Overexpression of ClRAP2.4 in Chrysanthemum enhances tolerance to cold stress

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Abstract

The apetala/ethylene responsive factor (AP2/ERF) family is one of the largest plant-specific transcription factors and plays a vital role in plant development and response to stress. The apetala 2.4 (RAP2.4) gene is a member of the AP2/ERF family. Whether RAP2.4 is involved in low-temperature stress in chrysanthemum is unknown. Here, we cloned the ClRAP2.4 cDNA fragment containing an open reading frame of 768 bp. Phylogenetic analysis showed that ClRAP2.4 was most closely related to AT1G22190 and belonged to the DREB subfamily. The ClRAP2.4 protein is localized in the cell nucleus and promotes transcriptional activation in yeast. We obtained four overexpression lines (OX-1, OX-2, OX-7, and OX-8) using the Agrobacterium-mediated leaf disc method. The overexpression of ClRAP2.4 in chrysanthemum increased tolerance to cold stress. The activities of superoxide dismutase (SOD) and peroxidase (POD) and the proline content in the transgenic chrysanthemum leaf were higher than those in the wild type (WT) under cold stress, but electrical conductivity and malondialdehyde (MDA) content in transgenic plants increased rapidly with decreasing temperature. We have identified 390 differentially expressed genes (DEGs) between the transgenic plants and wild type using RNA-Seq, among which, 229 were upregulated and 161 were downregulated. The number of ABRE, LTR, and DRE cis-elements in the DEGs promoter were 175, 106, and 46, respectively. The relative expression levels of CICOR, CIAPX, CIAP, CINCL, CIPLK, CIFAD, CIMYB in transgenic plants were higher than those in WT plants at low temperatures. Collectively, these data suggest that ClRAP2.4 may increase chrysanthemum tolerance to cold stress.

Introduction

Plant growth and development are often accompanied by abiotic and biotic stresses, such as low temperatures, drought, waterlogging, pests, and diseases. Among these factors, low-temperature stress has an important influence on the yield, quality, and geographical distribution of plants (Hossain et al. 2018). At low temperatures, the plant leaves wilt, flowers show brown spots, and severe plant death occurs. Plants change their physiological and biochemical attributes to improve their survival (Qi et al. 2020). These changes include membrane structural components, cellular water content, sugar content, amino acids, and soluble proteins (Yao et al. 2020). Thus far, the most extensive signaling pathway for cold stress is the inducer of CBF expression (ICE)-CBF/dehydration-responsive element-binding factor (DREB)- cold regulated gene (COR) pathway (Wang et al. 2017). The CBF/DREB genes were activated by ICE combined with cis-element C-repeat/DREB in their promoters. Subsequently, the CBF protein recognizes and binds to the cis-element C-repeat (CRT)/dehydration–responsive element (DRE) upstream of the COR gene promoter during cold tolerance (Morran et al. 2011; Wang et al. 2017). Apart from the ICE-DREB/CBE-COR cold response pathway, the abscisic acid (ABA) signaling pathway is widely involved in cold stress in plants (Nakashima et al. 2009). The ABA-responsive element (ABRE)-binding protein (AREB)/ABRE-binding factor (ABF) activates downstream ABA-dependent gene expression, including DREB/CBF transcription factor expression of the COR upstream (Garg and Kumar 2016; Sun et al. 2009).

The apetala/ethylene responsive factor (AP2/ERF) gene family is one of the largest transcription factors in plants (Krishnaswamy et al. 2011). According to DNA binding regions, AP2/ERF has been divided into
five subfamilies: AP2, ERF, DREB, RAV, and SOLOIST (Sakuma et al. 2002). The AP2/ERF proteins are widely involved in flower development, organ identity, and abiotic and biotic stress (Licausi et al. 2013). DREB subfamily genes bind to the C-repeat or dehydration response element (DRE) in the promoter-activated expression of related cold stress genes (Hao et al. 1998). DREB is further divided into five types: A (1–5) (Nakano et al. 2006). RAP2.1, from the DREB subfamily (A5), is strongly induced by cold stress through an ABA-dependent pathway. *Arabidopsis* overexpression of RAP2.1 shows enhanced sensitivity to cold (Dong and Liu 2010). The RAP2.4 of the DREB subfamily (A6), is induced by drought and salt (Feng et al. 2005). The drought tolerance of overexpressed AtRAP2.4 in alfalfa was improved compared to that of WT (Zhang et al. 2005). Further studies showed that RAP2.4 activated the expression of β-ketoacyl-CzoA synthase (KCS2) and Eeriferum1 (CER1) to improve *Arabidopsis* wax synthesis under drought stress conditions (Yang et al. 2020). The RAP2.4 gene is also involved in plant senescence. The overexpressed RAP2.4 lines showed increased chlorophyll degradation rates and leaf senescence (Xu et al. 2010). However, it is not yet clear if CIRAP2.4 is involved in cold tolerance in chrysanthemum.

