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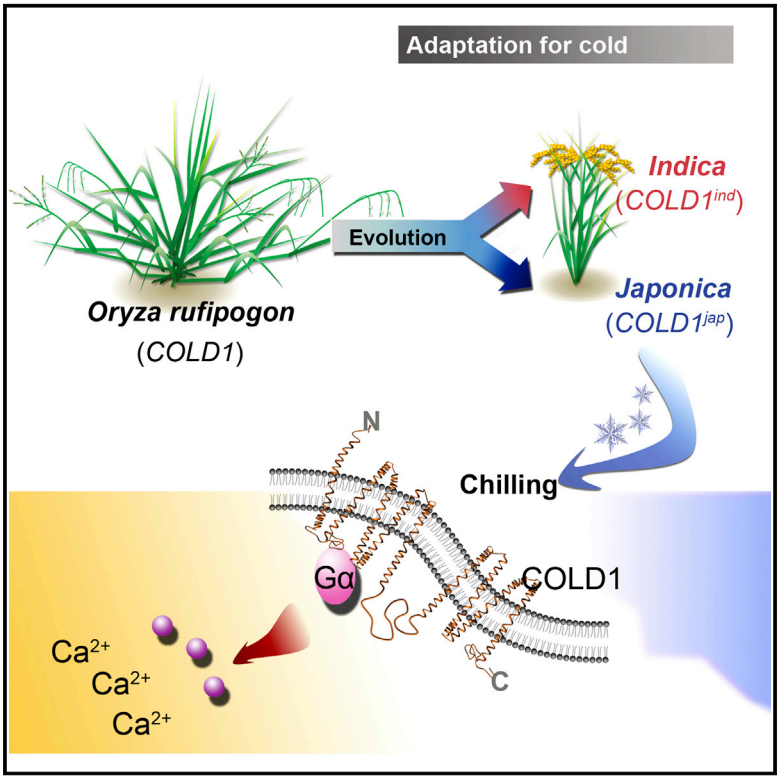
COLD1 Confers Chilling Tolerance in Rice

Graphical Abstract



Highlights

- QTL *COLD1* regulates G-protein signaling to confer chilling tolerance in rice
- SNP2 in *COLD1*$^{jap/ind}$ enhances its ability to activate G-protein α GTPase
- COLD1 interacts with G protein to activate the Ca^{2+} channel for temperature sensing
- The SNP2-containing allele is selected during *japonica* rice domestication

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In Brief

COLD1 regulates G-protein signaling to confer chilling tolerance in rice, and a SNP in *COLD1* underlies the adaptation to cold environment in *japonica* rice.

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Cell

COLD1 Confers Chilling Tolerance in Rice

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SUMMARY

Rice is sensitive to cold and can be grown only in certain climate zones. Human selection of *japonica* rice has extended its growth zone to regions with lower temperature, while the molecular basis of this adaptation remains unknown. Here, we identify the quantitative trait locus *COLD1* that confers chilling tolerance in *japonica* rice. Overexpression of *COLD1*^{jap} significantly enhances chilling tolerance, whereas rice lines with deficiency or downregulation of *COLD1*^{jap} are sensitive to cold. *COLD1* encodes a regulator of G-protein signaling that localizes on plasma membrane and endoplasmic reticulum (ER). It interacts with the G-protein α subunit to activate the Ca^{2+} channel for sensing low temperature and to accelerate G-protein GTPase activity. We further identify that a SNP in *COLD1*, SNP2, originated from Chinese *Oryza rufipogon*, is responsible for the ability of COLD$^{jap/ind}$ to confer chilling tolerance, supporting the importance of COLD1 in plant adaptation.

INTRODUCTION

Rice, which is both a model plant and one that feeds more than half of the world's population (Sasaki and Burr, 2000), evolved in tropical and subtropical areas and is sensitive to chilling stress (Kovach et al., 2007; Saito et al., 2001; Sang and Ge, 2007). Extreme temperature thus represents a key factor limiting global rice plant distribution. Super hybrid rice cultivars produce high yields in tropical or subtropical climates but are frequently harmed by chilling. Therefore, molecular genetic tools have been urgently sought to improve rice chilling tolerance in order to maintain rice production in current regions and expand it into northern areas with lower yearly temperatures.

