TaCOLD1 defines a new regulator of plant height in bread wheat

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Summary

Plant height is among the most important agronomic traits that influence crop yield. However, in addition to the Rht-1 alleles, the molecular basis of plant height in bread wheat remains largely unclear. Based on wheat gene expression profiling analysis, we identify a light-regulated gene from bread wheat, designated as TaCOLD1, whose encoding protein is homologous to cold sensor COLD1 in rice. We show that TaCOLD1 protein is localized to the endoplasmic reticulum (ER) and plasma membrane. Phenotypic analyses show that overexpression of a mutated form of TaCOLD1 (M187K) in bread wheat cultivar Kenong199 (Rht-B1b) background resulted in an obvious reduction in plant height. Further, we demonstrate that the hydrophilic loop of TaCOLD1 (residues 178–296) can interact with TaGα-7A (the α subunit of heterotrimeric G protein) protein but not TaGα-1B, and the mutation (M187K) in TaCOLD1 remarkably enhances its interaction with TaGα-7A. Physical interaction analyses show that the C-terminal region of TaGα-7A, which is lacking in the TaGα-1B protein, is necessary for its interaction with TaCOLD1. Intriguingly, the C-terminal region of TaGα-7A is also physically associated with the TaDEP1 protein (an atypical Gγ subunit). Significantly, we discover that TaCOLD1 and mTaCOLD1 (M187K) can interfere with the physical association between TaGα-7A and TaDEP1. Together, this study reveals that TaCOLD1 acts as a novel regulator of plant height through interfering with the formation of heterotrimeric G protein complex in bread wheat and is a valuable target for the engineering of wheat plant architecture.

Introduction

Bread wheat (Triticum aestivum) is a major staple crop worldwide. By the year 2050, the world population is expected to reach 9.3 billion and global demand for bread wheat is increasing. To guarantee global food security, people have been seeking elite agronomic traits of bread wheat to improve its yield. Height reduction has been associated with yield increases and yield stability in a number of different crop species (Peng et al., 1999). During the Green Revolution, substantial increase in bread wheat (T. aestivum) yield was realized, at least in part, through the introduction of the Reduced height (Rht)-B1b and Rht-D1b semi-dwarfing alleles encoding mutant gibberellin response modulators (Li et al., 2012; Peng et al., 1999; Van De Velde et al., 2017; Wu et al., 2011). However, in addition to the Rht1 alleles applied in the Green Revolution (Peng et al., 1999), new types of genes that determine bread wheat plant height remain to be identified. Therefore, with the continued growth of world population, it is an urgent need to develop better suited plant height based on the understanding of genetic basis of plant height in bread wheat.

Heterotrimeric G proteins, comprising Gα, Gβ and Gγ subunits, are intracellular membrane-attached signal transducers and involved in regulating shoot, root and epidermis development, as well as sugar sensing, hormone responsiveness and abiotic and biotic stress tolerance (Botella, 2012; Jones and Assmann, 2004; Jones et al., 2011; Perfus-Barbeoch et al., 2004; Urano and Jones, 2014; Urano et al., 2016). In Arabidopsis thaliana, there is one Gα (GPA1) (Ma et al., 1990), one Gβ (AGB1) (Weiss et al., 1994), and three Gγ subunits (Chakravorty et al., 2011; Mason and Botella, 2000, 2001). Plant heterotrimeric G-proteins are known to be involved in a myriad of physiological and developmental processes (Assmann, 2005; Chen, 2008; Jones, 2002; Jones and Assmann, 2004; Urano et al., 2013). Previous studies have shown that different subunits of heterotrimeric G proteins regulate crop plant height. For instance, rice Gα mutants displayed a dwarf phenotype (Fujisawa et al., 1999) and maize Gα gene mutant compact plant 2 (ct2) displayed a shorter stature phenotype (Bommert et al., 2013). In addition, rice DEP1 (DENSE AND ERECT PANICLES 1) gene encodes an atypical Gγ subunit, which interacts with both the Gα (REGA1) and Gβ (RGB1) subunits (Sun et al., 2014). Loss-of-function mutations of DEP1 in rice, or DEP1 homolog in barley caused dwarf phenotype (Huang et al., 2009; Wendt et al., 2016). Moreover, in the case of TaDEP1, the tadep1-aabbdd mutant wheat plants generated by CRISPR/Cas9-based genome editing exhibited a dwarf phenotype, suggesting that TaDEP1 is an important regulator of wheat plant architecture (Zhang et al., 2016).

In this study, we identify a light-regulated bread wheat gene, here designated as TaCOLD1, encoding a trans-membrane protein with high similarity to rice cold sensor COLD1 (Ma et al., 2015). Transgenic analyses indicate that TaCOLD1 plays an important role in regulating bread wheat plant height. Furthermore, we show that the central hydrophilic loop of TaCOLD1 (TaCOLD116) can interact with the C-terminal region of TaGα-7A protein. Remarkably, the mutation (M187K) in TaCOLD1 could enhance its interaction with TaGα-7A. Intriguingly, the C-terminal region of TaGα-7A can also physically associate with TaDEP1. We
reveal that both TaCOLD1 and mTaCOLD1 (M187K) can interfere with the physical association between TaGαx and TaDEP1. Thus, this study uncovers a molecular mechanism underlying the modulation of heterotrimeric G protein signalling in controlling bread wheat plant height.