Chrysanthemum (*Chrysanthemum morifolium* Fisch. ex Trautv.) is among the four most popular cut flowers in the world, with high ornamental value. In northeast China, low temperatures and long periods during winter seriously limit the growth and development of chrysanthemum. Therefore, we cloned and characterized the CIRAP2.4 transcription factor in *C. lavandulifolium* and investigated the cold stress of CIRAP2.4-overexpressing lines, and the molecular mechanism of cold tolerance CIRAP2.4-overexpressing chrysanthemum was elucidated by transcriptome.

**Materials And Methods**

**Plant materials and growth conditions**

Chrysanthemum and transgenic lines were maintained by the Chrysanthemum Institute at Yanbian University, Yanji, China. Chrysanthemums were cultivated in a greenhouse, and the temperature was maintained at 25±2 °C with a relative humidity of 70% for 12 h of photoperiod.

**Cloning of CIRAP2.4 and sequence analyses**

Total RNA was extracted from young leaves of *C. lavandulifolium* using RNAiso reagent (TaKaRa, Tokyo, Japan). First-strand cDNA synthesis was performed using 1 μg of total RNA with SuperScript III reverse transcriptase (TIANGEN) according to the manufacturer’s instructions. Based on *Chrysanthemum seticuspe* expressed sequence tags, a primer pair was designed to amplify the open reading frame (ORF) of CIRAP2.4. The PCR products were subjected to gel electrophoresis and the objective frame was cut and recovered using the AxyPrep™ Biospin Gel Extraction Kit (Axygen, Hangzhou, China), ligated into the pMD19-T easy vector, and cloned into *Escherichia coli* DH5α, followed by sequencing. The CIRAP2.4 amino acid sequence was aligned with those of other plant homologs using BLAST software (http://www.ncbi.nlm.gov/blast). Phylogenetic trees were constructed using the neighbor-joining method and MEGA software (version 5.0). The internal branching support was estimated using 1000 bootstrap replicates.
Subcellular localization and transcriptional activity of the ClRAP2.4 protein

The ClRAP2.4 ORF sequence was amplified using specific primers containing BamHI and NotI sites. The PCR products and pENTR™1A dual selection vector (Invitrogen) were digested with BamHI and NotI and ligated to generate a set of pENTR™1A-ClRAP2.4 fusions through T4 ligase. The fusion constructs were recombined with the binary vector pMDC43 to form the p35S::GFP-ClRAP2.4 constructs using the LR Clonase™ II enzyme mix (Invitrogen). The p35S::GFP-ClRAP2.4 plasmids were introduced into onion epidermal cells using biolistic bombardment (PDS-1000; Bio-Rad, USA) to enable its transient expression; the pMDC43 plasmid (containing p35S::GFP) served as the control. Onion epidermal cells containing plasmids were incubated on solidified Murashige and Skoog medium for 20 h at 25 °C in the dark, and GFP activity was detected using confocal laser scanning microscopy (LeicaSP2, Germany).

Transcriptional activity assays were performed using the Matchmaker GAL4 One-Hybrid System (Clontech, Mountain View, CA, USA). The previous pENTR™1A-ClRAP2.4 plasmid was inserted into pDEST-GBKT7 to obtain GBKT7-ClRAP2.4. The pGBKT7-ClRAP2.4 plasmid was inserted into the yeast strain Y2H Gold (Clontech), following the manufacturer's protocol, with pCL1 and pGBKT7 plasmids as the positive and negative controls, respectively. These transformed yeast cells were confirmed using PCR and streaked on synthetic dropout (SD) medium lacking histidine and adenine medium (SD/-His-Ade) for two days at 25 °C in the dark.

