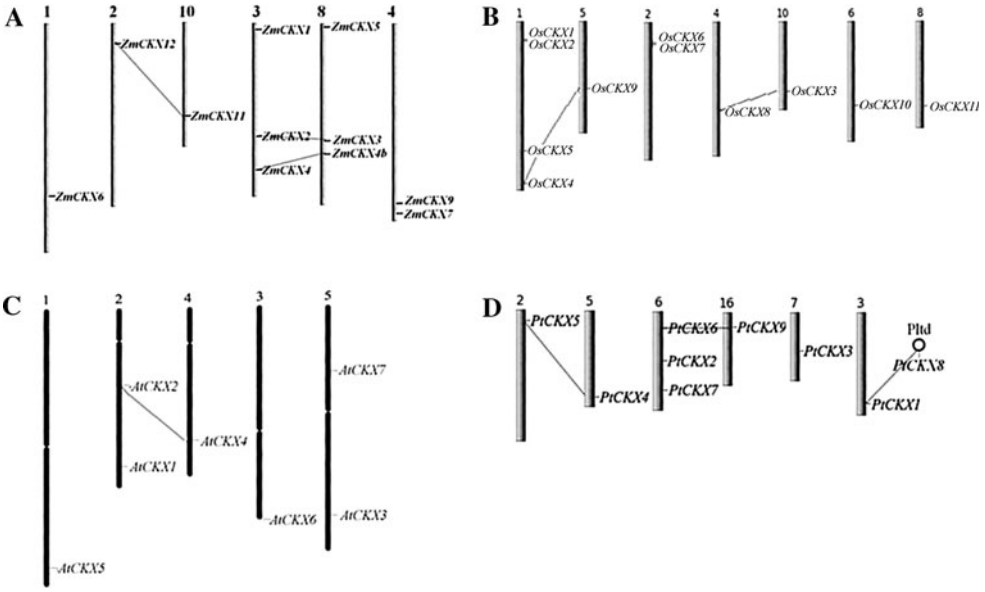

Fig. 3 Chromosomal location of *CKX* genes in maize (**a**), rice (**b**), *Arabidopsis* (**c**), and poplar (**d**). The localizations of *ZmCKX8* and *ZmCKX10* were not available from the database. A gene pair located in a duplication segment is indicated by a dotted line. The chromosome number is indicated on the top of each chromosome