Asian cultivated rice (*Oryza sativa*) was domesticated from its wild relatives *Oryza nivara* and *O. rufipogon*. It consists of two major subspecies, *indica* (*O. sativa* ssp. *indica*) and *japonica* (*O. sativa* ssp. *japonica*) (Kovach et al., 2007; Sang and Ge, 2007). Typical *japonica* cultivars, called *temperate japonica*, are grown in regions with lower yearly temperatures and generally exhibit stronger chilling tolerance than do *indica* cultivars. By contrast, some *japonica* cultivars that moved southwest to southeast Asia became tropical ecotypes, referred to as *javanica* or *tropical japonica*. Divergence between *indica* and *japonica* was driven by divergent natural selection imposed by contrasting environmental temperatures (Kovach et al., 2007; Sang and Ge, 2007). During human selection, cultivated rice has undergone significant changes in agricultural traits, such as grain yield, as well as environmental tolerance (Huang et al., 2012; Xu et al., 2012). Several developmental trait-related genes, such as *SH4* and *PROG1*, with signatures of domestication in cultivated rice have been identified using genetic mapping for quantitative trait loci (QTLs) and genome-wide association studies (GWAS) (Huang et al., 2012; Xu et al., 2012). The QTLs responsible for chilling tolerance in rice were mapped, revealing that the corresponding genes affect either seed germination or male sterility (Saito et al., 2001, 2010; Fujino et al., 2008; Koseki et al., 2010), but less is known about the molecular basis of the divergence between the two subspecies in terms of adaptation to the environment and geographical distribution.

Plant cellular adaptations to temperature differences are dependent on specific molecular cellular pathways including Ca^{2+}-mediated signal transduction. Cyclic nucleotide-gated channels (CNGCs) are nonspecific cation channels; in *Arabidopsis*, CNGCs form a family with 20 members and contribute to Ca^{2+} fluxes in various stress responses (Finka et al., 2012; Steinhorst and Kudla, 2013; Swarbreck et al., 2013). In mammals, Ca^{2+} channels interact with heterotrimeric guanine nucleotide-binding protein (G protein) complexes to function in stress responses (Wang and Chong, 2010). The transition of the mammalian G-protein α subunit between an activated sate and an inactivated is regulated by G-protein-coupled receptors (GPCRs), which mediate exchange (GDP release and GTP binding), and by regulator of G-protein signaling (RGS), which promotes GTP hydrolysis. Unlike animal G proteins, plant

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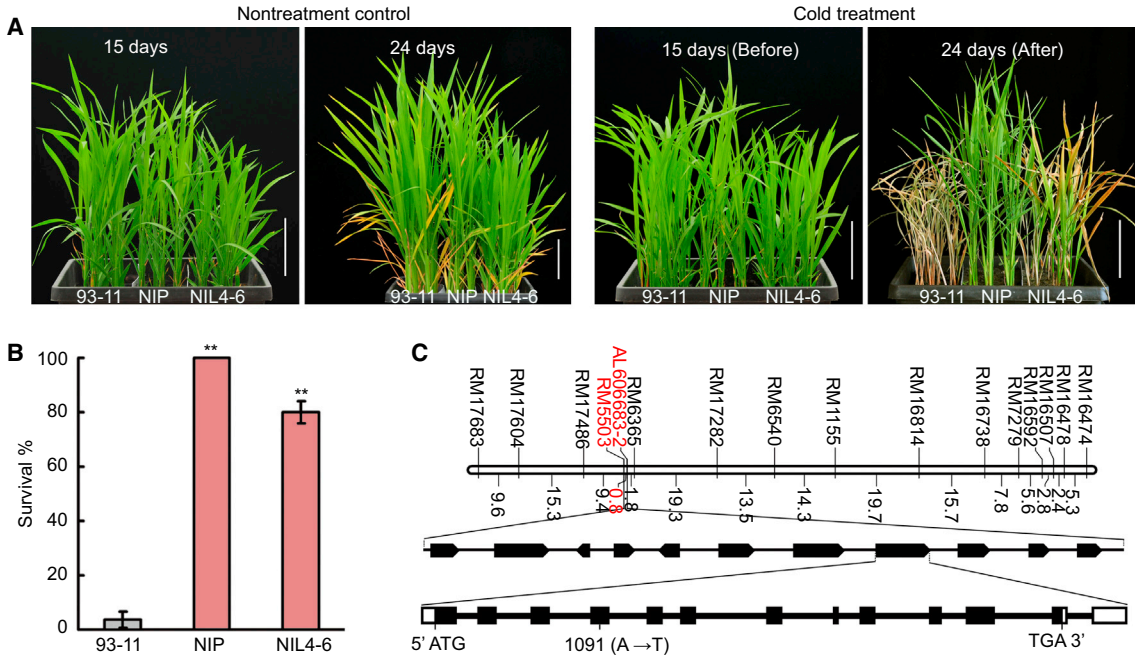


Figure 1. Map-Based Cloning of *COLD1*

(A) Phenotypic response to chilling in 93-11, Nipponbare (NIP), and the homozygote NIL4-6. Scale bars, 5 cm.