Plasmid construction and transformation of chrysanthemum

The previous pMDC43-ClRAP2.4 plasmids were transformed into Agrobacterium tumefaciens strain EHA105 by freeze-thaw transformation. Agrobacterium strains harboring pMDC43-ClRAP2.4 were cultured in liquid yeast mannitol (YEB) media containing kanamycin and rifampicin. The genetic transformation method of chrysanthemum was described in our previous publication (Gao et al. 2018). DNA and RNA were isolated from the putative transgenic lines using a kit to identify the transgenic plants. The primer pairs, pMDC43-F/R and ClRAP2.4-RT-F/R, were designed for PCR detection (Table S1). Three transgenic and three WT plants were planted in pots in a greenhouse.

Physiological changes of transgenic chrysanthemum under cold treatment

For the cold tolerance evaluation, the five- to six-leaf of the overexpressed chrysanthemum (OX) and the non-transgenic chrysanthemum (WT) were placed in a culture incubator (MIR-154, Sanyo). The temperature was set to 20 °C, 15 °C, 10 °C, and 5 °C for 2 h. Each treatment included three OX plants and three WT plants. After cold treatment, the second to sixth leaves from the top of each plant were collected. The samples were rapidly frozen in liquid nitrogen and stored at −80 °C for physiological and molecular index measurements. The antioxidant enzyme SOD, POD activity, proline, and MDA content were determined in accordance with previously described methods (Yue et al. 2020). OX and WT leaves were exposed to 8 °C, 4 °C, 0 °C, −4 °C, and −8 °C for 1 h. Each treatment included six leaves and three biological replicates. The relative electrical conductivity (REC) was measured using a conductivity meter and calculated using the following equation: REC = (EC before boiling/EC after boiling) × 100%.
Gene expression characterization using RNA-Seq

Three overexpressed chrysanthemum and three WT plants with five to six leaves were subjected to 10 °C for 2 h. Leaves from each plant were collected, frozen immediately in liquid nitrogen, and stored at -80 °C for RNA isolation. The library was constructed using overexpression plants and WT plants (including three replicates). The library was sequenced using an Illumina Hiseq2000 platform (Biomarker Biotechnology Co. Ltd., Beijing, China). Clean reads were checked, in terms of quality, by Q₃₀>99.999 and GC content. Clean reads were mapped to the reference genome (http://www.amwayabrc.com/) using TopHat 2 software under the criterion of no more than two mismatches in the alignment. Gene annotations were obtained through BLAST searches using the NCBI non-redundant protein (Nr) database and the Swiss-Prot protein database. Gene expression quantification was expressed as fragments per kilobase of transcript per million fragments mapped (FPKM). Differentially expressed genes (DEGs) were identified in terms of log₂ |FC| > 2.0, and false discovery rate (FDR) < 0.05. The fold change (FC) was the ratio of the average FPKM of the two groups.

Quantitative Real-time PCR

To analyze the expression of the cold stress-responsive genes CICOR, CIAPX, CIPX, CINCL, CIPLK, CIFAD, and CIMYB, total RNA was extracted from chrysanthemum at 0 °C, 5 °C, and −5 °C for 2 h. cDNA synthesis was performed using the Reverse Transcriptase FastQuant RT kit (TianGen). Each 20 µL qRT-PCR sample included 200 ng cDNA, 100 nM of each primer, and 10 µl SYBR Green PCR master mix. qRT-PCR amplification was performed as described by Yue et al. (Yue et al. 2020). The specific primers for cold-related genes and elongation factor 1α (EF1α) gene primers are shown in Table 1S. Three biological replicates were used for each sample. The data were processed using the 2⁻ΔΔCT method.

Results

Cloning and sequence analysis of ClRAP2.4

The AP2/DREB transcription factor ClRAP2.4 was isolated from chrysanthemum. The gene consisted of 1010 bp with an 870 bp ORF encoding a 290-amino acid protein. The comparison of ClRAP2.4 with CmDREB1-3, LsRAP2-13, HaRAP2-4, and CcRAP2-4 shows that they have a conserved AP2 domain (Fig. 1a). We performed a phylogenetic analysis to further evaluate the relationship between ClRAP2.4 and AP2/DREB proteins in Arabidopsis thaliana. The AP2/DREB family is divided into five classes, including DREB, ERF, RAV, AP2, and Soloist, and ClRAP2.4 belongs to the DREB subfamily, which is closely related to AT1G22190 (Fig. 1b).

