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Full Length Research Paper

Isolation and expression pattern of *COR15b* and *KIN1* genes in watermelon and pumpkin

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***COR15b* and *KIN1* (COR 6.5) genes encode polypeptides of 15 KDa and 6.5 KDa, respectively. They are involved in the dehydration tolerance mechanisms and play important role under cold stress. cDNA sequences of *COR15b* and *KIN1* genes were firstly isolated from leaves of watermelon (*Citrullus lanatus*) and pumpkin (*Cucurbita moschata*). Sequencing results indicated that the open reading fragments (ORF) of *COR15b* in watermelon (*ClCOR15b*) and *COR15b* in pumpkin (*CmCOR15b*) were 348 and 426 bp, which encoded 116 and 141 amino acids, respectively. The putative amino acids of *CmCOR15 b* shared 98.58% identities to *COR15b* in Arabidopsis (*AtCOR15b*), but *ClCOR15b* had only 80.85% identities to *AtCOR15b* because there appeared two mutations at the positions of 220 (C/T) and 418 (T/A) in *ClCOR15b* and T/A mutation produced a transcript end codon (TAA), which led to a lack of 26 amino acids. Similar with *KIN1* in Arabidopsis (*AtKIN1*), ORFs of both *KIN1* in watermelon (*ClKIN1*) and *KIN1* in pumpkin (*CmKIN1*) were 198 bp, encoding two short polypeptides of 65 amino acids. The putative amino acids of *ClKIN1* and *CmKIN1* shared 98.48 and 90.51% identities to *AtKIN1* respectively, although they also contained some mutation sites. Real-time quantitative PCR results indicated that, during cold stress condition, transcripts of *CmCOR15b*, *ClKIN1* and *CmKIN1* significantly increased, suggesting that they could take part in the cold tolerance. However, *ClCOR15b* kept stable during cold stress, implying that its role during cold stress could be changed because of the lacked sequence.**

Key words: *Citrullus lanatus*; *Cucurbita moschata*, *COR15b*, *KIN1*, cold tolerance.

INTRODUCTION

Plants have evolved diverse adaptive mechanisms that enable them to tolerate abiotic stresses, such as low temperature. It has been reported that cold acclimation could increase the cold tolerance in response to low, non-freezing temperatures (Thomashow, 2001). And many studies indicates that the enhancement of cold tolerance that occurs during cold acclimation is due, in part, to the action of cold-regulated genes and CBF/DREB transcription factors are key regulators for expression of many cold-regulated genes (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Nakayama et al., 2007).

Some of cold-regulated genes have been assigned to known classes of proteins. For example, many cold-induced hydrophilic polypeptides belong to the hydrophilin family, which includes late embryogenesis abundant (LEA) proteins. These hydrophilins may protect enzymes against the effects of water limitation *in vitro* (Reyes et al., 2005). In *Arabidopsis*, two small hydrophilic polypeptide, designated as *COR15* and COR 6.5, have been widely elucidated.

COR15a encodes a 15 KDa protein with substantial similarities in its amino acid sequence to those encoded by LEA genes. It is located in the stromal compartments of chloroplasts and is involved in the dehydration tolerance mechanisms of cold-stressed plants. Over-expression of the *COR15a* gene can reduce the propensity of membranes to form hexagonal-phase lipids during freezing stress and enhance the cold tolerance (Lin and Thomashow, 1992; Artus et al., 1996; Steponkus et al., 1998; Zhou et al., 2009). A homolog of the *COR 15a gene (COR15b*) (with 82% amino acid similarity to *COR15a*) has also been discovered in Arabidops thaliana. And transcripts for both *CORl5b* and *CORl5a*

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increase dramatically in response to low temperature (Wilhelm and Thomashow, 1993).

KIN1 is an up-regulated gene during cold acclimation and *KIN1* from Arabidopsis thaliana is particularly interesting because it codes for a 6.5 KDa polypeptide that bears some compositional similarity to the fish Ala-rich antifreeze proteins. This similarity as well as its increased expression during cold acclimation has led to the speculation that *KIN1* might be involved in cold tolerance in plants (Wang et al., 1994).