(B) The survival rate of 93-11, NIL4-6, and NIP after chilling treatment (96 hr). Values are expressed as mean ± SD, n = 3, **p < 0.01. See also Figure S1.

(C) The *COLD1* gene was mapped to the interval between the molecular markers AL606683-2 and RM5503 in chromosome 4. The gene was further delimited to a 77.33-kb genomic region on a BAC. Black arrows represent predicted genes. Black rectangles represent exons of *COLD1*.

See also Table S1 and Figure S1.

heterotrimeric G proteins are self-activating and do not utilize GPCRs in converting to the GTP-binding state (Urano et al., 2013). Instead, the RGS with activity of GTPase-accelerating protein (GAP) activity for GTP hydrolysis is more important for G-protein signaling in plant cells. In response to mild heating shock, Ca^{2+}-permeable channels mediate signals that lead to an influx of Ca^{2+} into plant cells (Saidi et al., 2009). Ca^{2+} signaling in plant cells also occurs during cold shock (Knight et al., 1996), although less is known about how the cold shock is linked to Ca^{2+} signaling. Overall, it is well established that Ca^{2+} signaling pathways and the resultant changes in gene transcription are involved in responses to altered temperature in plant cells (Dai et al., 2007; Lee et al., 2009; Ma et al., 2009). However, it is unknown how the signaling pathway in response to cold stimulation evolved during the divergence between rice subspecies *indica* and *japonica*.

Here, we provide evidence that a QTL gene, *CHILLING-TOLERANCE DIVERGENCE 1* (*COLD1*), is associated with divergence in chilling tolerance of rice cultivars. We further demonstrate that a single-nucleotide mutation at *COLD1* confers adaptation of *japonica* rice to chilling and originated from the Chinese wild populations of *O. rufipogon*. COLD1 localized at the plasma membrane, and endoplasmic reticulum (ER) is involved in sensing cold to trigger Ca^{2+} signaling for chilling tolerance. These findings reveal the importance of *COLD1* in plant adaptation and its great potential for rice molecular breeding.

RESULTS

COLD1 Confers Chilling Tolerance in Rice

Chilling tolerance of rice cultivars is regulated by QTLs derived from the subspecies *japonica* (Saito et al., 2001). To identify the genes involved in the increased chilling tolerance found in cultivars from growth regions with low yearly temperatures, we carried out a QTL analysis for *chilling-tolerance divergence* (*COLD*) in recombinant inbred lines (RILs) generated from a cross between chilling-tolerant Nipponbare (*japonica*) and chilling-sensitive 93-11 (*indica*) cultivars, testing for chilling sensitivity using the cold treatment (4°C) (Figure 1A). Using 151 RILs, we detected five QTLs, on chromosomes 1, 2, 4, 6, and 8 (Table S1). One of them, *COLD1*, was defined between markers RM6365 and RM5503 on the long arm of chromosome 4 (Figure 1C; Table S1). This locus explained 7.23% of the variance in chilling tolerance and shared the same locus with the QTL *Ctb2* despite slight differences in the crossed populations (Saito et al., 2001). The *COLD1* locus displayed much lower interaction with other QTLs for chilling tolerance (p = 0.0363, 0.0242) than did the other loci, such as *COLD4* (p = 0.0002) and *COLD5* (p = 0.0006) (Table S1).

To evaluate whether the Nipponbare (NIP) locus, $COLD1^{NIP}$, contributes to chilling tolerance, we generated three near-isogenic lines (NILs) containing the $COLD1^{NIP}$ locus in the 93-11 genetic background, which is one of the parental lines of the Chinese super hybrid rice. The homozygous $COLD1^{NIP/NIP}$ lines NIL4-1 and NIL4-6 showed remarkably higher tolerance