Subcellular Localization And Transcriptional Activation Of Clrap2.4

The localization of ClRAP2.4 expression was investigated using the pattern of transient expression in onion epidermal cells expressing GFP-ClRAP2.4. The 35S::GFP fusion protein (positive) and green fluorescence were dispersed throughout the cells. However, green fluorescence was restricted to the
nucleus in GFP-*CIRAP2.4* protein expression (Fig. 2a). This finding showed that CIRAP2.4 activity was located in the cell nucleus. The transcriptional activation ability of CIRAP2.4 was investigated using yeast one-hybrid assays. Figure 2b shows that the pGBK7-*CIRAP2.4* and pGBK7 (negative control) strains did not grow, whereas the pCL1 (positive control) strains grew well on SD media lacking histidine and tryptophan. The results suggested that CIRAP2.4 exhibited no transcriptional activity in yeast.

**Estimate of CIRAP2.4 overexpression in transgenic Chrysanthemum**

The plasmid pMDC43-*CIRAP2.4* was genetically transformed into Chrysanthemum using Agrobacterium tumefaciens-mediated transformation. Ten seedlings were obtained by hygromycin screening. Specific primers were designed for PCR to identify the transgenic lines. The results of gel electrophoresis showed that only OX-1, OX-2, OX-7, and OX-8 lines had expected fragment sizes, whereas WT and other lines had no specific fragments (Fig. 3b). The relative expression level of *CIRAP2.4* in overexpression chrysanthemum (OX1, OX2, OX7, and OX8) was more than twice as likely as the WT, and the relative expression level of OX7 was the highest at 8.04 (Fig. 3a).

**Effects of CIRAP2.4 gene on cold-tolerant adaption**

The *CIRAP2.4* overexpression and WT plants were placed in a low-temperature incubator to determine cold stress. The axil angle (angle between the leaf blade and stem) of WT plants was larger than that of *CIRAP2.4*-overexpression lines at 10°C. When the temperature dropped to 5°C, the angle of axil increased and leaves began to drop in WT plants, but the *CIRAP2.4*-overexpressed line leaves weakened. No morphological differences were found between *CIRAP2.4* overexpression and WT plants at temperatures above 15°C (Fig. 4). The SOD activity of the *CIRAP2.4*-overexpression and WT showed a trend of increasing first and then decreasing at low temperatures (15°C, 10°C, and 5°C). The SOD activity of the *CIRAP2.4* overexpression was higher than that of the WT at the corresponding temperature. The POD activity decreased gradually with a decrease in temperature, and the POD activity of WT decreased faster than that of the *CIRAP2.4*-overexpression lines at 5°C. *CIRAP2.4*-overexpressed lines OX2, OX7, and OX8 proline contents were 846.8 µg.g\(^{-1}\), 806.9 µg.g\(^{-1}\), and 906.3 µg.g\(^{-1}\), respectively, and higher than that of the WT at 5°C. However, the relative conductivity and MDA content increased gradually with the decrease in temperature, and the relative conductivity and MDA content of WT were higher than those of *CIRAP2.4*-overexpressed lines at the corresponding temperature (Fig. 5).

**Transcriptome analysis of CIRAP2.4 overexpression Chrysanthemum**

The *CIRAP2.4*-overexpressed chrysanthemum was analyzed using RNA-Seq. An average of more than 54 million (5,480,637,098) clean reads for each sample with a Q30 of 94.06% and GC content above 44.05% were obtained from the data (BioProject: PRJNA755738, https://dataview.ncbi.nlm.nih.gov/object/PRJNA755738). The total clean reads were compared with the Chrysanthemum genome, and the mapped library ranged from 60.71–63.69% (Supplementary Table S2). Pairwise comparisons of A (*CIRAP2.4*-overexpressed lines) versus B (WT) yielded 390; among these yields, 229 were upregulated and 161 were downregulated in FDR = 0.05 FC = 2. The number of the DEG
promoter region cis-element containing ABRE (core motif ACGTG), LTR (core motif CCGAAA), and DRE (core motif GCCGAC) were 175, 106, and 46, respectively (Table 1 and Table S3). These cis-elements are associated with cold stress. CIRAP2.4 likely combines with these cis-elements to regulate the expression of DEGs. These results further show that CIRAP2.4-overexpressed chrysanthemum tolerated cold compared with WT plants.