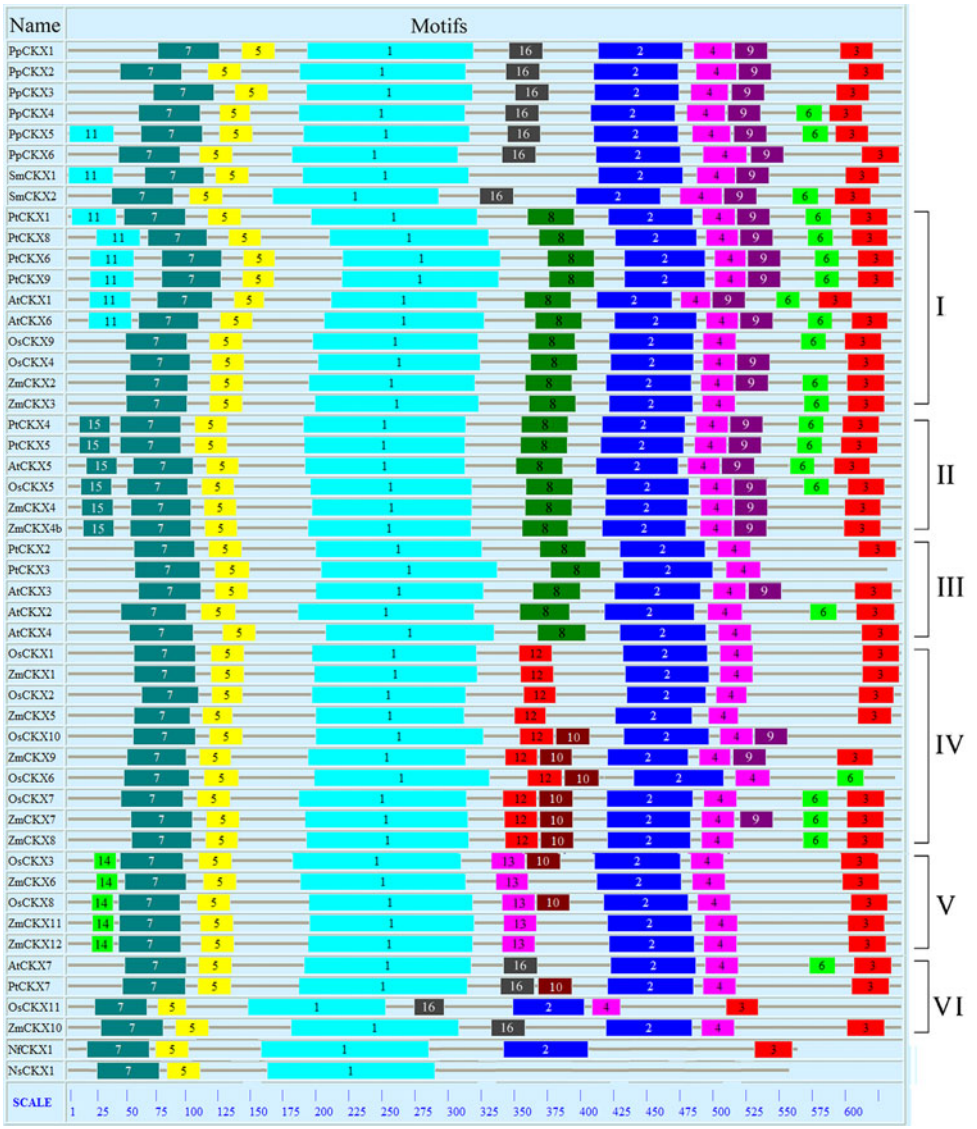

Fig. 4 Motif organization of CKX proteins. Motifs were identified by MEME using amino acid sequences. The phylogenic group of CKX proteins is indicated at the right. Motifs 1 and 2 are representative of the oxidase FAD binding domain and cytokinin dehydrogenase 1, FAD/cytokinin binding domain, respectively

