Mechanism of CsGPA1 in regulating cold tolerance of cucumber

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Abstract

G proteins function directly in cold tolerance of plants. However, the framework of the Gα subunit in regulating cold tolerance remains to be explored. Here, we used protein interaction techniques to elucidate cold-related pathways regulated by CsGPA1. Suppression of CsGPA1 decreased the cold tolerance of cucumber. Further protein interaction experiments showed that CsGPA1 interacted with Csa_4G663630.1 located in the cell membrane and nucleus and with CsCOR413PM2 located in the cell membrane. Csα was named Cα in its 71% protein sequence similarity to AtCDL1 located in cold stress. Principal component and linear regression analyses showed that expressions of CsGPA1 and brassinolide-related genes were positively correlated. Suppression of CsCOR413PM2 also decreased cold tolerance of cucumber. The expression and protein content of CsCOR413PM2 and CsGPA1 in CsCOR413PM2-RNAi and CsCOR413PM2-RNAi lines were determined under cold tolerance. Only CsGPA1 silenced affected the expression and protein content of CsCOR413PM2 during cold stress. Moreover, suppression of CsGPA1 or CsCOR413PM2 decreased Ca2+ influx at low temperature and then decreased the expression of CsICE-CsCBF. These results indicated that the CsGPA1–CsCOR413PM2–Ca2+ axis regulated the expression of CsICE–CsCBF during cold stress. In conclusion, Our results provide the first framework of CsGPA1 in regulating cold tolerance of cucumber, laying the foundation for further mechanistic studies of cold tolerance for Gα in cucumber.

Introduction

The G protein heterotrimer, a protein complex consisting of Gα, Gβ, and Gγ subunits, is a core component of plant signal transduction [1]. The Gα subunit is involved in several signal transduction pathways, and in particular functions in morphogenesis and abiotic stress [2–4]. A breakthrough study found that the plasma membrane and endoplasmic reticulum local G protein regulator, CHILLING TOLERANCE DIVERGENCE1 (COLD1), conjugates rice G protein α subunit 1 (RGA1) to participate in the rice cold stress response by regulating the Ca2+ signal [5]. The COLD1–RGA1 complex mediates cold-induced intracellular Ca2+ influxes, leading to activation of cold-regulated (COR) genes [5].

Plant cells rely on special signaling pathways, such as calcium and brassinolide (BR), to enhance cold tolerance. Abiotic stresses, such as low and high temperature and salt stress, activate Ca2+ osmotic channels, leading to increased concentrations of free intracellular Ca2+, and these calcium signals transform external signals into various intracellular biochemical reactions, enhancing plant adaptation to abiotic stresses [6, 7]. Cold stress changes the fluidity of the plant cell membrane, while calcium signaling and other lipid membrane proteins can sense changes in cell membrane fluidity and activate the cold stress response, leading to enhanced expression of ICE, CBF and COR genes [8]. Therefore, calcium signaling is essential for the cold stress response of plants.

In addition to calcium, the BR signal is also important for cold tolerance in plants. In Arabidopsis, BR signaling enhances cold tolerance by activating the ICE–CBF–COR signaling axis [9]. Another study in Arabidopsis showed that, during the early stage of cold stress, BR signals alter the stability of ICE1 protein by controlling the activity of brassinosteroid-insensitive 2 (BIN2), thus balancing the cold tolerance of plants [10]. In tomato, inhibition of the BR signal could lead to increased reactive oxygen species, while spraying epibrassinolide or overexpression of DWRF improved the cold tolerance of tomato [11]. Moreover, BR signaling enhanced cold tolerance of tomato by activating RBOH1 through the BZR1 transcription factor [12]. In cucumber, BR-mediated H2O2 signaling increased antioxidant enzyme activity to increase cold tolerance of seedlings [13–15]. Therefore, BR signals...
are widespread in the regulation of cold tolerance in plants. In plants, the COR genes respond to low temperature [16]. COR is a type of gene that regulates cold tolerance. These genes encode osmotic and antifreeze proteins to protect plants from injury [17, 18]. Around 4000 COR genes have been identified in Arabidopsis, of which only a few hundred are regulated by C-repeat binding factors (CBFs). The promoters of these COR genes have cis-elements capable of recognizing CRT/DRE by CBF proteins, which contain conserved CCGAC sequences [19–21]. Other COR genes are regulated by CBF-independent proteins, such as HsfC1, ZAT12, and BZR1 [22–24]. CBF-independent pathways may therefore be required for regulating COR gene expression. In plants, most cold-regulated proteins are localized in the cytoplasm and nucleus. However, cold-regulated proteins located in the plasma membrane of cells are necessary for cold tolerance in plants [25]. Plasma membrane cold-regulated proteins include COR413 [26], COR47 [27], and WCOR410 [28]. COR413 includes five potential transmembrane domains (TMDs) [29]. The COR413 gene encodes several transmembrane proteins, such as COR413PM in the plasma membrane of the cell and COR413TM in the locus chloroplast [30]. Recently, cor413pm1 mutants of Arabidopsis was found to be less tolerant to low temperature than the wild type (WT) [31]. Overexpression of the PsCOR413PM2 of Phlox subulata increased the expression of cold-stress-related genes COR and CBF in Arabidopsis [32]. Overexpression of SiskCOR413PM1 enhanced the cold and drought tolerance of transgenic tobacco [33]. In addition, overexpression of LeCOR413PM2 improved the cold tolerance of tomato seedlings, and inhibiting LeCOR413PM2 expression using RNA interference (RNAi) decreased the cold tolerance of seedlings [26].