<table>
<thead>
<tr>
<th>Up/down regulated</th>
<th>Number of DEGs in transgenic Chrysanthemum</th>
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<tbody>
<tr>
<td></td>
<td>Total numbers/with cis-element (%)</td>
</tr>
<tr>
<td>Up</td>
<td>58.7 (390/229)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Down</td>
<td>41.3 (390/161)</td>
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**Discussion**

AP2/ERF is a large family that is extensively involved in various biological processes related to growth and development, as well as various response stresses in plants. We identified a DREB subfamily gene, CIRAP2.4 from chrysanthemum. Sequence analysis showed that it contains an AP2 domain, and the amino acid of CIRAP2.4 was similar to RAP2.4 (AT1G22190) in Arabidopsis (Fig. 1). CIRAP2.4-overexpressed lines were incubated at different temperatures to further understand the function of CIRAP2.4 in response to cold stress. Compared with the CIRAP2-overexpression lines, the axil angle of WT plants increased and leaves dropped at 5°C (Fig. 4), indicating that CIRAP2.4 can improve the cold tolerance of Chrysanthemum. A member of the DREB subfamily RAP2.4 activated the ABA and ethylene signaling pathways and responds to abiotic stresses (Rudnik et al. 2017). Overexpression of AtRAP2.4 plants improved multiple stress responses, such as drought tolerance and salt tolerance, and inhibited hypocotyl growth through ABA and ethylene signaling (Lin et al. 2008). The overexpression of RAP2.4 has been shown to activate wax production and improve drought tolerance in Arabidopsis (Yang et al. 2020). The RAP2.6 promoter contains (ABRE, DRE, and MYBR) cis-elements; hence, expression of AtRAP2.6 level enhanced cold stress at low temperatures (4°C for 3 h) through the ABA pathway (Zhu et al. 2010). Cold stress causes the accumulation of reactive oxygen species (ROS), leading to a remarkable increase in oxidative damage to plants. The antioxidant metalloenzymes (SOD and POD) scavenge the toxicity of ROS to reduce the damage caused by adversity (Wang et al. 2019). The activities of POD and SOD in the CIRAP2.4-overexpressed lines were higher than those in WT plants at low temperatures. Proline is considered a major osmolyte that protects cells from damage caused by various stresses in plants (Wang et al. 2021). MDA is the final product of membrane lipid peroxidation, and its content can be used as an indicator to evaluate the severity of cell stress (Kaya 2020). The proline content in CIRAP2.4-overexpressed lines was higher than that in the WT. In contrast, the MDA content in WT plants
was higher than that in ClRAP2.4-overexpressed lines (Fig. 4), further indicating that ClRAP2.4-overexpressed plants were better adapted to low temperatures. Plant hormones, including ABA, salicylic acid (SA), and gibberellin (GA), are essential for response to changing environments. ABA plays an important role in abiotic stresses, such as cold, salt, drought, and hot tolerance (Sinha et al. 2015; Qu, et al. 2020). Transcriptional data analysis showed that 173 genes from the DEG promoter contained cis-acting ABRE (Table 1). ABA-inducible genes share the (C/T) ACCTGGC consensus and cis-acting ABA-response element in their promoter region. CBF transcription could be involved in cold stress through the activation of ABRE by ABA (Garg and Kumar 2016; Knight et al. 2004). Spraying ABA to grapevine buds increased the expression of VvCBF2, VvCBF3, VvCBF4, and VvCBF6 at low temperatures and the acquisition of freezing tolerance (Rubio et al. 2019).

The LTR elements (CCGAAA) have been identified as cis-acting promoter elements that regulate gene expression in response to cold stress. Receptor-like protein kinases (RLKs), which contain LTR cis-elements, characterize an extracellular domain and a cytosolic kinase domain and are connected through a single-pass transmembrane helix; they play important roles in cell–cell and cell–environment communications (Li 2010; Wu et al. 2015). Leucine-rich repeat (LRR), the largest PLK subfamily, contains multiple copies of leucine-rich repeats in their extracellular domains. The LRR responded to biotic and abiotic stresses, especially cold tolerance. GsLRPK expression was induced by cold and showed Ser/Thr protein kinase activity in Glycine soja. Overexpression of GsLRPK in Arabidopsis can improve cold stress (Yang et al. 2014).