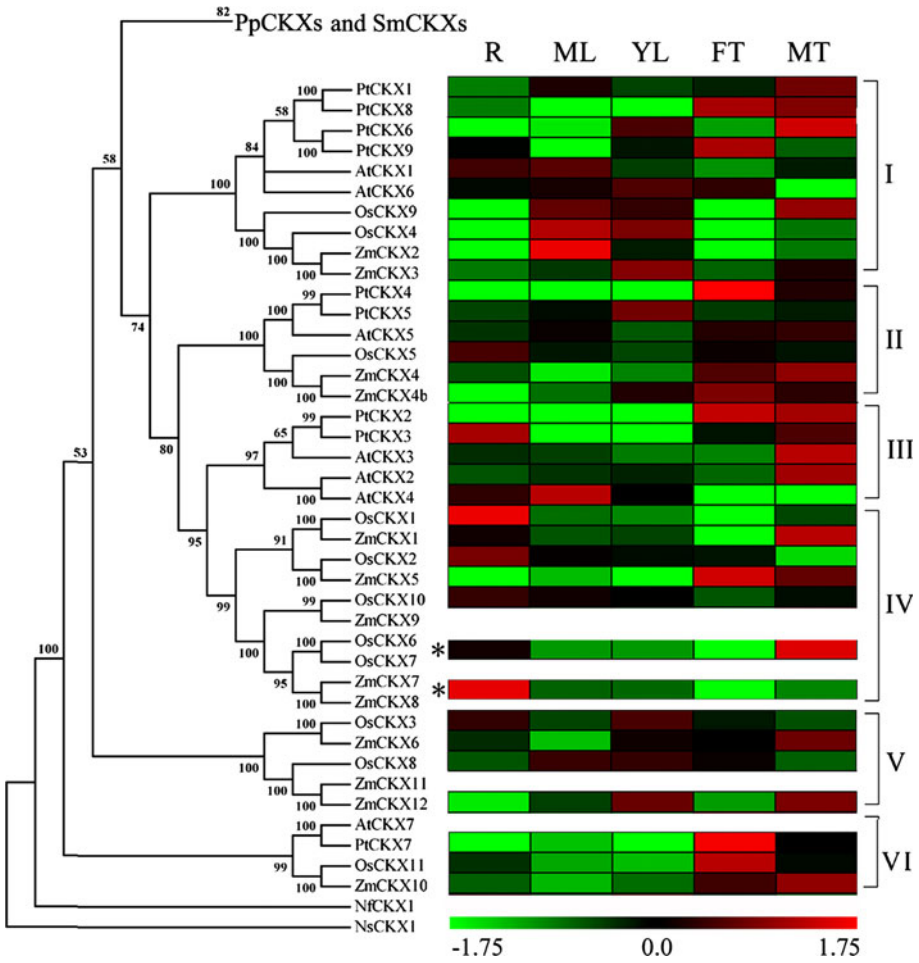

Fig. 5 Expression profiles of maize, rice, *Arabidopsis*, and poplar *CKX* genes in vegetative and reproductive organs displayed by their phylogenetic relationships. Relative gene expression data (Tables 1, 2) were genewise normalized by subtraction of the mean level of the five tissues. * represents the additive expression level of *OsCKX6* and *OsCKX7*, *ZmCKX7* and *ZmCKX8*, respectively. Color scale represents the signal $\log_2$ value. *R* root, *ML* mature leaves, *YL* young leaves or shoot, *FT* female tissues, *MT* male tissues

To explore the protein property of plant CKX, we first analyzed their isoelectric point (pI) using pI/Mw software (Fig. 2). Most members of plant CKX proteins had a neutral pI (5.5-8.4), except for a distinguishing acid pI (4.78-5.94) in those of group VI. In addition, two *Arabidopsis* CKXs in group I, AtCKX1 and AtCKX6, had the most alkaline pI (9.37 and 8.94). Besides the isoelectric point, the subcellular localization of CKX proteins was also predicted by TargetP software (Fig. 2). Most CKX proteins were predicted in the secreted pathway with reliability from 0.27 to 0.98. AtCKX1 and ZmCKX7, two proteins belonging to different groups, were both predicted to localize in mitochondrion with modest reliability. By contrast, the subcellular localization of all the CKX members in group II and at least one member of *P. patens* and *S. moellendorfii*, similar to the bacterial CKXs, could not be predicted due to their absence of any putative signal peptide.

Comparative Expression of *CKX* Genes in Higher Plants

To associate the biological function of *ZmCKX*s with specific developmental processes, we conducted comparative expression analysis of *AtCKX*s, *PtCKX*s, *OsCKX*s, and *ZmCKX*s in various tissues (Fig. 5). Using qPCR analysis of *CKX* members in maize and digital Northern analysis of those in *Arabidopsis*, poplar, and rice (Tables 1, 2), their tissue-dependent expression patterns were genewise normalized and displayed with respect to their phylogenetic relationships (Fig. 5). In general, gene expression patterns of examined *CKX*s were frequently conserved within each phylogenetic group. The gene members in groups III and VI were preferentially expressed in reproductive organs, especially in male tissue in group III. In vegetative organs, transcripts of *CKX* genes within group IV were more abundant in roots except *ZmCKX5*, and genes in group V were relatively highly expressed in young leaves. In contrast to other groups, expression levels of *CKX* members within groups I and II were more diverse, representing a preferential expression in root, leaves, or reproductive organs.

Discussion

Comparative phylogenetic analysis is a powerful method to identify homologous genes, including both orthologs and

paralogs. Orthologs are genes in different species that evolved from a common ancestor and which typically retain the same function (Tatusov and others 1997). Thus, identification of orthologs across species is essential to predict gene function in newly sequenced genomes. By contrast, paralogs are genes derived from duplication events within a genome. The genes may perform part of the original function (subfunctionalization) or even new functions (neofunctionalization) if they are not silenced (nonfunctionalization) (Shan and others 2007). Phylogenetic analysis has been widely used to classify gene families and predict their functional orthologs (Yang and others 2006, 2008). Moreover, cross-species expression profiling, which provides supportive information on gene function, has been combined with phylogenetic analysis to generate successfully the functional categories within large gene families such as WRKY and bHLH transcriptional factor families and CCCH zinc finger family (Li and others 2006; Mangelsen and others 2008; Wang and others 2008).