Based on these studies, cold tolerance of plants is clearly related to COR413 protein in the plasma membrane.

Cucumber (Cucumis sativus L.) is an important cultivated horticultural crop in northern China, and its sensitivity to cold in winter has always been an urgent problem to be solved [34]. It is of theoretical and practical significance for cucumber breeding to improve its cold tolerance. Although the function of Cα in cold tolerance has been studied in Arabidopsis [35], rice [5], and tomato [36], the framework of CsGPA1 in regulating cold tolerance of cucumber has not been reported.

Here, we explored low-temperature-related pathways regulated by CsGPA1 by screening proteins that interacted with CsGPA1. We found that (i) CsGPA1 interacted with CsCDL1, a key BR signal transduction gene, and CsGPA1 suppression weakens BR signaling in the regulation of cold tolerance; and (ii) CsGPA1 interacted with CsCOR413PM2 and suppression of CsGPA1 decreased the expression and protein content of CsCOR413PM2; however, there were no differences in the expression and protein content of CsGPA1 in CsCOR413PM2-RNAi lines compared with WT during cold stress. Furthermore, suppression of CsGPA1 or CsCOR413PM2 both decreased Ca²⁺ influx at low temperature and then decreased the expression of CsICE–CsCBF. Therefore, the CsGPA1–CsCOR413PM2–Ca²⁺ axis regulated the expression of CsICE–CsCBF during cold stress.

**Results**

**Suppression of CsGPA1 decreased cold tolerance of cucumber**

To investigate whether CsGPA1 contributes to cold tolerance in cucumber, we obtained two independent transgenic RNAi lines to suppress CsGPA1: CsGPA1-RNAi-9 and CsGPA1-RNAi-10 (Fig. 1a). Real-time PCR (qPCR) and western blot (Fig. 1b, c) confirmed that the expression and protein content of CsGPA1 were significantly decreased compared with WT in these RNAi lines. We then examined the response to cold stress in these knockdown lines by exposing WT and RNAi plants to 6°C for 60 hours. The results showed that while CsGPA1 expression was significantly lower than that of WT at all timepoints, CsGPA1 expression steadily increased over 24 hours, indicating that CsGPA1 expression was indeed elevated in response to low temperature (Fig. 1d). The phenotypic effects associated with CsGPA1 suppression were then examined in cucumber leaves by comparing the RNAi lines with WT, which revealed severe wilting under cold stress (6°C, 60 hours), whereas WT showed no obvious wilting (Fig. 1e). Determination of the relative electric conductivity (REC) and malondialdehyde (MDA) of cucumber seedlings (Fig. 1f and g) showed that REC was significantly higher in both RNAi lines compared with WT, as were the MDA values for RNAi-10, under ambient temperature (25°C). We then observed that both REC and MDA were significantly increased in the RNAi lines compared with WT following cold treatment. Together, these results suggested that CsGPA1 contributed to cold tolerance in cucumber.

**Validation of CsGPA1 interaction candidate proteins and its bioinformatic analysis**

Since CsGPA1 reached the highest expression levels in cucumber seedlings at 24 hours of cold treatment, leaves were collected at 24 hours and used to generate a cDNA library, which subsequently formed the basis for CsGPA1-cold-related protein interaction screens. Given previous studies showing that CsGPA1 was localized in the cell membrane [2], we performed split-ubiquitin yeast two hybrid (Y2H) assays to identify proteins that potentially interacted with CsGPA1. We identified 27 proteins interacting with CsGPA1. Among these 27 proteins, we speculated that Csa_4G663630.1 or CsCOR413PM2 might be involved in cold stress by bioinformatic analysis.

Based on our screening of the CsGPA1 interaction library, we next verified the potential interactions between CsGPA1 with CsCOR413PM2 or CsCDL1 (Fig. 2). We first verified their interaction in split-ubiquitin yeast Y2H assays, and found that CsCOR413PM2 or CsCDL1 and CsGPA1 could grow on deficient media, indicating
that CsGPA1 could interact with CsCOR413PM2 or CsCDL1 in vivo (Fig. 2a and b). Then we conducted a pull-down assay to determine whether they directly bind to one another in vitro. When tested with His antibody, only GST-CsGPA1/His-CsCOR413PM2 and Flag-CsGPA1/His-CsCDL1 combinations hybridized to produce bands, indicating that CsGPA1 interacted with CsCOR413PM2 or CsCDL1 (Fig. 3a and b).