The relative expression levels of CIPLK, which encodes a Na⁺/Ca²⁺ exchanger protein CinCL from overexpression Chrysanthemum was higher than that of WT at 5°C (Fig. 5). The cis-element DRE is essential for induction of the COR expression in Arabidopsis under low temperature and drought stress (Sakuma et al. 2002). DRE motifs in promoters of multiple genes have been reported, including COR25, a protein kinase catalytic subunit alpha (KIN). BnCOR25 overexpression in yeast (Schizosaccharomyces pombe) or Arabidopsis conferred cold tolerance (Chen et al. 2011). KIN gene expression was induced by low temperatures. Liu et al. (2015) reported that KIN expression increased cold tolerance by regulating sucrose biosynthesis. The relative expression level of COR in transgenic chrysanthemum was higher than that in WT under low temperature conditions (Fig. 5). The expression levels of cytosolic ascorbate peroxidase (APX1) and peroxidase (PX) genes were enhanced from 20°C to 5°C (Fig. 5). Ascorbate peroxidases are enzymes that scavenge hydrogen peroxide to improve cold stress in plant cells. In addition, the expression levels of CIFAD (fatty acid desaturase) and CIMYB genes related to cold tolerance were increased at low temperatures (Fig. 5). These data also further suggest that overexpression of ClRAP2.4 in transgenic chrysanthemum improves cold tolerance by stress-related genes with promoters containing ABRE, DRE, and LTR cis-elements.

Declarations

Author contributions
Conceived and designed the experiments: Ri Gao, Performed the experiments: Manrong Ren, Wenting Yang, Jianing Zhang, Li Zhao and Yanan Xu Analyzed the data: Ri Gao, Li Zhao, Yingjie Quan, Fageng Zhang, Ming Yin and Yaoying Wang. Contributed reagents/materials/analysis tools: Ri Gao, Wenting Yang, Jianing Zhang and Manrong Ren. Wrote the paper: Ri Gao, Wenting Yang, Jianing Zhang, Manrong Ren and Yingjie Quan. All authors read and approved the final manuscript.

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Data availability

Datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Conflict of interest: The authors declare no competing interests.

Code availability: Not applicable.

References


Figures
Figure 1

Domain of CIRAP2.4 and phylogenetic analysis of AP2/DREB proteins.

a: Alignment of the putative amino acid sequence of CIRAP2.4 with homologous proteins, which include a highly conserved AP2 domain. CmDREB1-3 (AHZ12872.1), LsRAP2-13 (XP023756400.1), HaRAR2-4 (XP022039742.1), and CcRAR2-4 (XP024976784.1).

b: Phylogenetic analysis of the AP2/DREB family The sequence details are as follows:

AT4G25470 (AtCBF2), AT4G25480 (AtDREB1A), AT5G05410 (AtDREB2A), AT3G11020 (AtDREB2B), AT2G35700 (AtDREB), AT4G16750 (AtDREB), AT1G22190 (AtRAP2.4), AT1G50640 (AtERF3), AT3G20310 (AtERF7), AT3G14230 (AtRAP2.2), AT1G53910 (AtERF74), AT5G43410 (AtERF96), AT1G04370 (AtERF14), AT2G39250 (AtAP2), AT2G28550 (AtRAP2.7), AT3G54990 (AtAP2), AT3G54320 (AtAP2), AT4G37750 (AtAP2), AT4G13040 (AtSOLOIST), AT1G5120 (AtRAV), AT1G50680 (AtRAV), AT1G68840 (AtRAV2), AT1G13260 (AtRAV1), AT3G25730 (AtRAV)
Figure 2

Subcellular localization and transcription activation of CIRAP2.4.

a: Localization of transiently expressed CIRAP2.4 in onion epidermal cells. DIC, GFP, and merged represent bright-field images, green fluorescence at 488 nm, and combined bright-field and fluorescence images. Bar 50 μm. b: Transcriptional activation activity of CIRAP2.4. pCL1 and pGBKT7 were used as positive and negative controls, respectively.

Figure 3

Relative expression level of CIRAP2.4 in OX1, OX2, OX7, OX8, and WT.
Identification of *CIRAP2.4* overexpression in Chrysanthemum plants

a: Relative expression levels of *CIRAP2.4*, overexpressed lines OX1, OX2, OX7, OX8, and control WT. b: Gel electrophoresis image, M: Marker 2000 bp.

Figure 4
Morphological of *CIRAP2.4* overexpression and WT plants under low temperature.

Overexpressed lines OX1, OX2, OX7, OX8, and control WT, Bars=2 cm.

Figure 5

Physiological effects of cold stress on *CIRAP2.4*-overexpression and WT plants under low temperature.

Physiological included SOD, POD, MDA and proline, and relative electrical conductivity
Figure 6

Relative expression levels of genes between *CIRAP2.4*-overexpression and WT under low temperature

Data represent means and standard errors of three replicates

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- TableS2.docx
- TableS3.xlsx