So far, studies on *COR15b* and *KIN1* genes have mainly been focused on *Arabidopsis* plants. Here, cDNA sequences of *COR15b* and *KIN1* genes were cloned from watermelon and pumpkin plants and their transcript levels during cold stress period were further explored. This was the first report on *COR15b* and *KIN1* genes in watermelon and pumpkin plants.

MATERIALS AND METHODS

Plant materials and chilling stress

Seedlings of watermelon (*Citrullus lanatus* cv. Xiaolan) and pumpkin (*Cucurbita moschata* cv. Hongmiben) were grown in pots in soil and sand mixture (8:1), which were placed in plastic growth chambers (30/22℃ day/night, 75% of RH , 12-h photoperiod with a PPFD of 250 µmol $m^{-2}s^{-1}$). Three leaf stage of uniform and healthy watermelon and pumpkin seedlings (about 14 cm high) were selected for experiments.

Some watermelon and pumpkin seedling were transferred to a climatic chamber (a PPFD of 150 µmol $m^{-2}s^{-1}$, a 12 h photoperiod and a relative humidity of 70%) to subject cold stress for 3 d at 8℃.

RNA extraction and molecular cloning

Total RNAs of the last fully developed leaves after 1 day of cold stress were isolated using Trizol (Sigma). The primers for cloning cDNA sequences containing ORFs for *COR15b* and *KIN1* were designed according to *COR15b* (NM-129814) and *KIN1* (NM_121601) in Arabidopsis, respectively. The specific primers used for *COR15b* amplification were: ATCTCACTTTCTCCATCT (forward primer) and GGTTGAATCAGGACTTTG (reverse primer) and cycle parameters were 95℃ for 3 min, 35 cycles of 95℃ 30 s, 56℃ for 30 s, 72℃ for 1 min and an extension of 72℃ for 8 min. Primers used for *KIN1* were TCTCATCATCACTAACCAAAAC (forward primer) GACCCGAATCGCTACTTG (reverse primer) and cycle parameters were 95℃ for 8 min, 45 cycles of 95℃ 40 s, 58℃ for 40 s, 72℃ for 1 min and an extension of 72℃ for 10 min.

The amplified fragments were isolated from gels, purified using Geneclean (Takara) and cloned into pMD 20-T vector (Takara). Each product was completely sequenced using the Applied Biosystems 3710 DNA capillary sequence for three times.

Bioinformatics analysis

Similarity search was done with the BLASTX program (http://www.ncbi.nlm.nih.gov/blast /Blast.cgi). Multiple sequence alignments of *COR15b* and *KIN1* with other *COR15b* and *KIN1* proteins were conducted using the CLUSTAL X (version 1.81) program. The phylogenetic trees were constructed using the MEGA software 4.0. Sequences used here for phylogenetic analysis were selected according to their reported functions.

Quantification of the transcripts of *COR15b* and *KIN1* genes by real-time quantification PCR

The last fully developed leaves from cold-stressed and control (at 30/22℃) leaves of watermelon and pumpkin plants were harvested directly into liquid nitrogen and stored at -80℃. Three separate samples of cold stress treatment and control were used for real-time PCR.