**Results**

**Identification and molecular characterization of TaCOLD1 in bread wheat**

To identify the regulatory genes for wheat plant architecture, we examined the public wheat expression profiling data (https://wheat.pw.usda.gov/WheatExp/). We noticed that the expression of one wheat gene (Triticum monococcum 2AL_7A5AD3700), here designated as TaCOLD1 (see below for detail information), was significantly repressed by light, indicating that it might play a role in wheat plant photomorphogenesis. To confirm the expression pattern of TaCOLD1 genes in the hexaploid bread wheat, the 5-d-old seedlings of bread wheat cultivar Kenong199 (KN199) grown in the dark were exposed to light or kept in continuous dark conditions for different time points. Consistently, quantitative reverse transcriptase (qRT)-PCR assay indeed showed that the expression levels of TaCOLD1 could be reduced in a short time in response to light signal, while no obvious change in continuous dark conditions (Figure 1a). Here, we isolated three highly conserved homologous sequences separately located on chromosomes 2A, 2B and 2D from the hexaploid bread wheat KN199 (Figure S1). Using TaCOLD1 protein sequences as query, we found that the wheat TaCOLD1 proteins are homologs of COLD1 in rice (Ma et al., 2015), with 97% identity to the COLD1<sup>ind</sup> (Oryza sativa ssp. indica) and COLD1<sup>jap</sup> (Oryza sativa ssp. japonica) proteins (Figure 1b). Then we constructed a neighbour-joining phylogenetic tree with Clustal W by aligning the protein sequences of TaCOLD1 and their homologs from various plant species. Phylogenetic analysis showed that TaCOLD1 proteins were closely related to their homologs in Hordeum vulgare and Brachypodium distachyon (Figure 2a).

**TaCOLD1 might be evolutionarily conserved**

A recent study identified one SNP in rice COLD1<sup>gen</sup> gene, which results in Lys<sup>187</sup> in COLD1<sup>jap</sup> compared to Met<sup>187</sup> in COLD1<sup>ind</sup>, is associated with stronger chilling tolerance of japonica cultivars compared to indica cultivars (Ma et al., 2015), suggesting this amino acid site might be critical for the function of COLD1 homologs. To investigate if there exists a similar SNP in wheat, we performed sequence analyses for the COLD1<sup>gen</sup> from the rice genes from nine accessions of diploid (Qin et al., 2017), 22 accessions of tetraploid (Qin et al., 2017) and nine accessions of hexaploid wheat. Intriguingly, we found that this locus in all the examined COLD1 proteins is identical to COLD1<sup>ind</sup> but not COLD1<sup>jap</sup> (Figure 2b and Table S1). These results suggest that the wheat COLD1 proteins might be evolutionarily conserved.

**TaCOLD1 is a membrane protein**

Prediction of subcellular localization with a transmembrane domain hidden Markov model (Ma et al., 2015) suggested that TaCOLD1 proteins were typical transmembrane proteins with nine transmembrane domains (Figures 3a and S2). To experimentally confirm this prediction, the TaCOLD1-GFP fusion protein and Pip2-mCherry, a marker for the endoplasmic reticulum (ER) and plasma membrane (Lee et al., 2009), were co-expressed in wheat mesophyll protoplasts. The results showed that the fluorescence signal of TaCOLD1-GFP could be merged with that of Pip2-mCherry at ER with a reticular pattern and plasma membrane (Figure 3b,c), suggesting that TaCOLD1 proteins localize to the ER and plasma membrane.

**Overexpression of mTaCOLD1 (M187K) caused a dwarf phenotype in bread wheat**

To explore the biological role of TaCOLD1 in bread wheat, we generated the mTaCOLD1 (M187K, identical to COLD1<sup>ind</sup>) transgenic lines in bread wheat cultivar KN199 background which already contains a semi-dwarf gene Rht-B1b (Figure S3). Significantly, the different mTaCOLD1 transgenic wheat lines displayed reduced plant height compared with WT KN199 at both vegetative and mature stages (Figures 4a and S4). Statistical analyses showed that a dramatic decrease of plant weight from ~70 cm in WT KN199 to ~50 cm in transgenic lines (Figure 4b). Consistently, qPCR confirmed that the expression levels of mTaCOLD1 were obviously increased in the mTaCOLD1 transgenic lines (Figure 4c). Further, each internode of mTaCOLD1 transgenic plants was shorter than that in WT KN199 (Figure 4d). Therefore, we propose that TaCOLD1 may be a critical regulator of plant height in bread wheat.