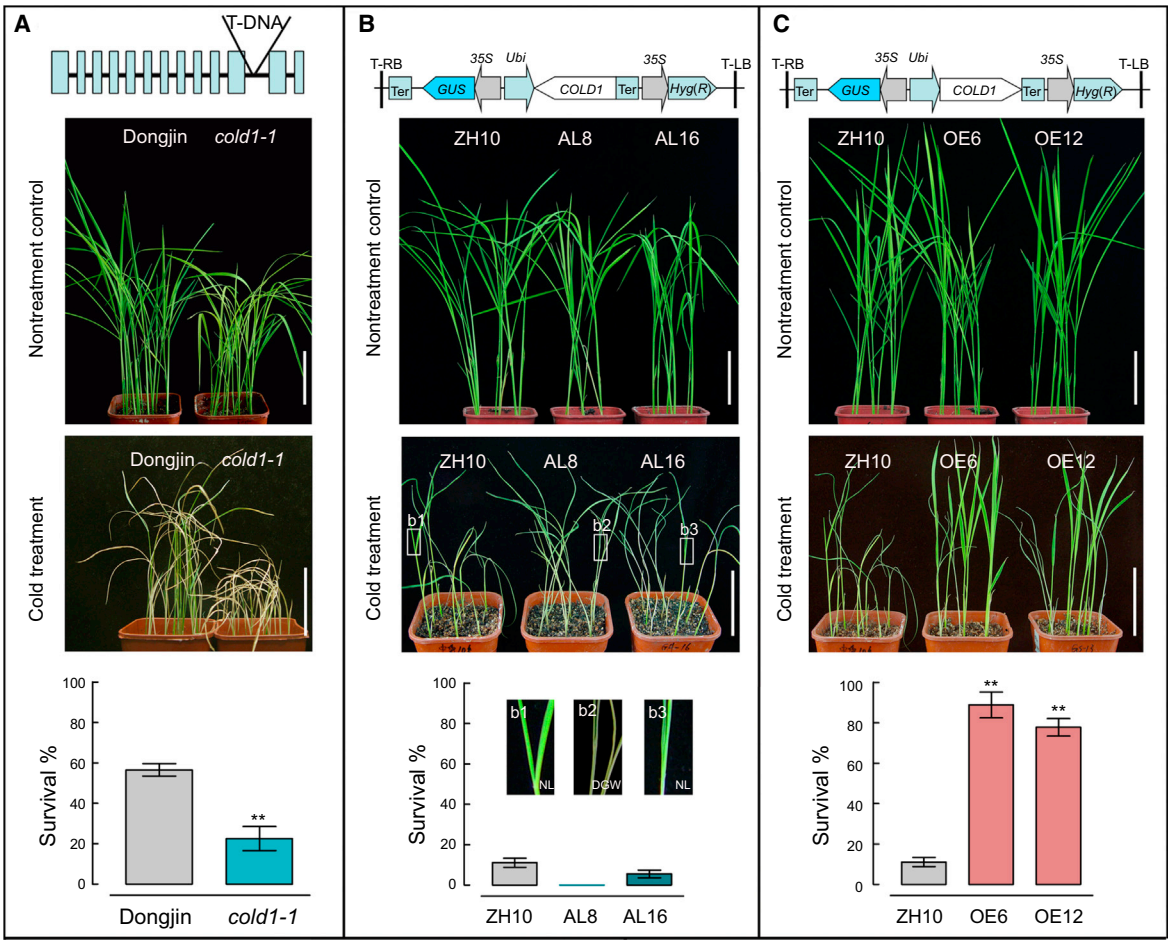


Figure 2. *COLD1* Is Essential for Chilling Tolerance

(A) The *cold1-1* mutant showed chilling sensitivity. The survival rate was determined after treatment at 4°C for 96 hr and subsequent recovery at 30°C for 7 days.

(B) The antisense transgenic rice lines (AL8 and AL16) showed chilling sensitivity. The survival rate was determined after treatment at 2°C–3°C for 96 hr and subsequent recovery at 30°C for 4 days. Panes are enlargements of plants showing live seedlings with new leaves (NL) and dead seedlings with dry green and white leaves (DGW).

(C) The overexpression transgenic lines (OE6 and OE12) showed chilling tolerance. The survival rate was determined after treatment at 2°C–3°C for 96 hr and subsequent recovery at 30°C for 4 days. The upper diagrams represent the T-DNA insertion or the transgenes used to generate the lines.

