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The systematic characterization of poplar CK2 α and its theoretical studies on phosphorylation of P-protein C-terminal domain

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Casein kinase 2 (CK2) is a ubiquitous serine-threonine protein kinase, which of specific substrates are involved in the regulation of several vital physiological processes in plants. Detailed studies of CK2 have focused on the herbaceous species of *Arabidopsis*, maize, however, it has remained largely unexplored in woody trees. In this study, we identified the four genes encoding CK2 α subunits in a search for *Populus* genome. Reorganization of CK2 α genes in *Populus* genome showed that its multiple copies are resulted from segmental duplication and tandem duplication events. Phylogenetic and *in silico* expression analysis indicated that, the divergence was present among poplar CK2 α genes, dividing into type I and type II, and each type represented by relatively consistent expression profile. Further docking and molecular dynamic simulations revealed that the poplar CK2 α specifically recognize the special pentapeptides (XES/T*DD) of natural substrates and then introduce the phosphorylation of C-terminal region of poplar P-protein. This study will provide the insight into the understanding of interacting mechanism of poplar CK2 α with their natural substrates, and be of valuable resource for further assessment of the function of phosphorylation of P-protein in woody plants.

Key words: *Populus trichocarpa*, phosphorylation, casein kinase 2, docking, silico simulation.

INTRODUCTION

Phosphorylation of protein mediated by protein kinases is a major post-translational modification mechanism for regulation of a broad spectrum of fundamental cellular

processes (Mann et al., 2002; de la Fuente van Bentem and Hirt, 2007). Casein kinase 2 (CK2, EC 2.7.11.1) is a typical and ubiquitous serine-threonine protein kinase with wide substrate profiles, and is highly conserved across all eukaryotes (Litchfield, 2003; Pinna, 2002). In comparison with other protein kinases, CK2 is a constitutively active enzyme, independent of second messengers, and can utilize either ATP or GTP as phosphate donors (Litchfield, 2003; Pinna, 2002). A vast amount of proteins (>300) are known as the substrates of CK2, involved in numerous vital cellular processes, such as cell proliferation, apoptosis and differentiation (Guerra and Issinger, 1999; Litchfield, 2003; Bibby and Litchfield,

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Abbreviations: CK2, Casein kinase 2; LG, Linkage Groups; CVFF, consistent-valence force-field; MD, molecular dynamic.

2005; Lou et al., 2008). In plants, phosphorylation of the specific substrates by CK2 are involved in the regulation of several vital physiological processes, such as seed and plant development (Moreno-Romero et al., 2008; Espunya and Martinez, 2003), auxin-signaling (Marques-Bueno et al., 2011), ABA signaling (Riera et al., 2004), plant defence (Kang and Klessig, 2005), circadian rhythms (Sugano et al., 1999; Sugano et al., 1998; Lee et al., 1999; Daniel et al., 2004), photomorphogenesis (Bu et al., 2011), translation (Aguilar et al., 1998; Dennis and Browning, 2009), transcription (Johnston et al., 2002; Hu et al., 2003) and transport (Lebska et al., 2010).

CK2 is a hetero-tetrameric complex formed by two catalytic subunits (α and α') and two regulatory subunits (β), which enhance the stability, activity and substrate specificity of CK2 hetero-tetramer (Litchfield, 2003). Moreover, previous studies had shown that, the individual catalytic subunits (CK2 α and CK2 α') could present the bioactivity without any regulatory subunits *in vivo*, and respectively exert different functions (Triodi et al., 2010; Domanska et al., 2005). With regard to the structure of CK2 α , it shares a core architecture, consisting of an N-terminal domain based on a central anti-parallel β -sheet and an α -helical C-terminal. Many eukaryotic CK2 α catalytic subunits gene had been identified in their individual genome, two genes (*CKA1* and *CKA2*) encoding for catalytic subunits in *Saccharomyces cerevisiae* (Domanska et al., 2005), two in human (Ackermann et al., 2005; Wirkner et al., 1994), two in mouse (Guerra et al., 1999; Chen et al., 2002). In contrast, in plants, genes encoding α subunits seem to be of multigene families, four genes encoding CK2 α in *Arabidopsis* (Salinas et al., 2006), three in *Zea mays* (Lebska et al., 2009). Although, genes encoding CK2 α catalytic subunits have been described in several plants (Salinas et al., 2006), there is still no systematic and comprehensive characterization of CK2 α in woody plant genome, especially for poplar, whose genome had been completely sequenced (Tuskan et al., 2006).