To investigate the dwarf phenotype of mTaCOLD1 transgenic lines in the cell level, we observed the longitudinal sections of KN199 and mTaCOLD1 transgenic lines using the uppermost internodes at vegetative stage. The cell length in mTaCOLD1 transgenic plants was greatly shorter compared with that in WT KN199 (Figure 4e,f). Taken together, we conclude that overexpression of mTaCOLD1 leads to a dwarf phenotype in bread wheat, at least in part, through reducing cell length.

**Identification of distinct Gα proteins in bread wheat**

In order to explore the molecular mechanism of TaCOLD1 in bread wheat, we planned to identify the partner proteins of TaCOLD1 in bread wheat. A recent study has shown that the homolog of TaCOLD1 in rice, COLD1<sup>jap</sup>, is physically associated with Gα protein (Ma et al., 2015). Moreover, the maize COMPACT PLANT2 (CT2) gene encodes the α-subunit (Gα) of heterotrimeric G protein, whose loss-of-function mutant displays a shorter stature phenotype (Bommert et al., 2013). These findings promoted us to ask whether TaCOLD1 is functionally associated with the heterotrimeric G protein in bread wheat. To test this idea, we first identified the TaGα genes based on the coding sequence (CDS) of ZmCT2 (LOC_Zm00001d027886) in maize and genome sequences of the bread wheat A and D genome donors (Jia et al., 2013; Ling et al., 2013). Then we isolated three homologous genes from bread wheat cultivar KN199 (Figure S5). Further, chromosomal locations of TaGα genes were determined by using the three TaGα sequences as query sequences to blast the wheat survey sequences, which include the chromosome-based draft sequence of the hexaploid wheat (http://wheat-urgi.versailles.inra.fr/blast) (Deng et al., 2007; International Wheat Genome Sequencing Consortium, 2014). The results showed that the three TaGα sequences were located on chromosomes 7A5, 7DS and 1BL, respectively, and here designated as TaGα-7A, TaGα-7D and TaGα-1B. Protein sequences alignment showed that TaGα-7A/7D/1B and ZmCT2 protein have ~93% identity (Figure 5a).

We next used the Chinese Spring nullisomic-tetrasomic lines to experimentally confirm the chromosomal locations of TaGα-7A, TaGα-7D and TaGα-1B. The results showed that target fragments produced by the TaGα-7A-specific primers could not be detected.
in the absence of chromosome 7A (N7AT7D) (Figure 5b). Similarly, target fragments produced by the TaGα-7D-specific primers could not be detected in the absence of chromosome 7D (N7DT7A); target fragments produced by the TaGα-1B-specific primers could not be detected in the absence of chromosome 1B (N1BT1A) (Figure 5b). In addition, phylogenetic analysis of the Ga proteins from various plant species showed that the three TaGα proteins were most closely related to their homolog in Hordeum vulgare (Figure 5c).

The central hydrophilic loop of TaCOLD1 interacts with TaGα-7A but not TaGα-1B.

To determine whether TaCOLD1 proteins physically interact with distinct TaGα proteins, we performed firefly luciferase complementation imaging (LCI) assays in Nicotiana benthamiana leaves. The central hydrophilic loop (HL, residues 178–296) of TaCOLD1 was selected for the physical interaction assay, which was fused with the nLUC to generate nLUC-TaCOLD1 HL.
TaCOLD1HL/cLUC-TaG accession numbers are MG748865 (TaCOLD1-2A), MG748866 (TaCOLD1-1B), MG748867 (TaCOLD1-2B). The evolutionary distances were computed in units of the amino acid substitutions per site. The species and protein names or numbers are given in the phylogenetic tree. The GenBank accession numbers are MG748865 (TaCOLD1-2A), MG748866 (TaCOLD1-2B), MG748867 (TaCOLD1-2D), XP_015633398 (COLD1<sup>ΔC</sup> N. benthamiana), A2XX57 (COLD1<sup>ΔC</sup> N. benthamiana), NP_176679 (AtGTG1), NP_194493 (AtGTG2).

Figure 2 Phylogenetic analysis of TaCOLD1. (a) The phylogenetic tree was constructed based on the neighbour-joining method by MEGA7 program. The evolutionary distances were computed in units of the amino acid substitutions per site. The species and protein names or numbers are given in the phylogenetic tree. The GenBank accession numbers are MG748865 (TaCOLD1-2A), MG748866 (TaCOLD1-2B), MG748867 (TaCOLD1-2D), XP_015633398 (COLD1<sup>ΔC</sup>n. benthamiana), A2XX57 (COLD1<sup>ΔC</sup> n. benthamiana), NP_176679 (AtGTG1), NP_194493 (AtGTG2). (b) Amino acid sequence alignment showing the conservation of amino acid Met (marked by red box) in bread wheat varieties.