35S, CaV 35S promoter; Ubi, maize ubiquitin promoter; T-RB, T-DNA, right border; T-LB, T-DNA, left border; GUS, β-glucuronidase; Hyg (R), Hygromycin B resistance, Ter, terminator. Values are means ± SD, n = 3. Scale bars, 5 cm. **<0.01. See also Figures S2 and S3.

to chilling compared to 93-11 (Figures 1B and S1). A dominance assay on the heterozygote *COLD1*$^{NIP/93-11}$ NIL2-5 showed that its chilling tolerance was similar to that of NIL4-1 and NIL4-6 (Figure S1). To fine-map *COLD1*, we analyzed 8,368 F_2 plants generated from NIL2-5 and narrowed the candidate region to 77.33 kb between AL606683-2 and RM5503. This region contains 11 predicted genes or open reading frames (Figure 1C; Table S1). Genomic DNA sequence comparisons between the candidate regions of the parents NIP and 93-11 showed that one single-nucleotide mutation at 15th nucleotide in the fourth exon of *COLD1* (A in NIP was changed into T in 93-11) (LOC_Os04 g51180, MSU Rice Genome Annotation (Osa1) release 7. http://rice.plantbiology.msu.edu) caused a change in an encoded amino acid (Lys in NIP was changed into Met in 93-11) (Figure 1).

To determine whether the *COLD1* gene underlies the QTL, we constructed *COLD1*^{jap}-overexpression (OE) and antisense (AL) transgenic rice lines in *japonica* cultivar Zhonghua 10 (ZH10) (Figures 2 and S2), and examined their chilling tolerance. In addition, we analyzed the *cold1-1* mutant, which has a T-DNA insertion in the 11th intron of *COLD1*, +3,707 bp downstream from the ATG in the *japonica* rice Dongjin (DJ) background, and which lacks the full-length transcript (Figure S2). Seedlings were exposed to chilling temperature (4°C) and subsequently returned to 30°C. Rice plants with chilling tolerance were defined as those that could re-differentiate new leaves or continue growing leaves when returned normal conditions after treatment with chilling stress. Clear phenotypic differences in the survival rate (percentage alive seedlings of the total tested plants) were observed among these lines (Figures 2 and S2). Seedlings of the *cold1-1*

mutant, as well as of the antisense lines (AL5, AL6, AL8, and AL16) were chilling sensitive compared to the wild-type (WT). By contrast, $COLD1^{jap}$-overexpression lines, such as OE6, OE12, OE1, and OE2, showed higher chilling tolerance than WT. The findings suggest that *COLD1* modulates chilling tolerance in rice.

SNP2 Is Associated with Chilling Tolerance

To test for association between *COLD1* alleles and chilling tolerance, we examined the chilling tolerance of 5 *indica* and 20 *japonica* cultivars, as well as 2 accessions of wild rice (Table S2). All *japonica* cultivars and 2 *O. rufipogon* accessions showed stronger chilling tolerance than did all *indica* cultivars (Figure 3A; Table S2). We then sequenced the full-length *COLD1* gene of 4.78 kb including the 5′ and 3′ untranslated regions in these samples and identified seven SNPs (Figure 3A), including a synonymous polymorphism in the first exon (SNP1), a nonsynonymous polymorphism only in the fourth exon (SNP2), and five substitutions in introns (SNP3, 4, 5, 6, and 7). We grouped the cultivars based on chilling sensitivity and examined whether chilling tolerance was associated with allelic differences (SNPs) in *COLD1*. Strikingly, all accessions with confirmed chilling tolerance, including 20 *japonica* cultivars and 2 *O. rufipogon* accessions, differed from the *indica* cultivars that lacked the chilling tolerance by the SNP in the fourth exon (SNP2). The nucleotide polymorphism of T/C versus A in the fourth exon resulted in Met^{187}/Thr^{187} in *indica* compared to Lys^{187} in *japonica* cultivars. At the remaining SNP sites, polymorphic nucleotides were found in cultivars both with and without chilling tolerance (Figure 3A and Table S3).

To determine whether SNP2 led to alteration of chilling tolerance, we generated transgenic lines overexpressing the gene from *indica* plants ($SNP2^{ind(T)}$) in the *japonica* ZH11 background (Figures 3 and S3). The $COLD1^{ind}$ transgenic lines were more sensitive to chilling compared to ZH11. In addition, the transgenic lines of $COLD1^{ind}$ ($SNP2^{ind(T)}$) in the *cold1-1* mutant background showed a similar chilling tolerance as *cold1-1*, but significantly weaker tolerance than wild-type DJ. By contrast, the transgenic lines of $COLD1^{jap}$ ($SNP2^{jap(A)}$) in the *cold1-1* background showed similar tolerance as wild-type after cold treatment (Figures 3B and S3). Together with the enhanced chilling tolerance observed in the $COLD1^{jap}$ ($SNP2^{jap(A)}$) transgenic lines in wild-type background and that in the *cold1-1* background for the genetic complementation (Figure 2C), this suggests that SNP2, resulting in a change of encoded amino acid, is responsible for chilling tolerance in *japonica* rice.