The substrates of phosphorylation by CK2 shared a consensus sequence feature as S/T-x-x-E/D/pS, and C-terminus of the acidic consensus E/D-x-D generally occurs on phosphorylation by CK2 (Meggio and Pinna, 2003; Zschoernig and Mahlknecht, 2009). Thus, several identified phosphorylated sites located on conserved C-terminal region of most poplar acid ribosomal proteins had been proved to be phosphorylated by CK2 *in vivo* (Liu et al., 2010; Niefind et al., 1998). To the best of our knowledge, the interaction mechanism of poplar CK2 α catalytic subunits with substrates had not been addressed.

Therefore, the aim of the present study was to evaluate the phosphorylation of the certain substrates actions of poplar CK2 α *in vivo* by the method of wide-genomic investigation of poplar CK2 α combined with *in silico* simulation. In this study, four genes encoding α subunits were identified across the complete *Populus* genome, and their multiple copies are resulted from segmental duplication

and tandem duplication events. On such basis, explicitly solvated docking and molecular dynamic (MD) methods were applied to investigate the interactions involving natural substrate with the poplar CK2 α . We proposed that further studies be carried out to understand the interacting mechanism and functions of phosphorylated P-protein in woody plants.

MATERIALS AND METHODS

Identification, chromosomal location and genomic structure of poplar CK2 α genes

Poplar CK2 α proteins were identified by running PSI-BLAST (position-specific iterated BLAST) iteration 3 with max 500 against protein database of *Populus trichocarpa* available in NCBI (<http://www.ncbi.nlm.nih.gov/>), and *Arabidopsis* CK2 α C sequence was selected as query sequence (Salinas et al., 2006).

CK2 α genes were located in the genome of *P. trichocarpa* by NCBI map viewer (<http://www.ncbi.nlm.nih.gov/projects/mapview/>). Identification of duplicated regions between chromosomes was completed as previous literature (Tuskan et al., 2006). The tandem gene duplication in poplar was determined according to the criteria that five or fewer gene loci occurred within a range of 100kb distance (Hu et al., 2010; Finn et al., 2006).

Gene structure display server (GSDS) program (<http://gsds.cbi.pku.edu.cn/>) was applied to illustrate exon/intron organization for individual CK2 α genes by comparison of the cDNA sequences and their corresponding genomic sequences.

Phylogenetic analysis and microarray analysis

Multiple sequences alignments of the full-length protein sequences were performed using ClustalW program in BioEdit software with default parameters (Hall, 1999). Based on these aligned sequences, the unrooted phylogenetic trees were constructed using MEGA 5.0 software (Tamura et al., 2011), by both Neighbor-joining method (Saitou and Nei, 1987) and Maximum Likelihood method with parameters (p-distance and completed deletion). The reliability of the phylogenetic tree was estimated using bootstrap value with 1000 replicates.

Probe sets corresponding to individual poplar CK2 α gene were identified using probe match tool available at NetAffxTM Analysis center (<http://www.affymetrix.com/analysis/index.affx>). For genes with more than one probe sets, non-redundant probe sets were considered as their assigned probe set. The transcript relative abundance value of all poplar CK2 α genes from various tissues was gained from the poplar transcript abundances datasets (Wilkins et al., 2009) in the website of the *Populus* electronic fluorescent pictograph browser (poplar eFP browser) (<http://bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi>). Dendrogram and heat map for display expression pattern were obtained using the Cluster 3.0 (de Hoon et al., 2004) for hierarchical clustering based on Pearson coefficients, and then Java Tree-View 1.1 program (Saldanha, 2004) for visualizing the analyzed datasets.

In silico modeling

All the docking and molecular dynamics (MD) simulations were performed with the different modules implemented under InsightII 2005 software package (InsightII Version 2005, 2005) on Linux workstations, using the consistent-valence force-field (CVFF).

Table 1. Characterization and identification of CK2 α subunits genes of poplar.