Meanwhile, TaG<sub>a</sub>-7A and TaG<sub>x</sub>-7A were fused with the cLUC to generate cLUC-TaG<sub>a</sub>-7A and cLUC-TaG<sub>x</sub>-1B, respectively. Interestingly, strong luminescence signals were observed in nLUC-TaCOLD1<sup>ΔC</sup>/cLUC-TaG<sub>a</sub>-7A co-expression samples and weak luminescence signals were observed in nLUC-TaCOLD1<sup>ΔC</sup>/cLUC-TaG<sub>x</sub>-1B co-expression samples, whereas no signal was detected in the negative controls (Figure 6a). Meanwhile, our qRT-PCR assays revealed that TaCOLD1 and TaG<sub>x</sub> were similarly expressed in different infiltrated samples (Figure 6b). Notably, we found that the C-terminal region of TaG<sub>x</sub>-7A is lacking in TaG<sub>x</sub>-1B (Figure 5a). To assess whether the C-terminal region of TaG<sub>x</sub>-7A is necessary for its physical interaction with TaCOLD1<sup>ΔC</sup>, the truncated TaG<sub>x</sub>-7A protein without C-terminal region (TaG<sub>x</sub>-7AΔC) was fused with the cLUC to generate cLUC-TaG<sub>x</sub>-7AΔC for LCI assays (Figure 6c). The results showed that obvious LUC activities were detected in nLUC-TaCOLD1<sup>ΔC</sup>/cLUC-TaG<sub>x</sub>-7AΔC co-expression samples, but no LUC activity was detected in nLUC-TaCOLD1<sup>ΔC</sup>/cLUC-TaG<sub>x</sub>-7AΔC co-expression samples (Figure 6c), which expressed similar transcript levels of TaCOLD1 and TaG<sub>x</sub> (Figure 6d), confirming that the C-terminal region of TaG<sub>x</sub>-7A is indeed required for the physical interaction between TaCOLD1<sup>ΔC</sup> and TaG<sub>x</sub>. Further, to investigate whether the mutation (M187K) of TaCOLD1 affects its interaction with TaG<sub>x</sub>-7A, we performed LCI assay in N. benthamiana leaves. The results indicate that the mutation (M187K) of TaCOLD1 enhances its interaction with TaG<sub>x</sub>-7A (Figure 6e–g).

To further evaluate the physical interaction between TaCOLD1 and TaG<sub>x</sub>-7A, we performed pull-down assays in vitro. As shown in Figure 7a, GST-TaG<sub>x</sub>-7A was pulled down by MBP-TaCOLD1<sup>ΔC</sup> but not MBP alone. Next, we performed the bimolecular fluorescence complementation (BiFC) assays in N. benthamiana to validate the interaction of TaCOLD1<sup>ΔC</sup> and TaG<sub>x</sub>-7A. The results showed that a strong YFP fluorescence signal was detected on the plasma membrane when nYFP-TaCOLD1<sup>ΔC</sup> was co-transformed with cYFP-TaG<sub>x</sub>-7A, and no fluorescence signal was detected in the negative controls (Figure 7b). Taken together, these results demonstrate that TaCOLD1 physically interacts with TaG<sub>x</sub>-7A but not TaG<sub>x</sub>-1B, through the central hydrophilic loop of TaCOLD1 and the C-terminal region of TaG<sub>x</sub>-7A, respectively (Figure 7c).}

TaG<sub>x</sub>-7A but not TaG<sub>x</sub>-1B physically associates with TaDEP1

A previous study has shown that OsDEP1 in rice functions as an atypical G protein γ subunit (Sun et al., 2014). Moreover, a recent study showed that the tadep1-aabbdd wheat mutant, generated by CRISPR/Cas9-based genome editing, displayed a dwarf phenotype during the vegetative and reproductive growth stages (Zhang et al., 2016). These findings promoted us to ask whether TaCOLD1 is functionally associated with TaDEP1. To this end, we first cloned a TaDEP1 gene from bread wheat cultivar KN199 (Figures S6 and S7). A neighbour-joining phylogenetic tree showed that TaDEP1 is closely related to HvDEP1 in Hordeum vulgare (Figure S8).

We hypothesized that TaDEP1, similar to OsDEP1 in rice, acts as a Gγ subunit and might associate with other subunits of G protein in bread wheat. To test this hypothesis, we evaluated the physical association between TaG<sub>x</sub> and TaDEP1 by LCI assays in N. benthamiana. The results showed that the samples co-expressing cLUC-TaG<sub>x</sub>-7A and nLUC-TaDEP1 displayed strong luminescence signals, whereas no signal was detected in the samples co-expressing cLUC-TaG<sub>x</sub>-1B and nLUC-TaDEP1 (Figure 8a). Our qRT-PCR assays revealed that the transcript levels of TaDEP1 and TaG<sub>x</sub> were similar in different infiltrated samples (Figure 8b). To further investigate the physical association between TaG<sub>x</sub>-7A and TaDEP1, we performed BiFC assays in N. benthamiana. The results showed that an obvious YFP fluorescence signal was detected on the plasma membrane when nYFP-TaDEP1 was co-transformed with cYFP-TaG<sub>x</sub>-7A, whereas no fluorescence signal was detected in the negative controls (Figure 8c). Moreover, the truncated TaG<sub>x</sub>-7A protein lacking its C-terminal region (TaG<sub>x</sub>-7AΔC; Figure 8c, upper panel) failed to associate with TaDEP1 in the LCI assays (Figure 8d). The qRT-PCR assays revealed that TaDEP1 and TaG<sub>x</sub> were similarly expressed in different infiltrated samples (Figure 8e). Thus, we concluded that the C-terminal region of TaG<sub>x</sub>-7A is responsible for its association with TaDEP1.