First strand cDNAs of the above samples were synthesized from 2 µg of total RNA using the first cDNA synthesis kit (Takara). A serial dilution of 100, 50, 5, 0.2 and 0.04 ng of first strand cDNA was used for all transcripts to generate a standard curve by plotting the threshold cycle (CT) values against log (ng cDNA) and to ensure that the efficiencies of the individual transcripts were equal. The log10 value of the dilution was plotted against the CT (threshold cycle) values obtained. For each sample, the amount of the *COR 15b* and *KIN1* transcript was expressed relative to the amount of *Actin* transcript. The copy number of *COR15b*, *KIN1* and *Actin* genes were calculated according to its molecular weight and then converted into the copy number based on Avogadro's number by the formula: number of copies = (amount (ng) × 6.022 $\times 10^{23}$)/ (length (bp) × 1 × 10^9 × 650). The pairs of specific primers were used to amplify the *Actin* in watermelon and pumpkin plants were TGGACTCTGGTGATGGTGTTA (forward primer) and ATGAG GGATGGCTGGAAAA3 (reverse primer).The pairs of specific primers used to measure the transcript levels of *COR15b* were TTTCGTGACGGATAAGA (forward primer) and TTCCTCAGTC GCAGTTT (reverse primer). And the pairs of primers were to measure the transcript levels of *KIN1* were TGTTCTGC TGGA CAAGG (forward primer) and ACCCGAATCGCTACTTG (reverse primer), respectively. Using these two pairs of primers, 103 and 143 bp cDNA fragments were amplified, respectively.

RESULTS

Cloning of *COR15b* and *KIN1* genes in watermelon and pumpkin plants and sequence analysis

Based on the reported *AtCOR15b* (NM-129814) and *AtKIN1* (NM-121601) gene, respectively, the predicated cDNA fragments of *COR15b* and *KIN1* were amplified from watermelon and pumpkin plants (Figure 1). The sequencing showed that the amplified fragments of *COR15b* from both watermelon (*ClCOR15b*) and pumpkin plants (*CmCOR15b*) were 505 bp. The fragments of *KIN1* from watermelon (*ClKIN1*) and pumpkin (*CmKIN1*) plants were 276 and 275 bp, respectively. ORF of *ClCOR 15b* and *CmCOR15 b* were 348 and 426 bp, respectively. ORFs of both *ClKIN1* and *CmKIN1* were 198 bp (Figures 2 and 3).

Compared with *AtCOR15b*, *ClCOR15b* cDNA sequence had two mutations at positions of 220 bp (C/T) and 418 bp (T/A). C/T mutation only resulted in an amino acid change (H/Y) at position of 50. However, T/A mutation formed a transcript end codon (TAA). Accordingly, putative protein sequence of *ClCOR15b* had only 115 amino acids, 26 amino acids fewer than those of *AtCOR 15b* (141 amino acids) (Figures 2 and 4). Accordingly, putative amino acids of *ClCOR15b* cDNA sequence had

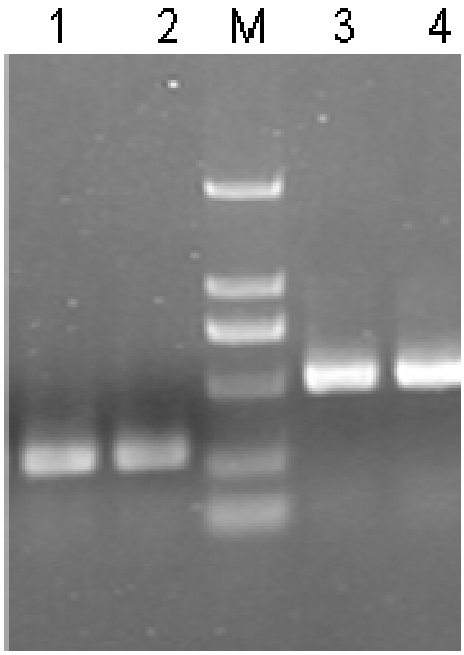

Figure 1. Amplification of cDNA fragments of *COR15b* and *KIN1* genes in leaves of watermelon and pumpkin. 1, *KIN1* of watermelon; 2, *KIN1* of pumpkin; 3, *COR15b* of watermelon; 4, *COR15b* of pumpkin; M, marker.

only 80.85% identity to those of *AtCOR15b* (Figure 4). Compared with *AtCOR15b*, there were also two mutations in *CmCOR15b* cDNA sequence at positions of 178 (G/A) and 355 (G/A), but the two mutations only brought about two amino acid changes (G/S and A/I, respecttively). And putative amino acids of *CmCOR15b* shared higher identity (98.58%) to those of *AtCOR15b* (Figure 4).