Gene name	The encoding proteins (GeneBank ID)	RNA (Refseq RNA ID)	Chromosome location
<i>PtrCK2α-1</i>	EEE79291.1	XM_002304276.1	LGIII-NC_008469(8031628-8035845)
<i>PtrCK2α-2</i>	EEE93496.1	XM_002306464.1	LGV-NC_008471(6084281-6089153)
<i>PtrCK2α-3</i>	EEE93497.1	XM_002306465.1	LGV-NC_008471(6097038-6101783)
<i>PtrCK2α-4</i>	EEE78372.1	XM_002303357.1	LGIII-NC_008469(8342118-8348911)

The crystal structures of the catalytic subunit of protein kinase CK2 (CK2 α) from *Z. mays* (PDB ID: 1LR4) (Yde et al., 2005) were recovered from the PDB database. All the hetero-atoms were removed from the protein structure (Zhang and Zhong, 2010). On such base, the poplar CK2 α proteins were generated with the Biopolymer module (InsightII, 2005) (InsightII Version 2005, 2005). All the obtained protein structures were saturated with hydrogen atoms based on the expected charge distributions of amino acids at physiological pH (InsightII Version 2005, 2005; Yang et al., 2009; 2010a; 2010b; 2011).

The structures of peptide-bougie (Table 2) were generated with the Builder module. Geometry and partial atomic charges of the substrate were conducted using the Discover 3.0 module by applying the BFGS algorithm (Head and Zerner, 1985) with a convergence criterion of 0.01 kcal mol⁻¹ Å⁻¹. Previous literatures revealed that (Yang et al., 2009; Yang et al., 2010b; Yang et al., 2010a), the docking and molecular dynamics (MD) simulations were performed to explore and understand the interactions between peptide-bougies and poplar CK2 α proteins, by the general protocols in the InsightII 2005 software packages (Affinity User Guide, 2005; Yang et al., 2009, 2010a, a). The MD trajectories were generated using a 1.0 fs time step for a total of 5000 ps, saved at 5.0-ps intervals. The interaction energies of peptide-bougies with poplar CK2 α proteins and the respective residues at the binding pocket were calculated by the Docking module (Park et al., 2001), over the 1000–5000 ps MD trajectories. More calculated details can refer to elsewhere (Yang et al., 2009).

RESULTS AND DISCUSSION

Identification and characterization of CK2 α subunits genes in poplar

Four genes for CK2 α subunits (α A, At5g67380; α B, At3g50000; α C, At2g23080; and *acp*, At2g23070) in *Arabidopsis* (Salinas et al., 2006), three genes in maize (Lebska et al., 2009), have been characterized previously. However, CK2 α has not been documented in poplar. In order to identify the CK2 α subunit genes present in the poplar genome, PSI-BLAST (position-specific iterated BLAST) program was exploited. As a result, four CK2 α proteins encoded by four CK2 α genes were gained from Nr protein database of *P. trichocarpa* in NCBI, respectively named *PtrCK2 α -1* (EEE79291.1), *PtrCK2 α -2* (EEE93496.1), *PtrCK2 α -3* (EEE93497.1) and *PtrCK2 α -4* (EEE78372.1) (Table 1). The *P. trichocarpa* genome encodes the same numbers of CK2 α gene members as *Arabidopsis* (Salinas et al., 2006), the similar as maize (Lebska et al., 2009). It appears that no significant expansion occurred from CK2 α gene members

of poplar. However, previous evidence revealed that many multi-gene families in poplar lineage were expanded and resulted from whole genome duplication events followed by multiple segmental duplication, tandem duplication, and transposition events (Tuskan et al., 2006).

The CK2 α genes were further *in silico* mapped to loci across all 19 Linkage Groups (LG). A schematic view of the chromosomal location of all genes is shown in Figure 1. It was found that all of the four poplar CK2 α genes were distributed across the duplicated blocks between the two Linkage Groups (LG), LGIII and LGV (Table 1, Figure 1); and the two genes of *PtrCK2 α -2* and *PtrCK2 α -3* were considered as the tandem duplication events that they were directly adjacent to each other on LGV without intervening annotated gene (Figure 1). Consistently, the similar segmental duplication and tandem duplication events also occurred in CK2 α genes of *Arabidopsis* (Salinas et al., 2006). In summary, unlike other multi-gene families of poplar, the expansion that poplar genome usually encodes more gene family members than *Arabidopsis* had not been presented in poplar CK2 α gene members, since they could possess similar segmental duplication and tandem duplication events.