Considering the interaction between CsGPA1 and Csa_4G663630.1 during response to cold stress and since Csa_4G663630.1 is annotated as a serine/threonine protein kinase, we next used Blastp to compare Arabidopsis non-redundant protein sequences to identify putative homologs. Csa_4G663630.1 shared 72% homology with Arabidopsis CDL1 (AT5G02800) in protein sequence (Supplementary Data Fig. S1), which has been shown to actively regulate BR signal transduction [37]. We therefore named Csa_4G663630.1 as CsCDL1.

To better understand the function of CsCOR413PM2, HMM software was used to search for proteins containing the WCOR413 (PF05562) domain in cucumber, tomato, rice, Arabidopsis, and Nicotiana benthamiana. Alignment of full-length sequences of the 15 COR413 proteins identified in these species were used to generate an unrooted neighbor-joining phylogeny (Supplementary Data Fig. S2). The three COR413 proteins in cucumber were separated into three different subfamilies in the tree. Cucumber has two COR413PM2 proteins, CsCOR413PM2 and CsCOR413PM2-2, but only CsCOR413PM2 (101203533) interacted with CsGPA1. Moreover, this protein belongs
to the same subfamily and shares high amino acid sequence similarity with OsCOR413PM2 from rice, which has not been functionally characterized. This result indicated that CsCOR413PM2 has not been previously described, nor has its function in the plant cold response been reported. In addition, we extracted the 1500 bp upstream of the 5′ UTR containing the CsCOR413PM2 promoter region and found that it did not contain typical cis-elements of CRT/DRE, indicating that CsCOR413PM2 may be a CBF-independent gene.

**Subcellular localization of CsCDL1 and CsCOR413PM2**

Previous studies have shown that CsGPA1 is in the cell membrane [2], so we studied the localization of a CsCOR413PM2/CsCDL1-GFP fusion protein in cells by agroinfiltration into leaves of *N. benthamiana*. Observation by confocal microscopy showed fluorescence signal from CsCDL1:GFP expression at both cell margins and nucleus (Fig. 4a). The fluorescence signal from CsCOR413PM2:GFP expression in leaf epidermal cells could be found at the cell margins (Fig. 4b). These observations showed that CsCDL1:GFP was localized in the nucleus and cell membrane and that CsCOR413PM2:GFP was localized in the cell membrane.

**CsGPA1 positively regulates the brassinolide signal to affect cold stress of cucumber**

There is a potential interaction between CsGPA1 and CsCDL1 (Figs 2b and 3b), a BR signal transduction protein during the response to cold stress. Furthermore, several studies have found that the BR signal can enhance cold tolerance in cucumber [13–15, 38]. We thus hypothesized that CsGPA1 could potentially affect BR signaling in cucumber by interacting with CsCDL1 under cold stress. To test this possibility, we first detected CsCDL1 expression under cold stress in both WT and CsGPA1-RNAi lines (Fig. 5a) and found that it reached maximum transcription in WT at 6 hours of cold treatment. CsCDL1 expression almost decreased in the most time points over the course of cold treatment in plants with suppressed CsGPA1.

We then used qPCR to investigate BR synthesis and signaling genes in cucumber seedlings (Supplementary Data Fig. S3) and found that BR-related gene transcription was upregulated in both WT and RNAi lines, generally reaching maximum transcription levels at 6 hours of cold treatment, suggesting that BR signaling participated in the cold stress response. Compared with WT, only the CsBZR1 and CsBZR2 mRNA levels were significantly lower in RNAi lines at 0 hours of cold treatment, and the expression of other genes was not significantly different between WT and RNAi lines. Notably, with prolonged cold treatment, the expression of most BR-related genes decreased significantly compared with WT at 6, 12, and 24 hours, which suggested that CsGPA1 suppression could result in decreased BR synthesis- and signaling-related gene expression during cold stress.

To further explore the relationship between CsGPA1 and BR signaling, we next conducted principal component analysis (PCA) of BR-related gene expression (Fig. 5b) which revealed that PC1, PC2, and PC3 contributed 60.04, 19.72, and 7.76%, respectively, to the effects on BR gene expression. The component matrix showed that there were 11 genes with loads >70% in PC1 (Supplementary Data Table S2), indicating that PC1 replaced the expression of BR-related genes. Finally, a significant positive correlation was found between CsCDL1 and PC1 (Fig. 5c), suggesting that CsCDL1 was closely related to BR. Furthermore, a significant positive
correlation was found between CsGPA1 and CsCDL1 and between CsGPA1 and PC1, further indicating that CsGPA1 may positively regulate the expression of BR synthesis and signaling genes at low temperature.