To map which domain of TaDEP1 is required for its association with TaG<sub>x</sub>-7A, we performed the LCI assays in N. benthamiana leaves. As shown in Figure 8f, TaDEP1 was divided into three parts: N terminus (residues 1-80) contains a GGL domain; middle domain (residues 81-180) contains a WFWC domain and C terminus (residues 181-285) contains a TNFR/NGFR domain. The three parts of TaDEP1 were fused with nLUC, respectively, for LCI assays. The results showed that obvious LUC activities were observed in cLUC-TaG<sub>x</sub>-7A/nLUC-TaDEP1-NT and cLUC-TaG<sub>x</sub>-7A/nLUC-TaDEP1-NC co-expression samples, whereas no signal was detected in the samples co-expressing cLUC-TaG<sub>x</sub>-1B and nLUC-TaDEP1-NT (Figure 8g). Thus, we concluded that the C-terminal domain of TaDEP1 is responsible for its association with TaG<sub>x</sub>-7A.
7A/nLUC-TaDEP1-MD co-expression samples, but no LUC activity was observed in cLUC-TaG a-7A/nLUC-TaDEP1-CT co-expression samples (Figure 8g). Taken together, these results showed that the GGL and vWFC motifs of TaDEP1 mediate its association with TaG a-7A.

TaCOLD1 interferes with the physical association between TaG a-7A and TaDEP1

It has been known that G a keeps its GDP tightly bound and forms an inactive heterotrimer with the G bG subunits at steady state (Gilman, 1987). The findings that the C-terminal region of TaG a-7A mediates its interaction with both TaCOLD1 HL and TaDEP1 (Figures 6c, 8d and 9a), led us to ask whether TaCOLD1 displays a competitive effect on the physical association between TaG a-7A and TaDEP1. To test this idea, we co-expressed TaCOLD1 HL-GFP with nLUC-TaDEP1 and cLUC-TaG a-7A proteins in N. benthamiana leaves. The results showed that the LUC intensities in cLUC-TaG a-7A/nLUC-TaDEP1/TaCOLD1HL-GFP co-expression samples (Figure 9b,c, co-infiltration 4) were dramatically decreased by more than 50% compared to those in the cLUC-TaG a-7A/nLUC-TaDEP1/GFP-MYC co-expression samples (Figure 9b,c, co-infiltration 3). Our qRT-PCR assays showed that

Figure 3 Transmembrane structure and subcellular localization analyses of TaCOLD1. (a) Topology prediction for TaCOLD1-2D using a transmembrane domain hidden Markov model (TMHMM version 2.0). (b) Subcellular co-localization of TaCOLD1 with ER marker in bread wheat mesophyll protoplasts. GFP signal of TaCOLD1-GFP was merged with that of ER marker in the ER. The B1, B2 and B3 images (lower panel) are enlargements of the regions framed in white (upper panel). Scale bars, 10 μm. (c) Plasma membrane localization of TaCOLD1-GFP. TaCOLD1-GFP signal was merged with that of the PIP2-mCherry marker at plasma membrane in wheat mesophyll protoplasts.
TaDEP1 and TaGα were similarly expressed in different infiltrated samples; meanwhile, the immunoblotting assay showed that the protein accumulation levels of TaCOLD1 and GFP-MYC were equal (Figure 9d,e). Similarly, mTaCOLD1 (M187K) could also attenuate the physical association between TaGα-7A and TaDEP1 (Figure S9). According to these data, we propose that TaCOLD1 might regulate the biological function of heterotrimeric G protein in bread wheat, at least partly, through interfering with the physical association between TaGα-7A and TaDEP1.

**Discussion**

Due to the rapid increasing of world population, food shortage is becoming a serious global problem. Improvement of grain yield has been the key focus of wheat breeding programmes over the past 50 years. Identification of key regulatory genes for important agronomic traits is of enormous significance for wheat molecular assisted breeding. However, map-based cloning and Genome Wide Association Study (GWAS) in the hexaploid bread wheat with complex genome still remain challenging. In this study, we identify TaCOLD1 as a novel regulator of bread wheat plant height, and uncover a molecular mechanism by which TaCOLD1 might regulate the function of heterotrimeric G protein.