Phylogenetic analysis of CK2 α gene members in poplar, *Arabidopsis*, and maize

To determine the phylogenetic relationship among the CK2 α proteins of poplar, *Arabidopsis* and maize, an unrooted tree was respectively constructed by both Neighbor-Joining (Saitou and Nei, 1987) and Maximum likelihood methods using MEGA 5.0 (Tamura et al., 2011). The tree topologies generated by the two methods were comparable without modifications at branches, and supported by their high bootstrap values of > 50, suggesting that a reliable unrooted tree topology was constructed.

Phylogenetic analysis identified the presence of 3 distinct CK2 α protein groups across these plants: type I, II and III (Figure 2), in which most of plant CK2 α proteins (11/15) were assigned to type I, a few (3/15) in type II including AtCK2 α acp (AEC07404.1), *PtrCK2 α -4* and *PtrCK2 α -3*, and only ZmCK2 α -4 (ACF80391.1) to type III (Figure 2). The result indicated that all CK2 α proteins of poplar were encoded by evolutionarily divergent genes,

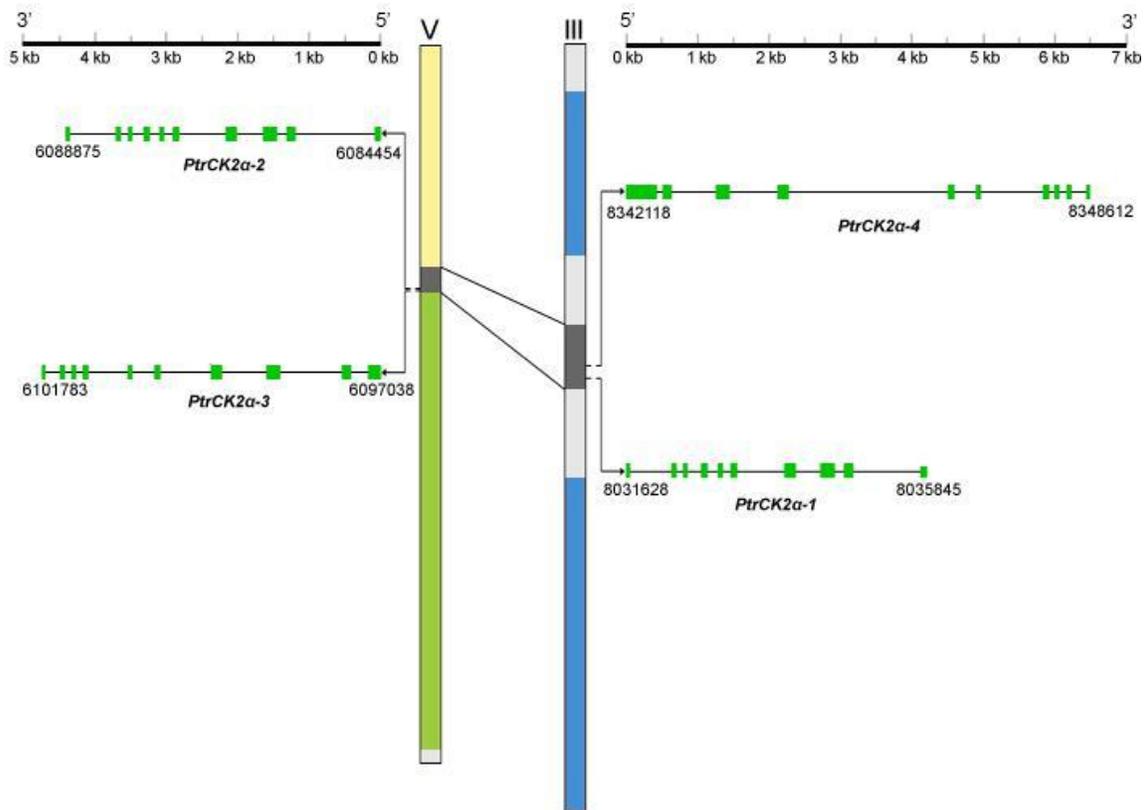


Figure 1. Schematic representation of chromosomal location for the *CK2α* genes over the *Populus trichocarpa* genome, and their Exon/intron structures. Dark grey regions linked by lines were indicated as segmental duplicated homologous blocks between LGIII and LGV chromosomes. Exons and introns of *CK2α* genes are represented by green boxes and black lines, respectively, and their sizes can be estimated by the scale at the top.

and the divergence occurred between two *CK2α* proteins “PtrCK2α-1” and “PtrCK2α-2”, and other two *CK2α* proteins “PtrCK2α-3” and “PtrCK2α-4”, which is consistent with previous report on *Arabidopsis* (Salinas et al., 2006) and mustard (Ogrzewalla et al., 2002). In addition, the divergence in *CK2α* proteins is supported by apparent differences in amino acid sequences of type I, II and III, especially in their N-terminal extension sequences that are predicted to be putative chloroplast signal peptides in type II, and mitochondrial signal peptides in type III, whereas, no any leader peptides in type I (data not shown). It indicates that independent duplication and divergence of *CK2α* genes are common across plants.