Compared with *AtKIN1* (NM_121601), the isolated *ClKIN1* cDNA sequence had also two mutations at positions of 72 bp (C/A) and 244 bp (T/C) (Figure 3), yet only C/A mutation resulted in an amino acid change (G/E) (Figure 5). Putative amino acids of *ClKIN1* shared higher identity to those of *AtKIN1* (98.48%). However, there were more base mutations in *CmKIN1* at positions of at positions of 27 (A/T), 50 (A/T), 103 (G/A), 162 (T/A), 168 (A/T), 178 (T/G), 179 (C/G), 180 (C/A), 202 (A/G), 218 (T/C) and 250 (A/G). And there was a base lack at position of 65 (A) (Figure 5). Because of these base mutations, putative amino acids of *CmKIN1* had lower identity (90.14%) to those of *AtKIN1*.

Phylogenetic analysis of ClCOR15b, CmCOR15b, ClKIN1 and CmKIN1 proteins

Phylogenetic tree generated from the amino acid sequences of some plant *COR* genes showed their division into three main classes (Figure 6). Both *ClCOR15b* and *CmCOR15b* belonged to Class I and they were clustered together with *AtCOR15b* (NM-129814), *TaCOR15b* (FJ5947 71), *CbCOR15b* (AY437888) and *BnCOR15b* (U14665). Compared with *CmCOR15b*, however, *ClCOR15b* had higher homology to *AtCOR15b* (NM-129814).

Phylogenetic tree from the amino acid sequences of some plant *KIN* genes also showed their division into three main classes (Figure 7). Both *ClKIN1* and *CmKIN1* belonged to Class I and they were clustered together with *AtKIN* (NM-121601), *AtKIN* (NM-121602) and *AtKIN* (X151474). *ClKIN1* had higher identity to NM-121601 and X151474, but *CmKIN1* had higher homology to NM-121602.

Expression Patterns of *COR15b* and *KIN1* genes in leaves of in watermelon and pumpkin plants during cold stress

Real-time quantification PCR was undertaken to investigate the transcript levels of *COR15b* and *KIN1* genes in watermelon and pumpkin plants under 8℃ cold stress condition. During cold stress periods, the transcripts of *CmCOR15b*, *ClKIN1* and *CmKIN1* genes in watermelon and pumpkin plants increased rapidly and significantly higher than those at 30/22 7℃ conditions (Figure 8). During cold stress periods, however, *ClCOR15b* transcripts increased slightly and kept stable.

DISCUSSION

In this paper, cDNA sequences of *COR15b* and *KIN1* genes were firstly isolated from watermelon and pumpkin plants (Figures 1, 2 and 3). Sequence analysis indicated that putative amino acids of *CmCOR15b*, *ClKIN1* and *Cm KIN1* shared high identities to *AtCOR15b* and *AtKIN1*, respectively (Figures 4, 5, 6 and 7), although there were some base mutations in their sequences. It has been reported that transcript levels of *AtCOR15b* and *AtKIN1* increased quickly in response to low temperature and were speculated to take part in the cold tolerance (Wilhelm and Thomashow, 1993; Wang et al., 1994). In this paper, during cold stress, transcript levels of *CmCOR 15b*, *ClKIN1* and *CmKIN1* genes also increased dramatically (Figure 8), suggesting they could take part in cold tolerance in these plants.

Compared with *AtCOR15b*, however, putative amino acid sequence of *ClCOR15b* lacked a fragment of 26 amino acids because of a mutation (T/A), which brought about a transcript end codon (TAA) (Figure 2). Accordingly, putative amino acids of *ClCOR15b* protein shared low identity to those of *AtCOR15b* (80.85%) (Figure 4). Its transcript expression patterns differed temporally from *CmCOR15b* (Figure 8) and *AtCOR15b* (Weretilnyk et al., 1993). This inferred that its role during cold stress could be changed because of the lacked sequence and this needed to be further explored.