The *CKX* gene family *in planta* consists of a great number of members that are expressed in different plant tissues and play essential roles in fine-tuning cytokinin levels for control of plant growth and development (Schmuelling and others 2003). The physiological function of an individual CKX isozyme in plants depends mainly on its protein property, subcellular localization, and expression manner (Schmuelling and others 2003; Werner and others 2003; Massonneau and others 2004). To classify functional orthologs, Schmuelling and his coworkers (2003) had constructed a phylogenetic tree of the *CKX* family, but a limited number of genes (*ZmCKX1*, 7 *AtCKX*s, and 5 *OsCKX*s) were examined. More recently, Vyroubalova and others (2009) collected most maize and rice *CKX*s and constructed a relatively informative phylogenetic tree together with all *Arabidopsis CKX* members. However, the functional categories of *CKX* members have not been discussed yet. In our study, *CKX* members from poplar and two lower-plant species, bryophyte (*Physcomitrella patens*) and lycophyte (*Selaginella moellendorfii*), were supplemented by phylogenetic analysis in addition to maize, rice, and *Arabidopsis CKX*s (Fig. 2), and a comparative gene expression analysis among the higher-plant species was also combined with their phylogenies (Fig. 5). As a result, a comprehensive relationship between these *CKX* members in relation to their gene sequences, protein properties, and expression patterns was revealed. This information not only provides an overview of the evolutionary development of the *CKX* gene family, it also aids in classifying functional categories of different CKX proteins and further expands *CKX* gene function from one species to the other.

The putative *CKX* gene sequences (*NsCKX1* and *RfCKX1*) have been discovered in the cyanobacterium *Nostoc* and the phytopathogen *Rhodococcus fascians* (Schmuelling and others 2003), which showed a large distance to plant members (Fig. 2). Although the importance of cytokinins during *Rhodococcus fascians* pathology was recently elucidated (Pertry and others 2009), the function of *RfCKX1* still remains unclear. Nevertheless, the presence of putative *CKX* sequences in cyanobacteria suggests that plant *CKX* genes may have evolved from ancient genes of chloroplasts probably originating from the endosymbiosis of cyanobacteria (Schmuelling and others 2003). We subsequently examined the genome sequences of green algae (*Chlamydomonas reinhardtii*, *Ostreococcus tauri*, and *Volvox carteri*), but none of the *CKX* sequences were found. Indeed, nothing is known about cytokinin oxidase activity in algae, and Stirk and others (2003) further suggested that macroalgae have different pathways for regulating cytokinin concentrations. However, six putative *CKX* genes were identified from the bryophyte *Physcomitrella patens*, which was the most basal land plant investigated in this study (Fig. 2). Meanwhile, the *IPT* gene, which catalyzes cytokinin biosynthesis, is also absent in green algae (Stirk and others 2003), six members of *IPT* are present in *Physcomitrella patens* (Yevdakova and von Schwartzenberg 2007; Sakakibara and others 2008). The coexistence of *IPT* and *CKX* genes in bryophytes may suggest an important evolutionary event, one that generates fine-tuning control of cytokinin homeostasis via biosynthesis and degradation processes. Most importantly, a recent study from Pils and Heyl (2009) showed that the complete set of proteins of the cytokinin signaling pathway also first appeared in this basal plant (Pils and Heyl 2009). Thus, the cytokinin metabolic pathway together with the signal transduction pathway makes *Physcomitrella patens* the earliest diverging species to conquer the land.