The gene structural display could provide us additional information for evolutionary relationship of multi-gene families (Guo et al., 2007). To further gain novel insight into the phylogenetic relationship of poplar *CK2α* genes, the exon/intron organization was illustrated for individual *CK2α* genes by comparison of the cDNA sequences and their corresponding genomic sequences (Figure 1). As a result, the closely related *CK2α* genes members from the same type in poplar, respectively exhibited a highly

conserved distribution of exon/intron, and the presence of some extent of variation between type I and type II. The similar gene architecture of *CK2α* genes in the same type might provide additional supports to the phylogenetic analysis. On the other hand, the differences of gene organization between different types might also support the divergence in *CK2α* genes of poplar.

Differential expression profile of poplar *CK2α* genes

Publicly available microarray data was often considered as a reliable means of studying gene expression profiles (Ohlrogge and Benning, 2000). To gain the expression pattern of all poplar *CK2α* genes, the poplar Affymetrix microarray data (Wilkins et al., 2009) were reorganized. All 4 poplar *CK2α* genes have their corresponding probe sets in the dataset and the expression profile was displayed as indicated Figure 3. Poplar *CK2α* genes showed distinct tissue-specific expression patterns that were preferentially expressed in c), root (R), and seedling under specific conditions, while few in mature leaf (ML), young leaf (YL) and xylem (X). Combined with the high