Figure 2. Comparison of cDNA sequences encoding *COR15b* among watermelon (*ClCOR15b*), pumpkin (*CmCOR15b*) and Arabidopsis (*AtCOR15b*, NM-129814). The transcript start codon (ATG) and end codon (TGA) are shown in asterisks or pane.

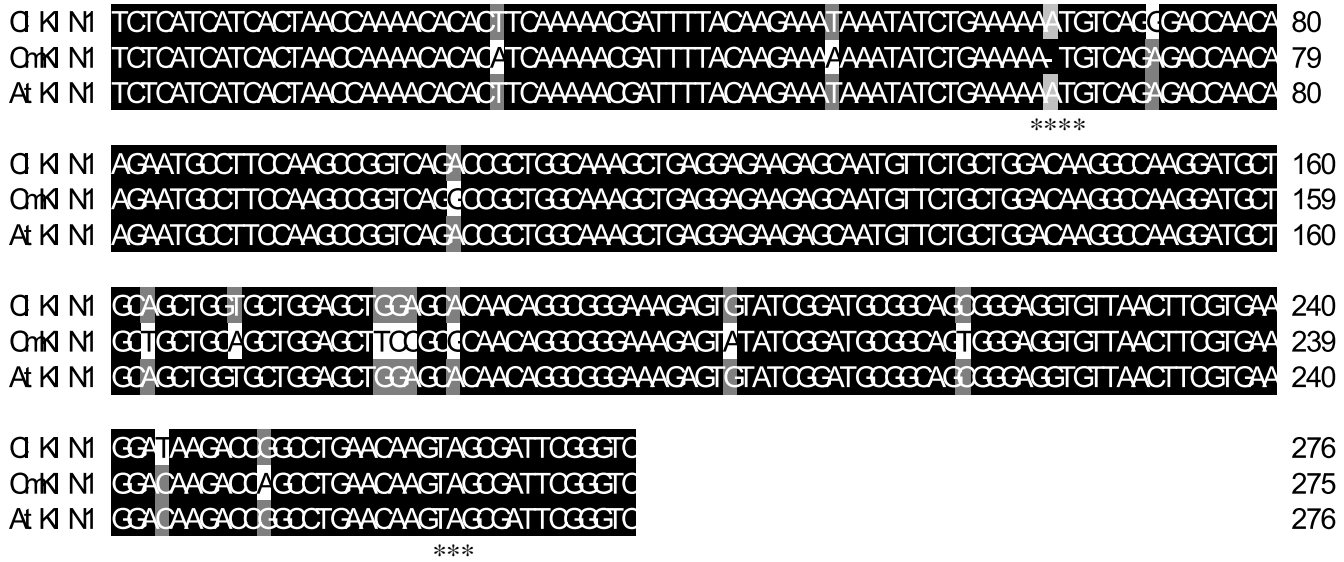

Figure 3. Comparison of cDNA sequences encoding *KIN1* among watermelon (*ClKIN1*), pumpkin (*CmKIN1*) and Arabidopsis (*AtKIN1*). The transcript start codon (ATG) and end codon (TGA) are shown in asterisks.

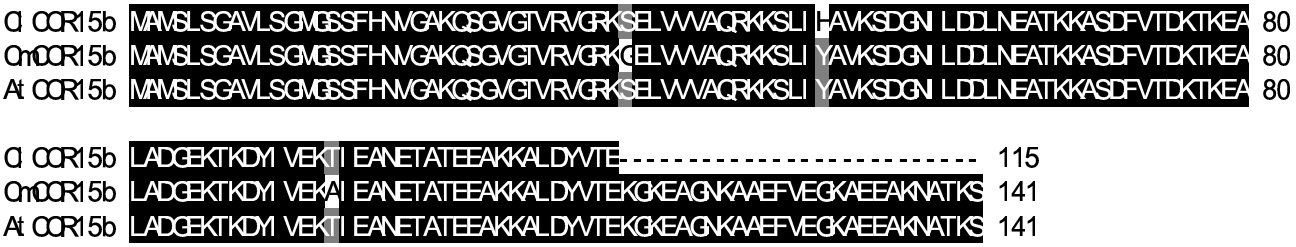

Figure 4. Comparisons on putative amino acid sequences of *COR15b* among watermelon (*ClCOR15b*), pumpkin (*CmCOR15b*) and Arabidopsis (*AtCOR15b*).