**Suppression of CsCOR413PM2 decreased cold tolerance of cucumber**

To verify whether CsCOR413PM2 was involved in cold tolerance regulation of cucumber seedlings, we generated CsCOR413PM2-RNAi-17 and CsCOR413PM2-RNAi-21 transgenic knockdown lines (Fig. 6a). Analysis by qPCR (Fig. 6b) and western blot (Fig. 6c) confirmed that the expression and protein content of CsCOR413PM2, respectively, were both significantly decreased compared with WT in these RNAi lines. We next determined that the expression of CsCOR413PM2 during cold stress was significantly lower in the RNAi lines compared with WT (Fig. 6d). Phenotypic analysis revealed that CsCOR413PM2 silencing in cucumber leaves resulted in wilting under cold stress (6°C, 60 hours), while WT plants showed no obvious wilting or other symptoms of cold stress (Fig. 6a; Supplementary Data Fig. S4). Furthermore, both REC and MDA were detected to be significantly higher than WT in both RNAi lines under ambient temperature.

Figure 3. Pull-down validation of proteins interacting with CsGPA1. **a** Pull-down between His-CsCOR413PM2 and GST-CsGPA1. **b** Pull-down between His-CsCDL1 and Flag-CsGPA1 proteins.

Figure 4. Subcellular localization of CsCDL1 and CsCOR413PM2. Subcellular localization of CsCDL1-GFP fusion protein (a) and CsCOR413PM2-GFP fusion (b). All fusions were expressed in N. benthamiana plants. Images at the far left show cells with GFP signal. In the middle are bright-field images of the same cells, and images at the end are overlays of the bright-field and fluorescence images. In (a) we labeled a total of five cells, and the five red triangles represent the nuclei of five cells. An irregular polygon surrounded by a green curve is the cell membrane (a, b).
CsCOR413PM2 as part of the cold stress response. CsGPA1 that suppression of cold stress (Fig. 7b and d). The above results suggested CsCOR413PM2 in the expression and protein content of (Fig. 7a and c). However, there were no differences of RNAi lines compared with WT during the cold stress. We found that the expression and protein content of CsCOR413PM2 and CsGPA1 in CsGPA1-RNAi and CsCOR413PM2-RNAi lines under cold stress (Fig. 7). We found that the expression and protein content of CsCOR413PM2 significantly decreased in CsGPA1-RNAi lines compared with WT during the cold stress (Fig. 7a and c). However, there were no differences in the expression and protein content of CsGPA1 in CsCOR413PM2-RNAi lines compared with WT during cold stress (Fig. 7b and d). The above results suggested that suppression of CsGPA1 only affects the regulation CsCOR413PM2 as part of the cold stress response.

Suppression of CsGPA1 or CsCOR413PM2 decreased Ca\(^{2+}\) influx under cold stress, resulting in decreased expression of cold-related genes

Since cell membrane fluidity is affected by cold conditions, and calcium signals can sense this change and activate cold-stress-related genes in plants [8, 39], we next examined the relationship between CsGPA1 or CsCOR413PM2 and Ca\(^{2+}\) influx in cucumber roots using non-invasive microtest technology. We found that Ca\(^{2+}\) influx in RNAi lines was lower than that in WT under cold conditions and CsGPA1-RNAi lines showed the lowest Ca\(^{2+}\) influx (Fig. 8a). In addition, the average Ca\(^{2+}\) influx of RNAi lines was significantly different from that of WT under cold conditions (Fig. 8b). Moreover, CsGPA1-RNAi lines showed the lowest average Ca\(^{2+}\) influx under cold conditions (Fig. 8b).

Increased Ca\(^{2+}\) influx increases the expression of ICE-CBF–COR under cold stress [5, 39]. Based on these findings, we next detected the expression of genes induced by Ca\(^{2+}\) influx. Analysis by qPCR revealed that the expression of cold-related genes increased in both WT and RNAi lines in response to cold treatment, with CsRD-29, CsICE1, CsCBF1, and CsCBF3 exhibiting maximum expression between 3 and 12 hours, while suppression of CsGPA1 or CsCOR413PM2 significantly decreased the expression of these four cold-related genes under cold stress (Fig. 8c and d).

Discussion

Low temperature is a major environmental factor affecting plant geographical distribution [25]. To survive at low temperatures, plants have evolved a complex set of physiological and biochemical processes to adapt to low-temperature stress [18]. The G protein \(\alpha\) subunit is involved in cold tolerance of Arabidopsis [35], tomato [36], and rice [5]. However, the framework of CsGPA1 in regulating cold tolerance of plants is still unclear. Here, cucumber, a cold-sensitive vegetable crop, was examined. We found that CsGPA1 interacted with CsCDL1, a key BR signal transduction gene, and suppression of CsGPA1 weakened BR signaling in the regulation of cold tolerance; CsGPA1 interacted with CsCOR413PM2 and CsGPA1 suppression decreased the expression and protein content of CsCOR413PM2. Furthermore, suppression of CsGPA1 or CsCOR413PM2 both decreased Ca\(^{2+}\) influx at low temperature and then decreased the expression of CsICE–CsCBF. Therefore, the CsGPA1–CsCOR413PM2–Ca\(^{2+}\) axis regulated the expression of CsICE–CsCBF during cold stress.