TaCOLD1 is a novel regulator of plant height in bread wheat

The widely cultivated hexaploid bread wheat (2n = 6x = 42, AABBDdd) is derived from the domestication processes, which had...
been important for the agricultural revolution and the establishment of human civilization. Since the 1970s, green revolution wheat breeding project, characterized by \( \text{Rht-B1b} \) or \( \text{Rht-D1b} \) loci, has developed a number of high-yielding wheat varieties, which are more resistant to damage by wind and rain (Peng et al., 1999). Among several pleiotropic functions of \( \text{Rht-B1b} \), \( \text{Rht-D1b} \) and \( \text{Rht-B1c} \) genes, some are advantageous for plant improvement and some are disadvantageous. For example, \( \text{Rht-B1c} \) plants can delay heading date and resist to sprouting (Wu et al., 2011). However, other regulators of wheat plant height remain to be identified for ideal wheat plant architecture. In this study, we showed that overexpression of \( \text{mTaCOLD1} \) (M187K) can cause an appropriate reduction of plant height in the bread wheat cultivar KN199 containing the \( \text{Rht-B1b} \) allele (Figures 4a and S4), suggesting that \( \text{TaCOLD1} \) acts as a new regulator of bread wheat plant height potentially independent of \( \text{Rht1} \).

Therefore, manipulation of \( \text{TaCOLD1} \) orthologues in temperate cereals like bread wheat and barley through genome engineering using the CRISPR/Cas9-mediated genome editing technology might be applicable to facilitate the breeding of new crop varieties with ideal plant architecture. A haplotype analysis of the critical region of \( \text{TaCOLD1} \) locus (Figure 2b and Table S1) suggested that the \( \text{TaCOLD1} \) gene might have not been used to breed elite wheat varieties.

A molecular mechanism underlying \( \text{TaCOLD1} \) actions in bread wheat

In this study, our results revealed that the central hydrophilic loop of \( \text{TaCOLD1} \) could interfere with the formation of heterotrimeric G protein complex in bread wheat. First, we showed that the central hydrophilic loop of \( \text{TaCOLD1} \) differentially interacts with distinct \( \text{TaG} \) nature variants, such as \( \text{TaG} \)-7A and \( \text{TaG} \)-1B (Figure 6a). Second, domain mapping showed that the C-
TaCOLD1 physically interacts with TaGa-7A but not TaGa-1B. (a) LCI assay demonstrating that TaCOLD1 could directly interact with TaGa-7A but not TaGa-1B in N. benthamiana. (b) The expression levels of TaCOLD1 and TaGa in the infiltrated N. benthamiana leaf areas were determined by qRT-PCR (mean ± SD, n = 5). Results were normalized to NbACT1 (NbACT1). **P < 0.01 (Student’s t test). (c) LCI assay showing that the C terminus of TaGa-7A was essentially required for the interaction with TaCOLD1. Upper panel, schematic representation of the truncated TaGa-7A proteins used for the LCI assays. The full coding sequence of TaGa-7A protein contains 1-393 amino acids, while the truncated TaGa-7AΔC protein contains 1-362 amino acids. Lower panel, a representative image of LCI assay showing that TaCOLD1ΔC interacts with TaGa-7A but not TaGa-7AΔC in N. benthamiana. (d) qRT-PCR determination of the expression levels of TaCOLD1 and TaGa in the infiltrated N. benthamiana leaf areas shown in (c). The data were normalized to NbACT1. (Mean ± SD, n = 4). (e) LCI assay indicating that mTaCOLD1 enhances the interaction with TaGa-7A. (f) Quantification of the relative luminescence intensities shown in (e), (n = 16). The values in combination 3 were defined as “1”. Error bars indicate SD among three independent replicates. **P < 0.01 (Student’s t test). (g) qRT-PCR assay showing the expression levels of TaCOLD1 and TaGa in the infiltrated N. benthamiana leaves shown in (e). The data were normalized to NbACT1. (Mean ± SD, n = 4). In (a), (c) and (e), four independent tobacco leaves were used for the assays and three independent biological replications were performed with similar results.

Figure 7 TaCOLD1 interacts with TaGa-7A in vitro and in vivo. (a) Pull-down assay showing the interaction between TaCOLD1 and TaGa. Anti-MBP and anti-GST antibodies were used for the immunoblotting. (b) BiFC assay detecting the physical interaction between TaCOLD1 and TaGa-7A in N. benthamiana. The YFP fluorescence signals were detected 48 h post infiltration (hpi). BF, bright field. Scale bars, 10 μm. (c) Schematic showing the interaction between TaCOLD1 and TaGa.

terminal region of TaGa-7A protein is necessary for its association with TaCOLD1 (Figure 6c), whereas this region is lacking in the TaGa-1B protein. Third, the C-terminal region of TaGa-7A protein also mediates its association with TaDEP1 (an atypical G protein subunit) (Figure 7d). Finally, we uncovered that TaCOLD1 might interfere with the binding of TaGa-7A and TaDEP1, revealing a molecular mechanism for the TaCOLD1-mediated regulation of bread wheat plant height. In addition, a recent study reported that the rice COLD1 protein, an orthologue of bread wheat TaCOLD1, could activate Gα GTPase activity (Ma et al., 2015). These findings lead us to propose that TaCOLD1 might regulate the function of heterotrimeric G protein through distinct mechanisms in bread wheat.