The CKX proteins of lower plants were more similar to each other than to those of any other higher-plant species (Fig. 2), which is similar to the phylogeny of the cytokinin receptors and phosphotransmitter proteins (Pils and Heyl 2009). The drastic expansion of CKX family members occurred in higher plants and subsequently diversified to several clades (Fig. 2). *CKX* members in group VI are highly conserved and present the largest distance from those genes in other groups of higher plants (Fig. 2). These *CKX* members revealed a special gene structure with first and second exons of distinct length (Fig. 2). In contrast to the existence of duplication events in other groups, group VI contains only a single gene from each species. Meanwhile, unique motif 16, which originated from lower plants, is retained only in the CKX proteins of this group (Fig. 4). Importantly, most CKX enzymes were predicted to be secreted proteins with neutral pI, but the proteins in group VI represented distinguished acid pI and were not predicted to be in any cell compartments (Fig. 2). Indeed, a

recent study showed that one member of this group, ZmCKX10, was localized in cytosol as a nonsecreted enzyme (Smehilova and others 2009). These authors also showed that *ZmCKX10* has an organ-specific expression pattern distinct from that of *ZmCKX1*, and our comparative expression analyses among four plant species further support the finding of their dominant expression in reproductive organs (Fig. 5). Considering also that ZmCKX10 had distinct electron acceptors and preferential substrates (Smehilova and others 2009), group VI could be a special class of CKX enzymes with distinct physiological function in higher plants.

In contrast to the conserved *CKX* members in group VI, the *CKX* members are highly diverse in groups I and II. These two groups contain a large number of *CKX* genes of both dicot and monocot plants (Fig. 2). Many gene duplication events were observed, supporting their expansion of gene numbers within these two groups (Fig. 3). Three principal evolutionary patterns were attributed to gene duplication: segmental duplication, tandem duplication, and transposition (Kong and others 2007). In plants, segmental duplication occurs most frequently because most plants are polyploids and retain numerous duplicated chromosomal blocks within their genomes (Cannon and others 2004). Thus, it is expected that the *CKX* gene expansion occurred mainly through segmental duplications in maize, rice, and poplar (Fig. 3). Gene duplication events are important to gene family evolution because duplicated genes could provide the raw materials for the generation of new genes which, in turn, facilitate the generation of new functions (Kong and others 2007). Interestingly, in groups I and II, a distinct tissue expression pattern was observed between the paralogs of *CKX* genes, which were derived from duplication events (Fig. 5). *CKX* paralogous genes with altered manners of expression would probably cover the full range of functions of ancestral genes, which is known as the subfunctionalization process. Taken together, CKX members in groups I and II may have similar enzyme properties but play diverse functions in the different developmental and physiological processes due to their varying expression manners.

Groups III, IV, and V contain either dicot- or monocot-specific genes, suggesting that gene expansion within these groups occurred after the dicot/monocot split, which is common in many gene families (Yang and others 2008). Group III contains dicot genes that are preferentially expressed in male tissue (Fig. 5). Interestingly, both monocot-specific groups IV and V have three to four exons, which might be evolutionarily derived from their common ancestors of five exons (Fig. 2). Reducing the number of exons is likely in be useful to enhancing gene expression levels in monocots (Chiaromonte and others 2003). These two monocot-specific groups also differed from each other because each contains unique motifs and has distinct gene expression patterns (Figs. 4 and 5). *CKX* genes of group IV are expressed mainly in root, whereas genes of group V are expressed mainly in young leaves. Moreover, the *CKX* genes in group IV were strongly induced by external BA supply (Fig. 1), showing cytokinin-dependent regulation activity, which is further supported by the previously reported *ZmCKX1* expression patterns (Brugiere and others 2003) and the digital data of *OsCKX2* (www.genevestigator.ethz.cn). Within group IV, *ZmCKX1* and *OsCKX2* genes have been shown to play an essential role in reproductive organs (Brugiere and others 2003; Ashikari and others 2005). However, in the present study both genes were also found to be highly expressed in roots, suggesting their potential physiological function in roots in addition to that in grains. In this way, the combination of phylogenetic characterization of *CKX* genes and their subsequent comparative expression patterns would provide a solid basis for further functional characterization of the *CKX* family *in planta*.

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