CsGPA1 affected cold tolerance of cucumber by regulating BR signal and CsCOR413PM2

G protein \(\alpha\) subunit interacted with many proteins [4]. However, few studies have investigated the function and interactions of the \(\alpha\) subunit with cold-related proteins,
Figure 6. Functional verification of CsCOR413PM2 under cold stress. a Acquisition of CsCOR413PM2-RNAi lines and functional verification of CsCOR413PM2. Three treatments were set up: WT, CsCOR413PM2-RNAi-17, and CsCOR413PM2-RNAi-21, with five biological replicates for each treatment. b, d Expression of CsCOR413PM2 in WT and CsCOR413PM2-RNAi lines. c Protein content of CsCOR413PM2 in WT and CsCOR413PM2-RNAi lines. Western blot of WT and different transgenic lines for CsCOR413PM2-RNAi using anti-CsCOR413PM2 and anti-actin antibodies. The red numbers indicate the protein content. e REC in WT and CsCOR413PM2-RNAi lines. f MDA content in WT and CsCOR413PM2-RNAi lines. All measured indices were set up with three biological replicates per treatment and time point. *P < .05 significant difference between CsCOR413PM2-RNAi lines and WT (Tukey HSD).

except for COLD1 in rice, which conjugates rice G protein α subunit 1 (RGα1) to participate in rice cold stress response by regulating calcium signaling [5]. The COLD1–RGα1 complex mediates cold-induced intracellular calcium influxes, leading to COR gene activation [5]. This study is the only report on the mechanism of RGα regulating cold stress in plants. Therefore, the mechanism of CsGPA1 in regulating cold tolerance remains to be explored. In this study, suppression of CsGPA1 significantly affected cold tolerance of cucumber, indicating that CsGPA1 was involved in regulating cucumber cold tolerance (Fig. 1).

By screening the interaction proteins of CsGPA1, we explored the cold-stress-related pathways regulated by CsGPA1. CsGPA1 interacted with Csa_4G663630.1 (a serine lysine kinase) (Figs 2b and 3b) located in the cell membrane and nucleus (Fig. 4a). We further compared the protein sequence of Arabidopsis and found that Csa_4G663630.1 was 71% similar to AtCDL1, a positive BR signal gene, so it was named CsCDL1 (Supplementary Data Fig. S1). Further experiments showed that CsGPA1 silencing leads to decreased CsCDL1 expression (Fig. 4a). With the prolonged cold treatment, the expression of most BR-related genes decreased significantly compared with WT at 6, 12, and 24 hours, indicating that CsGPA1 regulates BR synthesis and signal at low temperatures (Supplementary Data Fig. S2). Furthermore, the PCA and linear regression analysis showed a significant positive correlation between BR signal and the expression of CsGPA1 (Fig. 5b and c). Taken together, these results suggest that CsGPA1 can actively regulate the expression of BR synthesis and signaling genes at low temperatures. Our study is the first to show that the Gα subunit affected cold tolerance of plants by regulating BR signaling. Moreover, BR signaling is involved in regulating tolerance to cold stress [13–15, 38]. Therefore, we proposed that CsGPA1 may mediate BR signaling under cold stress to regulate cold tolerance of cucumber. Additionally, studies have also shown that G protein α subunit affected U-box E3 ubiquitin ligase (TUD1) in Arabidopsis, and TUD1 mediated BR signaling transduction, regulated cell proliferation, and promoted plant growth and development [46, 47]. Previously, we have already found that, compared with WT seedlings,
Figure 7. Relationship between CsGPA1 and CsCOR413PM2 in transcript and protein levels. a Expression of CsCOR413PM2 in WT and CsGPA1-RNAi-10 during cold stress. b Expression of CsGPA1 in WT and CsCOR413PM2-RNAi-17 during cold stress. c Protein content of CsCOR413PM2 in WT and CsGPA1-RNAi-10 during cold stress. d Protein content of CsGPA1 in WT and CsCOR413PM2-RNAi-17 during cold stress. Western blot of WT and different transgenic lines (CsGPA1-RNAi-10 and CsCOR413PM2-RNAi-17) using anti-CsCOR413PM2, anti-CsGPA1, and anti-actin antibodies. The red numbers indicate the protein content. All measured indices were set up with three biological replicates per treatment and time point. *P < .05, significant difference between RNAi lines and WT (Tukey HSD).

suppression of CsGPA1 inhibits seedling growth and development and CsGPA1 controls hypocotyl elongation and root growth by promoting meristem and cell size of hypocotyl and root tip cells in cucumber seedlings [2]. In this study, we also found that suppression of CsGPA1 decreased expression of CsBZR1 and CsBZR2, two BR-signaling-related genes under normal temperature, indicating that CsGPA1 may affect growth and development of cucumber seedlings at normal temperature by influencing the BR signal. In general, the above results suggested that the G protein α subunit in plants mediated BR signaling to affect plant growth, development, and cold stress.