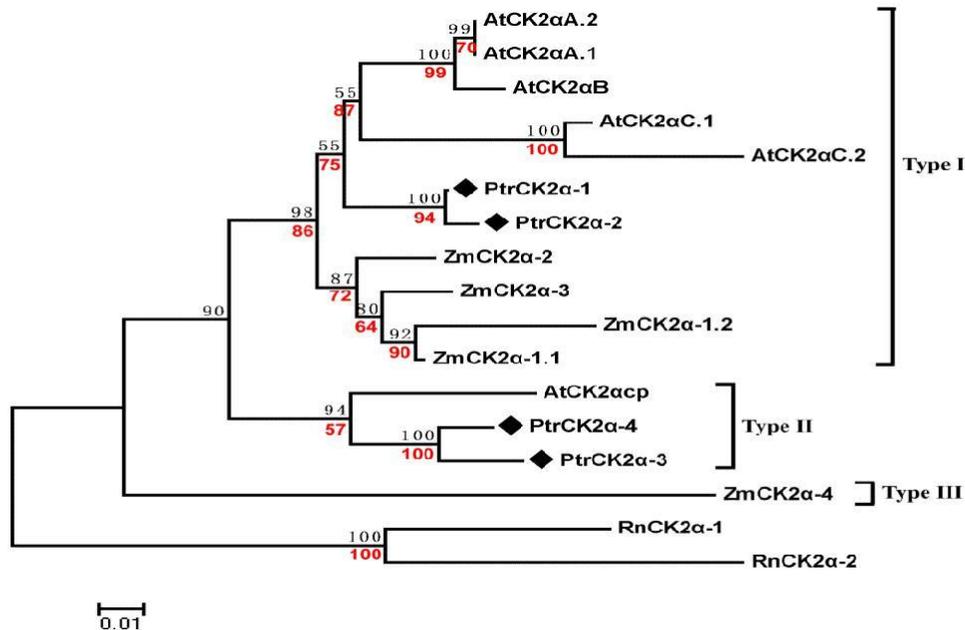


Figure 2. Phylogenetic analysis of CK2 α proteins. Multiple sequence alignment of 17 full-length amino acid sequences of CK2 α were performed by ClustalW and a phylogenetic tree was constructed using MEGA version 5.0 (Tamura et al., 2011) by both Neighbor-joining method (Saitou and Nei, 1987) and Maximum Likelihood method with 1000 bootstrap replicates. Branch lengths are proportional to the amino acid distances along each branch. Neighbor-joining bootstrap values for clans supported above the 50% level are indicated in black font above branches, whereas Maximum Likelihood bootstrap values above the 50% level are shown in red font below the branches. The black diamonds are highlighted in the front of all CK2 α protein subtypes from poplar. All CK2 α protein names and their individual corresponding ID number for phylogenetic analysis are listed as follow: ZmCK2 α -1.2 (AAF76187.1); ZmCK2 α -1.1 (P28523.1); ZmCK2 α -3 (AAG36872.1); ZmCK2 α -2 (CAA72290.1); ZmCK2 α -4 (ACF80391.1); AtCK2 α A.1 (AED98335.1); AtCK2 α A.2 (AED98334.1); AtCK2 α B (AEE78615.1); AtCK2 α C.1 (AEC07406.1); AtCK2 α C.2 (AEC07405.1); AtCK2 α cp (AEC07404.1); PtrCK2 α -1 (EEE79291.1); PtrCK2 α -2 (EEE93496.1); PtrCK2 α -3 (EEE93497.1); PtrCK2 α -4 (EEE78372.1); RnCK2 α -1 (AAH91130.1); RnCK2 α -2 (EDL87283.1).

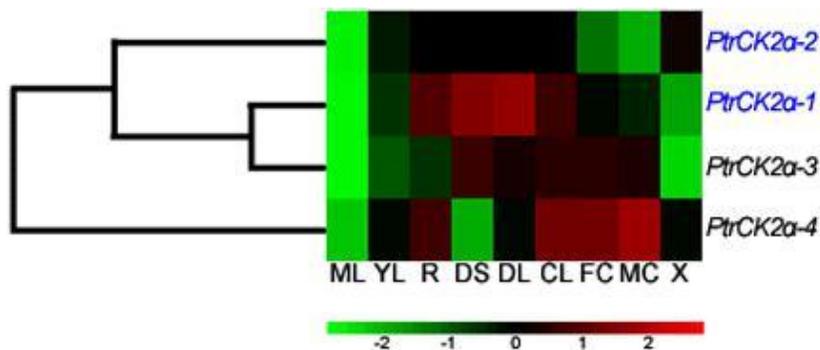


Figure 3. Relative transcript abundance profiles of poplar CK2 α genes across different tissues. A heat map displaying the transcript abundance is produced using the genome-wide microarray data generated by Wilkins and coworkers (Wilkins et al., 2009). The transcript abundance levels for the poplar CK2 α genes were clustered using hierarchical clustering based on Pearson correlation. Color scale at the bottom of each dendrogram represents log₂ expression values, green color represents low level and red color represents high level of transcript abundances. The same type of poplar CK2 α genes are marked in the same color. Symbols represent as follows: ML, mature leaf; YL, young leaf; R, root; DS, dark-grown seedlings; DL, etiolated dark-grown seedling transferred to light for 3h; CL, continuous light-grown seedling; FC, female catkins; MC, male catkins; X, xylem.

Table 2. The two selected phosphopeptides for docking study on their interaction with poplar CK2 α subunits.

Poplar P-Proteins	JGI Protein ID	Identified phosphopeptides	Residue	Segment	Mr (Da)	Charge	Score
RPP2B	832971	KEEKVEEKEES ^a DDDMGFSLFD	S103	92-113	2585.04	2	170
RPP2C	836661	KEEKVEEKEDT ^a DDDLGFSLFD	S104	93-114	2070.77	2	150
RPP2A	567964	KEEKVEEKEDT ^a DDDLGFSLFD	T100	90-110	2070.77	2	150

^aPhosphorylated residues. The two identified phosphorylation peptides are referred from our previous identification (Liu et al., 2010).