SNP2 Arose during *japonica* Domestication

To examine the evolutionary origin of the alleles, we sequenced the full-length *COLD1* gene in an additional 100 accessions of cultivated and wild rice, including 36 *indica*, 15 *japonica*, and 15 *javanica* accessions, and 14 *O. nivara* and 19 *O. rufipogon* individuals as well as one *O. barthii* individual (Table S2). All *japonica* accessions, except for two samples displaying heterozygosity, had nucleotide A at the SNP2 site, whereas the *indica* accessions had either T or C, and *javanica* had A or T or C at this site. The five *O. rufipogon* samples originated from China had A at this site, and one *O. rufipogon* sample from Hainan province in China had W, whereas the remaining wild rice samples including 15 *O. rufipogon* samples from outside of China, 14 *O. nivara* samples and one *O. barthii* sample had either T or C (Table S2).

Geographically, 33 *japonica* cultivars, one *javanica* and the Chinese *O. rufipogon* samples with A at SNP2 were distributed in the northern area of China, Japan, Korea, and the United States, or at higher elevations of the southeast zone of Asia (Figure 3C). By contrast, all samples without A at SNP2, including 41 *indica* and 15 *O. rufipogon* samples from outside of China, were distributed in southern and southeastern Asia, regions with higher yearly temperatures. For *javanica*, 14 samples with nucleotide diversities at the site were distributed in regions of higher yearly temperature, such as southern area of China and the Philippines. Phylogenetic analysis of the *COLD1* sequences of the 72 accessions sampled (Table S2) indicated that all *japonica* accessions and the Chinese *O. rufipogon* samples carrying the chilling-tolerance $SNP2^{A}$ were grouped together with 60% bootstrap support (Figure 3D). These observations indicate that the *COLD1* allele with the mutation at $SNP2^{A}$ is likely to have originated from Chinese *O. rufipogon* during *japonica* rice domestication.

To examine whether selection has acted on *COLD1*, we analyzed nucleotide diversity across the sequenced region in 72 accessions (Table S2), including the original 27 accessions tested for chilling tolerance. A comparison of the nucleotide diversity among *indica*, *japonica*, *javanica*, *O. nivara*, and *O. rufipogon* indicated that on average, *japonica* exhibited much lower diversity (θ = 0.0004; π = 0.0002) than *indica* (θ = 0.0014; π = 0.0013), *javanica* (θ = 0.0025; π = 0.0017), and the two wild rice species (θ = 0.0014–0.0022; π = 0.0010–0.0020). Significantly negative Tajima's *D* values were observed only for *japonica* cultivars (Table S3), consistent with selection at the *COLD1* locus.

To determine further whether the reduction in nucleotide diversity in *japonica* rice could be caused by artificial selection, we conducted MLHKA tests on *COLD1* sequences for all six taxa (Table S3) in reference to seven neutral genes (Zhu et al., 2007). We found a significant value for *japonica* rice (p = 0.001), indicative of strong artificial selection on the *COLD1* locus during *japonica* domestication. To exclude the potential impact of demography on diversity reduction at *COLD1*, we further examined the nucleotide diversity for the ten genes within 400-kb region surrounding the *COLD1* locus in 43 accessions (Tables S2 and S3) because selection might lead to a selective sweep in the flanking region of the selected genes (Asano et al., 2011). As expected, we found that the average nucleotide diversity of the ten genes in *japonica* (π = 0.0003) was much lower than those of all other rice groups (π = 0.0027 for *indica*; π = 0.0020 for *javanica;* π = 0.0057 for wild rice) (Table S3), consistent with the selective sweep argument. A coalescent simulation using the ten surrounding genes revealed a significant lower *K* value (the severity of the bottleneck) in *japonica* (*K* = *0.06*) than that of neutral genes (*K* = 0.2) (p = 0.0097) (Table S3) (Zhu et al., 2007), indicating that the reduced diversity at the genes surrounding *COLD1* in *japonica* cannot be explained by a domestication bottleneck alone. Taken together, our data