In addition to CsCDL1, we also found that CsGPA1 can interact with CsCOR413PM2 a cold-related protein (Figs 2a and 3a) located in the cell membrane (Fig. 4b). The Gα subunit participated in cold tolerance in Arabidopsis [35], tomato [36], and rice [5], and COR413PM2 participated in cold tolerance in tomato [26]. However, the differences and the regulatory relationship between the two genes in plants have not been reported. In this study, we first verified the function of CsCOR413PM2 in cucumber and found that suppression of CsCOR413PM2 significantly decreased cucumber cold tolerance. We further compared phenotypes between CsGPA1-RNAi and CsCOR413PM2-RNAi lines after 60 hours of cold treatment (Figs 1e and 6a; Supplementary Data Fig. S4).

We found that CsGPA1 silencing led to more severe wilt and dehydration, suggesting that CsGPA1 might be involved in multiple, functionally redundant cold-response pathways, such as mediating the BR signal, whereas the relatively low impact of CsCOR413PM2 silencing suggests a comparatively minor role in cold tolerance. To further explore the regulatory relationship between CsGPA1 and CsCOR413PM2 in transcript and protein levels, we determined the expression and protein content of CsCOR413PM2 and CsGPA1 in CsGPA1-RNAi and CsCOR413PM2-RNAi, respectively, under cold stress (Fig. 7). We found that the expression and protein content of CsCOR413PM2 significantly decreased in CsGPA1-RNAi plants compared with WT during cold stress (Fig. 7a and c). However, there were no differences in the expression and protein content of CsGPA1 in CsCOR413PM2-RNAi lines compared with WT during the cold stress (Fig. 7b and d). The above results suggested that suppression of CsGPA1 only affects the regulation of CsCOR413PM2 as part of a cold-stress response.

In general, we first indicated that CsGPA1 affected cold tolerance through regulating BR signal and CsCOR413PM2 in plants.

The CsGPA1–CsCOR413PM2–Ca²⁺ axis regulated the expression of CsICE–CsCBF during cold stress

The Gα subunit reportedly controls cold tolerance by mediating calcium signaling in rice [5] and studies also found that the calcium signal was essential for cold tolerance in cucumber [40, 41]. But it has remained unclear whether this pathway is conserved across other plant species, leading us to generate cucumber lines silenced for CsGPA1 to examine its effects on cold tolerance (Fig. 7). We found that CsGPA1 suppression significantly affected calcium influx at low temperatures (Fig. 8), suggesting that Gα-subunit-mediated calcium signaling in cold stress is conserved in both rice and cucumber. In addition, it is still not clear whether suppression of COR413PM2 affects calcium signaling in plants now. In
Figure 8. Ca\(^{2+}\) influx during low temperature and expression of cold-related genes in cucumber. 

**a** Ca\(^{2+}\) influx during low temperature for different treatments, including WT, CsGPA1-RNAi-9, CsGPA1-RNAi-10, CsCOR413PM2-RNAi-17, and CsCOR413PM2-RNAi-21. 

**b** Average Ca\(^{2+}\) influx for different treatments. 

**c, d** Expression of cold-related genes in WT, CsGPA1-RNAi, and CsCOR413PM2-RNAi plants. The negative sign (−) indicates Ca\(^{2+}\) influx. All measured indices were set up with three biological replicates per treatment and time point. *P < 0.05, **P < 0.001, significant difference between RNAi lines and WT (Tukey HSD).

In this study, suppression of CsCOR413PM2 significantly deceased Ca\(^{2+}\) influx during cold stress (Fig. 8). Further comparison of Ca\(^{2+}\) influx between CsGPA1-RNAi and CsCOR413PM2-RNAi shows that CsGPA1-RNAi lines show lower Ca\(^{2+}\) influx than CsCOR413PM2-RNAi lines (Fig. 8), which may be a reason why suppression of CsGPA1 resulted in weaker cold tolerance than suppression of CsCOR413PM2.