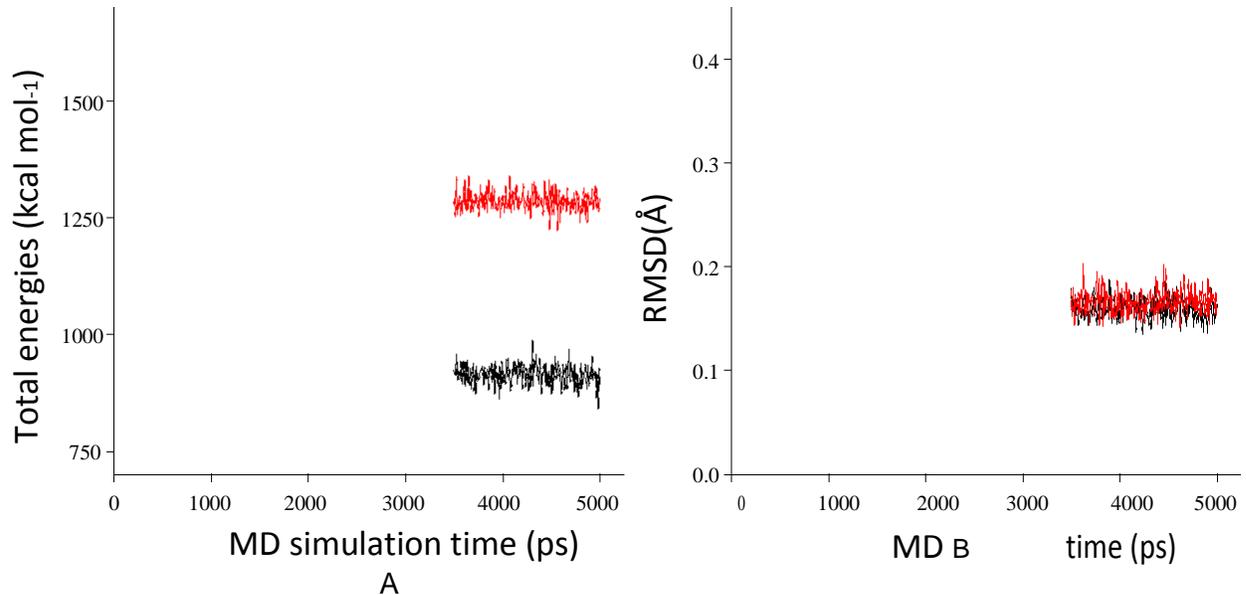


Figure 4. The time-evolution total energies (A) and backbone-atom root mean square deviations (RMSD, B) for the docked complexes during the MD simulations. The values of KEEKVEEKEES^aDDDMGFSLFD-CK2 α and KEEKVEEKEDT^aDDDLGFSLFD-CK2 α were represented by black and red lines, respectively.

activities of CK2 present in chloroplasts from *Arabidopsis* leaves (Salinas et al., 2006), it indicates that some translated CK2 proteins after transcription should be transported to chloroplasts. *PtrCK2 α -3* and *PtrCK2 α -4* genes from type II in phylogenetic tree have significantly similar expression pattern that high-level expression are mostly present in FC, MC, and continuous light-grown seedling (CL). The expression profile of *PtrCK2 α -1* and *PtrCK2 α -2* genes from type I in phylogenetic tree also showed relatively consistent that transcript accumulation focused on continuous light-grown seedling (CL), etiolated dark-grown seedling transferred to light 3h (DL), dark-grown seedlings (DS), and root (R). The fact that poplar CK2 α genes from the same type share similar expression patterns across tissues, provide an additional evidence for their phylogenetic relationship.

Previous studies on *Arabidopsis* implicated that CK2 play an important role in regulating light signaling and circadian rhythms (Sugano et al., 1999). However, little is known whether light signaling could regulate the expression of CK2 α genes. In the current study, it was found that the transcript abundance of *PtrCK2 α -1* and

PtrCK2 α -4 were induced by light, while *PtrCK2 α -2* and *PtrCK2 α -3* were negatively modulated by light. It seems that the mechanism should contribute to quick start phosphorylation of their extensive substrates under the conditions of dark and light transition.

***In silico* simulation on the interaction of CK2 α with the substrate**

In order to evaluate the interaction of these identified CK2 α catalytic subunits with C-terminal regions of poplar acidic ribosomal P-protein (Liu et al., 2010), two identified phosphopeptides (Table 2) from the C-terminal regions with high confidence are selected to explore the interaction with poplar CK2 α using explicit solvent docking and molecular dynamic (MD) simulations.