Figure 5. Comparisons on putative amino acid sequences of *KIN1* among watermelon (*ClKIN1*), pumpkin (*CmKIN1*) and Arabidopsis (*AtKIN1*).

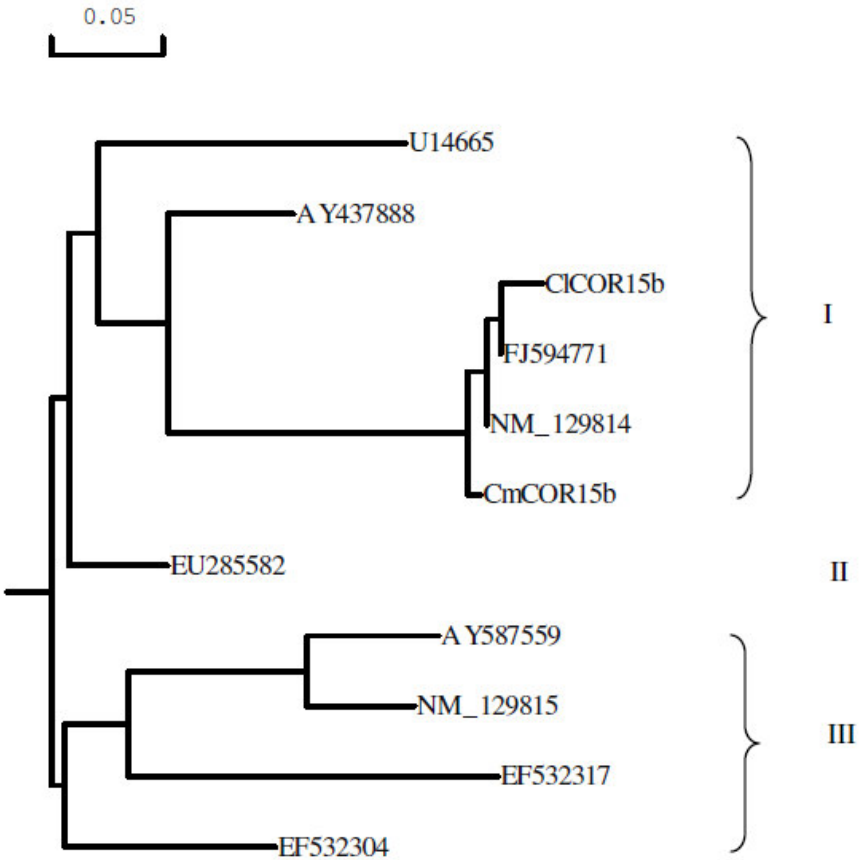

Figure 6. Phylogenetic tree of the predicted *ClCOR15b* and *CmCOR15b* proteins and other known COR proteins as created using the nerghborjoining method in DNAMAN. GenBank database accession numbers of the displayed COR genes are as follows: *Arabidopsis thaliana*, NM-129814 and NM-129815; *Thellungiella salsuginea*, EU285582; *Triticum aestivum*, FJ594771; *Capsella bursa-pastoris*, AY437888; *Draba alpina*, EF532304; *Arabis pumila*, AY587559; *Brassica napus*, U14665 and *Draba draboides*, EF532317.