However, CBF-dependent or -independent genes can regulate COR expression [22, 23, 49, 50]. In this study,
CsCOR413PM2 was found to regulate ICE–CBF expression. COR genes have also been found to regulate expression of CBF. For example, one study found that repression of COR27 and COR28 by blue light negatively regulated CBF expression through crosstalk with CCA1 function and with PRR5 expression via unknown mechanisms [48]. In addition, slyCOR413PM2 has been found to positively regulate ICE–CBF gene expression in tomato, but the mechanism is unknown [26]. However, in this study, suppressing CsCOR413PM2 affected Ca$^{2+}$ influx, and other studies also found that increased Ca$^{2+}$ influx resulted in an increase in expression of ICE–CBF–COR under cold stress in plants [5, 45] (Fig. 8). Therefore, we next detected the expression of cold-stress-related genes that are induced by Ca$^{2+}$ influx [5, 45]. CsGPA1 or CsCOR413PM2 knockdown decreased Ca$^{2+}$ influx then decreased the transcriptional regulation of these four cold-related genes under cold stress (Fig. 8c and d). Therefore, CsCOR413PM2 regulated ICE–CBF–COR expression through the Ca$^{2+}$ signal. Since CsGPA1 can control CsCOR413PM2 transcription and translation, and both CsGPA1 and CsCOR413PM2 can regulate Ca$^{2+}$ influx, the expression of ICE–CBF can be regulated by the CsGPA1–CsCOR413PM2–Ca$^{2+}$ signal axis to affect cold tolerance of cucumber.

Conclusions
We have provided the first framework of CsGPA1 regulating cold tolerance of cucumber. We found that (i) CsGPA1 interacted with CsCDL1, a key BR signal transduction gene, and suppression of CsGPA1 weakens BR signaling in the regulation of cold tolerance; and (ii) CsGPA1 interacted with CsCOR413PM2 and CsGPA1 suppression could decrease the expression and protein content of CsCOR413PM2. Furthermore, suppression of CsGPA1 or CsCOR413PM2 both decreased Ca$^{2+}$ influx at low temperature and then decreased the expression of CsICE–CsCBF. Therefore, the CsGPA1–CsCOR413PM2–Ca$^{2+}$ signal axis regulated the expression of CsICE–CsCBF during cold stress.

Materials and methods
Plant material culture and treatment
WT and transgenic cucumber seeds were soaked in hot water at 55°C and constantly stirred until the temperature dropped to 30°C. Next, the seeds were soaked for 4–6 hours and then placed in a Petri dish with a diameter of 9 cm. Two layers of filter paper were added and moistened with 5 mL of bactericidal distilled water to keep them moist. When most of the seeds had germinated, they were sown with substrate (quartz sand:vermiculite = 1:1) in a plastic basin (15 cm ×15 cm). Cucumber seedlings were grown under 14 hours light (25°C)/10 hours dark (18°C) photoperiods (600 μmol m$^{-2}$ s$^{-1}$) with 70% relative humidity. When most of the seedlings had grown two leaves and one heart, seedlings at the same growth level were selected for cold stress. The WT and transgenic cucumber seedlings were treated at a low temperature of 6°C. Cucumber leaves were collected at low temperature for 0, 3, 6, 12, 24, and 60 hours, and then frozen in liquid nitrogen and finally stored in a refrigerator at −80°C. These samples were used to measure physiological indices or expression of genes (samples at each time point had three biological replicates).

Real-time PCR
The methods of RNA extraction, cDNA synthesis, and qPCR were based on the previous study [2]. The qPCR primers are listed in Supplementary Data Table S1 (some of the qPCR primers were got from qPrimerDB). The gene expressions were calculated using the $2^{-ΔΔCt}$ method (reference gene: CsActin), and then R 4.02 software was used to analyze the significance of differences using Tukey’s HSD ($P < .05$). Prior to the PCA and regression analysis, the method of data standardization was based on our previously published paper [3]. PCA and regression analysis were performed on the gene expression data using R 4.02 software.

Gene cloning and construction of RNAi vector
CsGPA1 cloning and construction of CsGPA1-RNAi vector were according to our previously published paper [2]. The methods of CsCOR413PM2 cloning and construction of CsCOR413PM2-RNAi vector were as follows. Forward and reverse fragments (200 bp) of COR413PM2 were separately cloned, and then ligated to PHANNIBAL vector by using the NEBuilder® HiFi DNA Assembly Cloning Kit (NEB #E2621S). The vector was then transformed into Escherichia coli DH5α competent cells, blue-white spot screening was performed, and the correct positive E. coli clone detected by PCR was sequenced. We obtained PHANNIBAL-COR413PM2-hairpin, which was cut with SACI and PSTI enzymes, and then we cloned the fragment into the same locus of pCAMBIA1300 vector to obtain pCAMBIA1300-COR413PM2-hairpin. The primers used in the study are shown in Supplementary Data Table S1.

Subcellular localization
The full lengths of CsCOR413PM2 and Csa_4G663630.1 were amplified by using the specific primers shown in Supplementary Data Table S1. After digestion with HindIII and KpnI, the fragment was attached to the HindIII/KpnI site in Super-1300-GFP. Methods for subcellular localization were according to Huang et al. [42].

Determination of malondialdehyde and relative electric conductivity
The MDA content was determined based on the method of Murshed et al. [43]. The REC was determined according to the method of Jiang and Zhang [44]. The calculation formula was as follows:

$REC (\%) = (EC_1/EC_2) \times 100$
where EC₁ and EC₂ refer to the initial and final electrical conductivity, respectively. The data were analyzed using R 4.02 software for Tukey’s HSD (P < .05).