Experimental procedures

Plant materials and growth conditions

Bread wheat (T. aestivum L.) cultivar wild-type (WT) Kenong199 (KN199) was used to amplify gene sequences, generate transgenic wheat plants and analyze gene expression. The mutant TaCOLD1 gene sequence was ligated to pUbicas vector and transformed into one-month-old embryogenic calli of KN199 by using a PDS1000/He particle bombardment system (Bio-Rad, Hercules, CA) with a target distance of 6.0 cm from the stopping plate at helium pressure 1100 psi, as described previously (Shan et al., 2013).

The WT KN199 and pUbic:mTaCOLD1 transgenic lines were planted at the experimental station (39°57’N, 116°19'E) of the Institute of Crop Sciences, CAAS, Beijing. The seeds were planted at the beginning of October and harvested in mid-June next year. Plant heights were measured after harvest.

Nicotiana benthamiana were grown in a greenhouse under long-day conditions (16-h-light/8-h-dark) at 22 °C.

Generation of DNA constructs

For LCI assays, the constructs were based on ligation free cloning mastermix (Applied Biological Materials, E011-5-A) according to the manufacturer’s instruction. In brief, the amplified target genes were separately cloned into the pKpnI5saI digested p1300-35S-nLUC and KpnI/BamHI digested p1300-35S-cLUC vectors (Chen et al., 2008).

For BiFC assays, gateway cloning technology (Invitrogen, California, CA) was used. All the target genes were ligated into the entry vector pQBV3 and then introduced into the pEarleygate201-YN (nYFP) and pEarleygagate202-YC (cYFP) destination vectors (Lu et al., 2010), respectively.

For pull-down assays, the constructs were based on the vectors pMAL-c2X and pGEX4T-1. Briefly, the central
Figure 8 The C-terminal region of TaGα-7A physically associates with TaDEP1. (a) LCI assays showing that TaGα-7A but not TaGα-1B associates with TaDEP1 in N. benthamiana leaves. Empty vectors were used as negative controls. (b) qRT-PCR assay showing the expression levels of TaDEP1 and TaGα in the infiltrated N. benthamiana leaves (mean± SD, n = 5). Results were normalized to NbACT1. **, P < 0.01 (Student’s t test). (c) BiFC assay demonstrating the physical association between TaDEP1 and TaGα-7A in N. benthamiana. The YFP fluorescence signals were detected 48 h post infiltration (hpi). BF, bright field. Scale bars, 50 μm. (d) LCI assay showing that the C-terminal region of TaGα-7A is required for its association with TaDEP1. (e) qRT-PCR assay showing the expression levels of TaDEP1 and TaGα in the infiltrated N. benthamiana leaf areas shown in (d). The data were normalized to NbACT1. (Mean± SD, n = 5). (f) Schematic representation of the truncated TaDEP1 proteins used for the LCI assays. The N terminus of TaDEP1 (TaDEP1-NT) contains the 1-80 amino acids; the middle domain of TaDEP1 (TaDEP1-MD) contains the 81-180 amino acids; the C terminus of TaDEP1 (TaDEP1-CT) contains the 181-285 amino acids. GGL, G protein γ-like; vWFC, von Willebrand factor type C; TNFR/NGFR, tumor necrosis factor receptor/nerve growth factor receptor. (g) LCI assay showing that the NT and MD domains of TaDEP1 mediate its association with TaGα. Empty vectors were used as negative controls. Three biological replications were performed with similar results.

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hydrophilic loop of TaCOLD1 (residues 178–296) was cloned into BamHI/SalI digested pMAL-c2X to generate MBP-TaCOLD1 HL; the coding sequence of TaGα-7A was cloned into EcoRI/SalI digested pGEX4T-1 to generate GST-TaGα-7A.

The constructs for 35S:TaCOLD1-GFP and 35S:TaCOLD1HL-GFP were based on the gateway cloning technology (Invitrogen). The coding sequence of TaDEP1 and the central hydrophilic loop of TaCOLD1 (residues 178–296) were separately ligated into the entry vector pQBV3 and subsequently introduced into the destination vector pGWB5 (GFP-tagged) (Nakagawa et al., 2007).

All the primers used for the construction above are summarized in Table S2 and the constructs described above are summarized in Table S4.

RNA extraction and quantitative RT-PCR

Trizol reagent (Invitrogen) was used to extract the total RNA of WT KN199 or pUbi:mTaCOLD1 transgenic lines. About 2 μg of total RNA was applied to synthesize cDNA using the 5x All-In One RT MasterMix system (Applied Biological Materials) according to the manufacturer’s instructions. The cDNA was diluted in 1:5 ratios with distilled water, and 2 μl of the diluted cDNA were used as template. SYBR® Premix Ex Taq Kit (TaKaRa, Japan) was used for quantitative RT-PCR assays in a total volume of 10 μl. Each experiment was repeated with three biological replicates, and each sample was analysed in triplicate. The expression levels of target genes were normalized to TaGAPDH or NbACTIN1 (NbACT1). All the primers used for qRT-PCR assays are listed in Table S3.