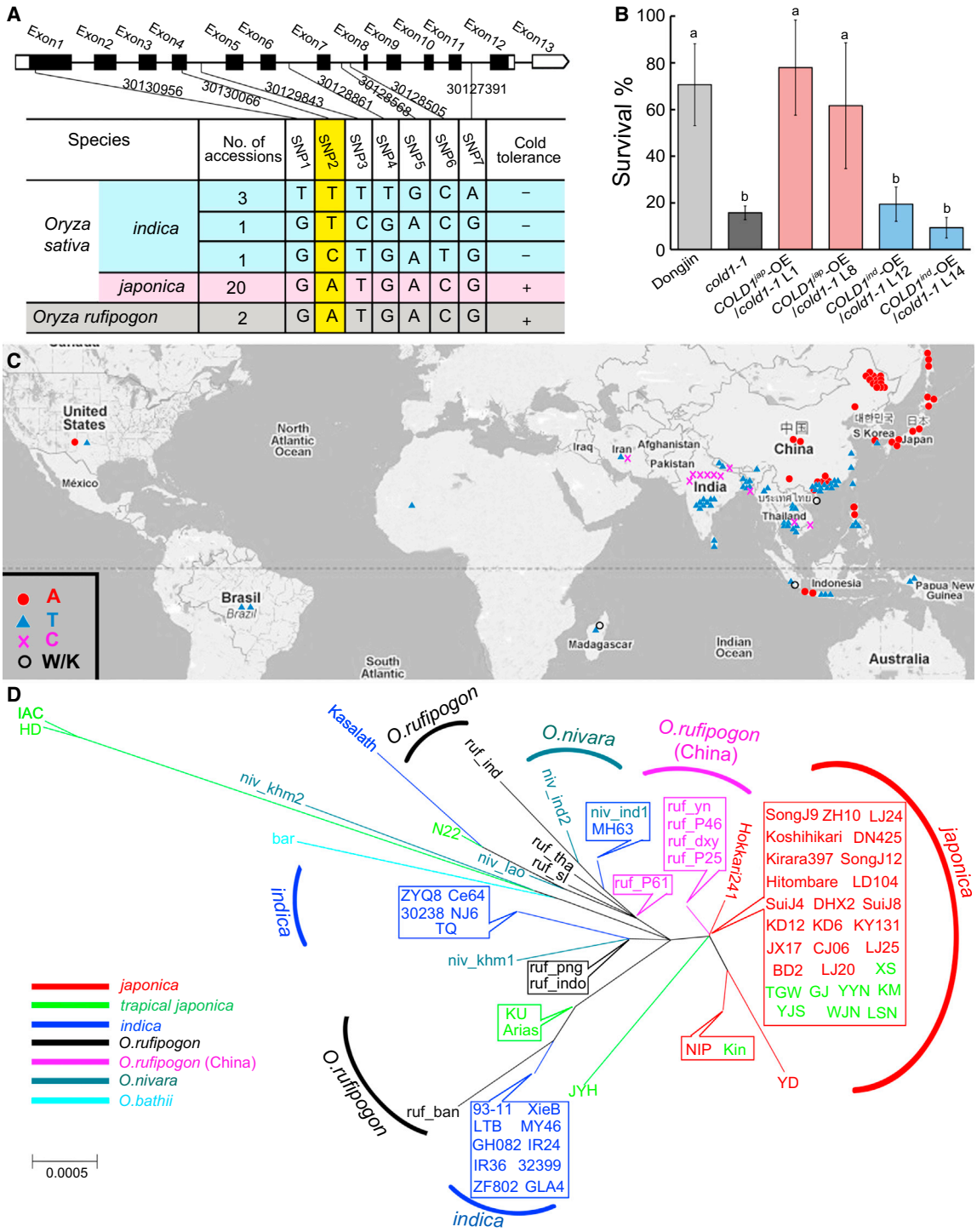


Figure 3. Association of SNPs in *COLD1* with Chilling Tolerance and Their Geographic and Phylogenetic Origins