As shown in Figure 4, total energies and backbone-atom RMSDs indicated that the two docked complexes reached equilibrium after about 1000 ps and remained rather stable afterwards. Accordingly, the geometric and energetic analyses were performed on the average

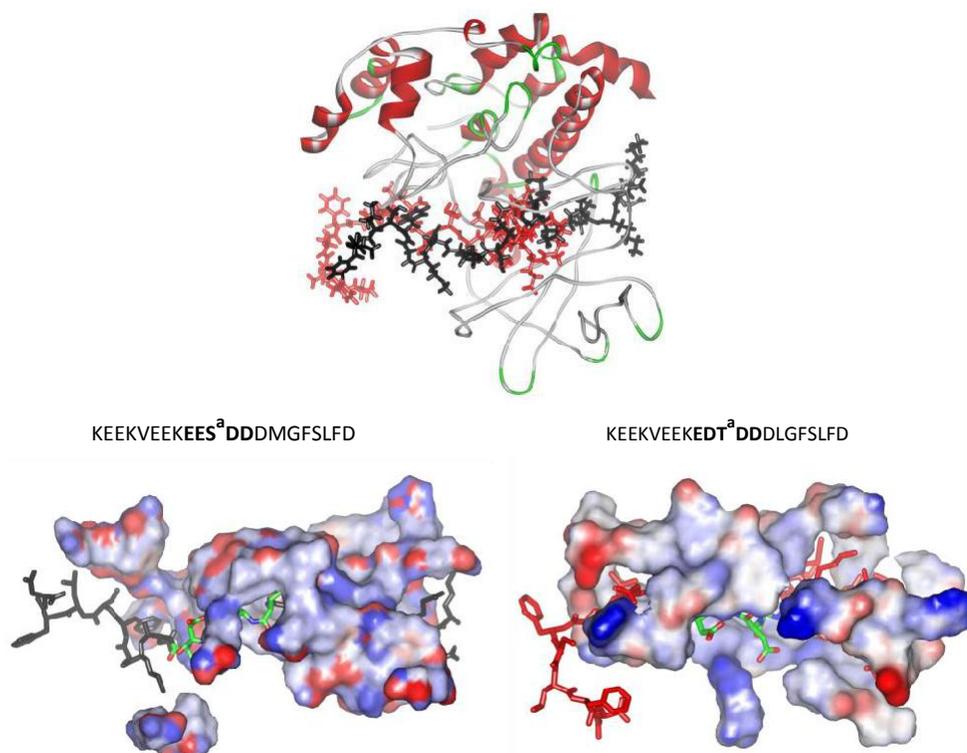


Figure 5. The modeled structures of KEEKVEEKEES^aDDDMGFSLFD (black) and KEEKVEEKEDT^aDDDLGFSLFD (red) bound to the CK2 α active site. The protein is in ribbon. The Connolly surfaces of the CK2 α binding pocket are created using the InsightII 2005 scripts. The ligands are represented by stick models. Ribbon colors: Helices, beta sheets, turns and random coils are in red, cyan, green and white, respectively. The hydrogens were avoided for readability.

structures of 1000~5000 ps MD trajectories. The superposed structures in Figure 5 showed that the two identified phosphopeptides occupy the proximity space at the binding pocket of PtrCK2 α -1, which is mapped to the loop region on the top of helix region within the protein (Dlagic, 2005). In addition, there are some common characteristics of the binding modes. The interaction energies (E_{inter}) of KEEKVEEKEES*DDDMGFSLFD or KEEKVEEKEDT*DDDLGFSLFD with PtrCK2 α -1 were calculated to be -1833.05 and -1676.66 kcal mol⁻¹. Electrostatic interactions rather than van der Waals interactions played a dominant role for the binding processes, contributing to more than 89%. As Figure 5 shows, the EES*DD of peptide 1 and EDT*DD of peptide 2 were buried in the binding pocket of enzyme, involving strong electrostatic interactions with them. As these fully or partially conserved residues are general for the phosphopeptides (Tchórzewski, 2002; Chang et al., 2005; Liu et al., 2010; Meggio and Pinna, 2003; Zschoernig and Mahlkecht, 2009; Dlagic, 2005), it commendably support a viewpoint that the CK2 α identifies the phosphopeptides through the XES/T*DD sequences and then introduces the phosphorylation at the site S/T, which is in good agreement with the previous experimental reports (Riera et al., 2001; Meggio

and Pinna, 2003; Liu et al., 2010). Taken together, it is likely that the special pentapeptides (XES/T*DD) are the identified sites for the CK2 α and therefore, further phosphorylation studies in the poplar are urgently needed to support this point of view.

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