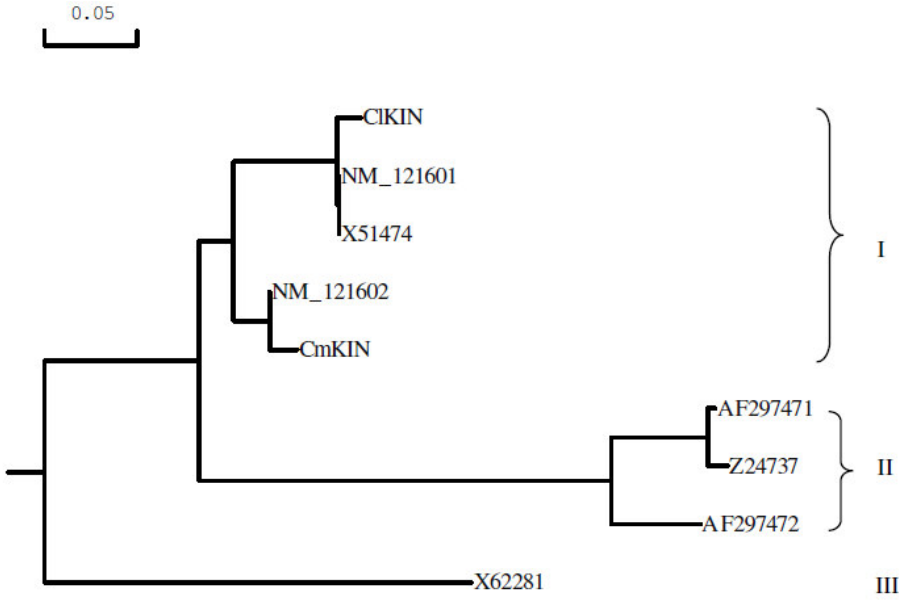

Figure 7. Phylogenetic tree of the predicted *ClKIN1* and *CmKIN1* proteins and other known *KIN* proteins as created using the nerghborjoining method in DNAMAN. GenBank database accession numbers of the displayed *KIN* genes are as follows: *Arabidopsis thaliana* NM-121601, NM-121602, X151474 and X62281; *Brassica campestris* Z24737; *Brassica napus* AF297471 and AF297472.

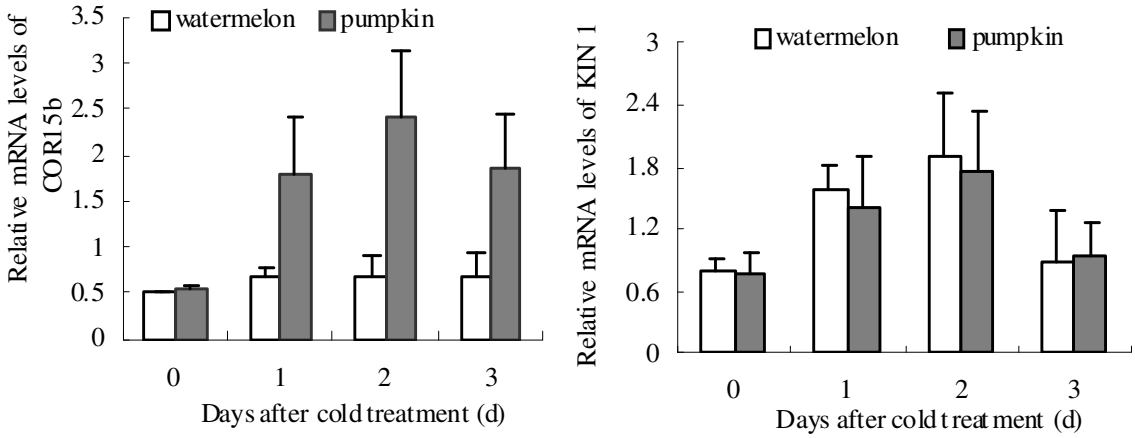

Figure 8. Transcript levels of *ClCOR15b*, *CmCOR15b*, *ClKIN1* and *CmKIN1* genes under 8℃ cold stress conditions. The relative levels of *ClCOR15b*, *CmCOR15b*, *ClKIN1*and *CmKIN1* transcripts were measured by densitometric scanning of the qutoradiogram. The expression of *ClCOR15b*, *CmCOR15b*, *ClKIN1*and *CmKIN1* transcripts were normalized to the expression of *β*-actin. The values are means (SD) of three replications and the means were tested using LSD at $P<0.05$.

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