Subcellular localization analysis in bread wheat protoplasts

TaCOLD1-GFP and a plasma membrane marker, PIP2 (PLASMA MEMBRANE INTRINSIC PROTEIN 2; At3g53420)-mCherry, were cotransfected into bread wheat mesophyll protoplast cells by the PEG-mediated method as described previously (Yoo et al., 2007). After 20 h-incubation under dark at 22 °C, protoplasts were examined by a confocal microscopy (Carl Zeiss, LSM880).

Protein expression and in vitro pull-down assay

The MBP-TaCOLD1HL, GST-TaGα and MBP proteins were separately expressed in Escherichia coli strain BL21 by induction with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18 °C overnight, and extracted with the column buffer [20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and 1x protease inhibitor (Roche 4693132001)]. Then, the crude protein extracts of TaGα-7A were mixed with MBP-TaCOLD1HL or MBP in equal volume and pulled down using the amylose resin (New England Biolabs) at 4 °C overnight. The
amylose resin was washed with column buffer for five times and resuspended in SDS/PAGE loading buffer for immunoblotting assays using the anti-GST (Cat# CW0144, CWbiotech, Beijing, China) and anti-MBP (Cat# CW0288, CWbiotech, Beijing, China) antibodies.

**Protein extraction and immunoblotting assays**

Total proteins of different infiltrated samples were extracted using the extracted buffer (125 mM Tris-HCl at pH 6.8, 4% SDS, 0.001% Bromophenol blue, 20% glycerol, 2% β-Mercaptoethanol). For the immunoblotting detection of TaCOLD1α-GFP/mTaCOLD1β-GFP and GFP-MYC fusion proteins, anti-GFP (1:2000; Roche, 11814460001) and anti-mouse IgG (1:75000, Sigma, A9044-2ML) antibodies were used. ACTIN was employed as the loading control with anti-ACTIN (1:5000; CWBIO, CW0264) antibodies.

**Firefly LUC assay**

The firefly LUC transient expression assay was performed as previously described (Sun et al., 2013). In brief, *Agrobacterium* strain GV3101 carrying the nLUC and cLUC derivative binary plasmids were coinfiltrated in 4-week-old *N. benthamiana* leaves. The corresponding empty vectors plasmids were coinfiltrated in 4-week-old *N. benthamiana* leaves. The empty vector was used as control. LUC activities were measured 48 h after infiltration with the NightSHADE LB 985 (Berthold).

**Bimolecular fluorescence complementation (BiFC) assay**

BiFC assay was performed as described previously (Liu et al., 2017). Briefly, *Agrobacteria* harbouring the nYFP and cYFP derivative constructs was used together with the *Agrobacteria* p19 strain for infiltration in *N. benthamiana* leaves. The YFP signal was observed by a confocal microscopy (Carl Zeiss, LSM880) after incubation at 22 °C for 48 h.

**Phylogenetic analysis**

The homologs of TaCOLD1, TaGα and TaDEP1 were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) under the GenBank database (http://www.ncbi.nlm.nih.gov/) under the accession numbers: Rht-B1b, MG681100; TaCOLD1-2A, MG748865; TaCOLD1-2B, MG748866; TaCOLD1-2D, MG748867; TaGα-7A, MG748862; TaGα-1B, MG748863; TaGα-7D, MG748864; TaDEP1, MG758053.

**Accession Numbers**

Sequence data from this study can be found in the GenBank database (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: Rht-B1b, MG681100; TaCOLD1-2A, MG748865; TaCOLD1-2B, MG748866; TaCOLD1-2D, MG748867; TaGα-7A, MG748862; TaGα-1B, MG748863; TaGα-7D, MG748864; TaDEP1, MG758053.

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**Author contributions**

J.S. conceived the original screening and research plans; H.D., S.Y., J.L. and P.L. performed the experiments; H.D. and J.S. wrote the article.

**Conflict of interest**

The authors have declared that no competing interests exist.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 The coding sequences of TaCOLD1-2A/2B/2D genes from bread wheat cultivar KN199.

Figure S2 Topology prediction for TaCOLD1-2A/2B proteins using a transmembrane domain hidden Markov model (TMHMM version 2.0).

Figure S3 The coding sequence of Rht-B1b gene from bread wheat cultivar KN199.

Figure S4 Phenotypes of WT KN199 and pUbi:mTaCOLD1 transgenic wheat lines grown in the field at vegetative stage.

Figure S5 The coding sequences of TaGα-7A/1B/7D genes from bread wheat cultivar KN199.

Figure S6 The coding sequence of TaDEP1 gene from bread wheat cultivar KN199.

Figure S7 Sequence alignment of DEP1 homologs.

Figure S8 Phylogenetic tree of DEP1 homologs.

Figure S9 The mTaCOLD1 (M187K) protein interferes with the physical association between TaGα-7A and TaDEP1.

Table S1 Conserved amino acid sequences of COLD1 homologs in rice, diploid and tetraploid wheat.

Table S2 Primers used in this study.

Table S3 Primers used for qRT-PCR in this study.

Table S4 Constructs used in this study.