**Ca²⁺ influx detection**
Calcium fluxes in roots were determined by using the non-invasive microtest (Younger LLC, Amherst, MA 01002, USA). Test solution at 0°C was used for the cold treatment. The data were analyzed using R 4.02 software for Tukey’s HSD (P < .05).

**Construction of a cold-related cDNA library of cucumber**
The expression of CsGPA1 reached its maximum after seedlings were treated with cold stress for 24 hours. Leaves at this point were collected to construct a cDNA library that might be associated with CsGPA1 in response to low temperature. The method of cDNA construction was based on our previous study [3].

**Split-ubiquitin yeast two-hybrid system**

pBT3-N-CsGPA1 bait vector construction and yeast transformation
The method was based on our previous study [3].

**Self-activation identification of decoy proteins**
The plasmid combination pBT3-N-CsGPA1/pPR3-N was co-transformed into yeast NMY51. The mixture was coated on an SD-Trp-Leu medium and grown for 2–3 days. After positive clones were obtained, the monoclones were inoculated into SD-Trp-Leu medium for shake culture overnight. The bacterial fluid was placed on SD-Trp-Leu–His liquid medium containing 0, 5, 7.5, or 10 mmol L⁻¹ 3-AT (3-amino-1,2,4-triazole), and then cultured at 30°C for 2 days.

**Yeast two-hybrid assay and identification**
The cDNA library plasmid of cucumber seedlings and bait pBT3-N-CsGPA1 plasmid were extracted and co-transformed into NMY51. The transformation product was coated on SD–Trp–Leu–His medium + 10 mmol L⁻¹ 3-AT. The plate was sealed with a membrane and cultured at 30°C for 3–4 days, and then colony growth was observed. Clones with β-galactosidase activity that could still grow normally on the SD–Trp–Leu–His–Ade + 10 mmol L⁻¹ 3-AT deficient medium after three lines were plated and identified as positive clones. After plasmids were extracted from positive yeast colonies and transferred to *E. coli*, the plasmids were extracted from prey and then transformed into NMY51 yeast again with pBT3-N-CsGPA1. Y2H was carried out, with the SD–Trp–Leu–His–Ade + 10 mmol L⁻¹ 3-AT deficient medium. Interacting genes were isolated from positive colonies growing on this medium. Additionally, pTSU2-APP and pNubG-Fe65 were co-transformed to NMY51 yeast as positive controls [45], while pTSU2-APP and pPR3-N were co-transformed to NMY51 yeast as negative controls [45].

**Pull-down assay**
We designed primers according to gene sequence information on CsGPA1, Csa4G663630. The RNA was extracted from leaves of cucumber seedlings, and cDNA was used as a template. After gel recovery, the target fragment was linked with pGEX4T1 and pET21a, and transformed into DH5α. The recombinant plasmids pET21a-CsCOR413PM2/CsORF413, and pGEX4T1-CsGPA1 were obtained by PCR identification and positive clone sequences. The recombinant plasmids pGEX4T1-CsGPA1 and pET21a-CsCOR413PM2/CsORF413 were separately transformed into strain BL21 (DE3) and we obtained the protein lysates. Finally, we used western blot to detect whether GST/FLAG/His-beads bind to His tag protein.

**Membrane protein extraction and western blot**
The details of western blot and membrane protein extraction followed a previously published paper [2]. In addition, the N-terminal peptide of CsCOR413PM2 (7-LKMVTDSDAADLISDSC-22 + 16-ADLSSDLREGNDARC-31) was synthesized by Beijing QiWei YiCheng Tech Co. Ltd. (Beijing, China) and used to produce a rabbit polyclonal antibody with CsCOR413PM2 (XP_004145347.1) as the antigen. ImageJ software (IJ152-WIN-Java8) was used to analyze the gray scale of western blot bands.

**Evolutionary tree construction**
Proteins containing the WCOR413 (PF05562) domain in cucumber, tomato, rice, *Arabidopsis thaliana*, and tobacco were searched using HMM software, and then unrooted phylogenetic trees of the full-length COR proteins of different species were inferred using the neighbor-joining method in MEGA 7.0 software.

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**Author contributions**
Y.Y., S.M.T., G.L.H., and Y.X.C. designed the study. Y.Y., F.Q., M.S., S.M.T., and D.Q.H. performed the experiments. Y.Y. collected the data. S.M.T. and Y.Y. performed all mapping and data analysis. S.M.T. and Y.Y. prepared and revised the manuscript. S.M.T., G.L.H., and Y.X.C. provided guidance on the whole study. All the authors approved the final manuscript.
Data availability
The data are presented within the paper and supplementary files.

Conflict of interest
There are no conflicts of interest to declare for authors.

Supplementary data
Supplementary data is available at Horticulture Research online.

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