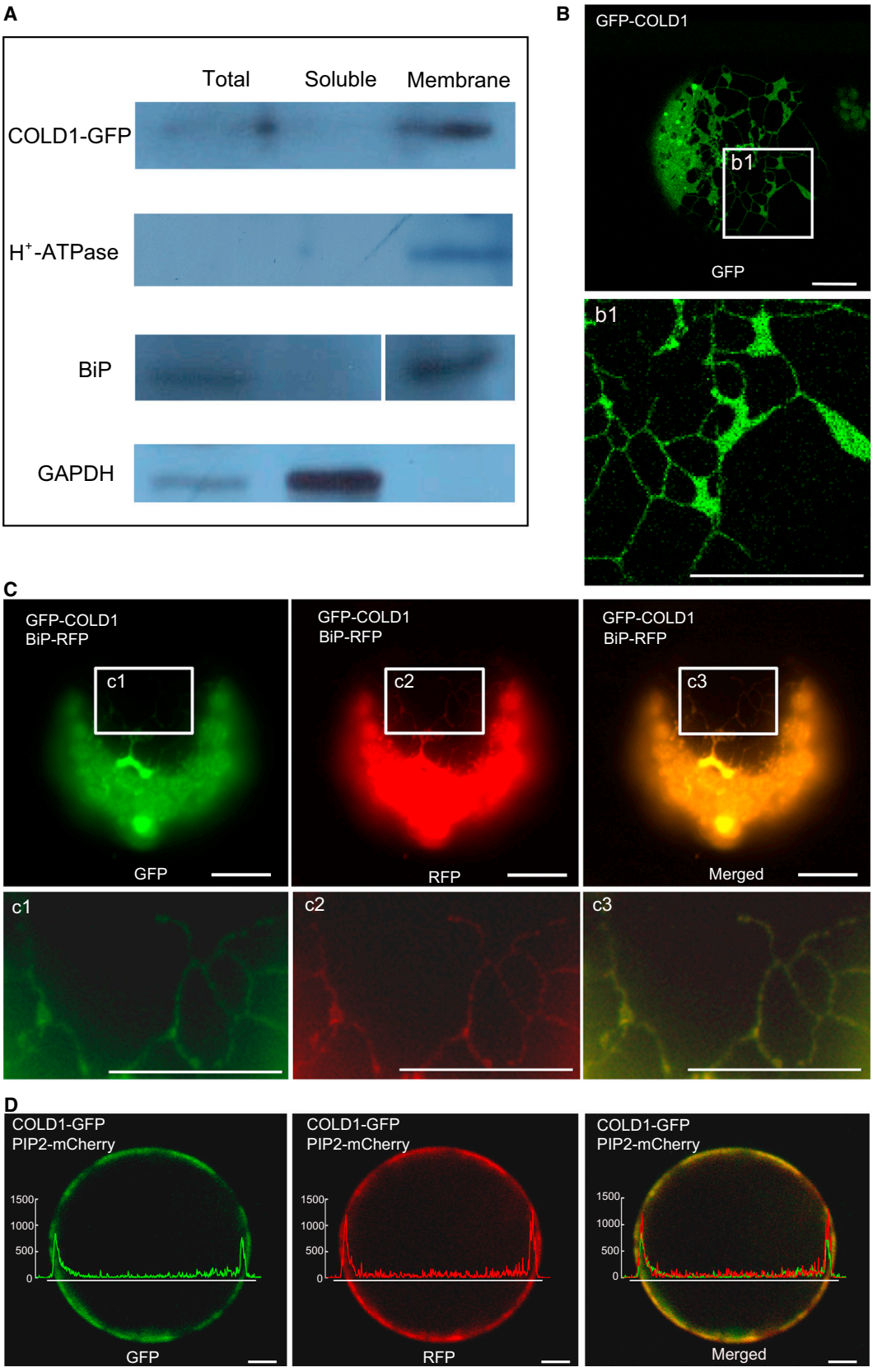
(A) SNPs and chilling tolerance in 27 accessions.

(B) Chilling tolerance response of *COLD1* complementation lines in the *cold1-1* genetic background. Values are expressed as means ± SD, n = 3. Statistically different values ($p < 0.05$) are indicated by different letters.

(C) Geographic distribution of 127 accessions tested (Table S2). The *japonica* and *O. rufipogon* samples carrying A at the SNP2 site are represented by red circles. The *indica* cultivars with T/C are denoted by blue triangles/purple crosses, respectively. The heterozygous cultivars [W (A or T)/K (G or T)] are represented by black rings.

(D) Neighbor-joining tree. Bootstrap values over 60% are given on the branches.

See also Tables S2 and S3.



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show that the A at the functional SNP2 of *COLD1* is associated with the development of chilling tolerance in cultivated rice and might represent an ancient allele preserved in the Chinese populations of *O. rufipogon* and selected during domestication of *japonica* rice.

COLD1 Localizes to the ER and Plasma Membrane

COLD1 was predicted to encode a 53-kDa protein with nine transmembrane domains. As expected, it was grouped with its orthologs from the monocotyledons in a phylogenetic tree (Figure S4). Immunoblotting assays on tissues expressing a *COLD1-GFP* fusion transgene showed signal from an anti-GFP antibody only in the membrane protein fraction, similar to the control membrane proteins H^+-ATPase and BiP, a marker of the endoplasmic reticulum (ER). No signal for COLD1-GFP was found in the soluble fractions, although the soluble control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein did show a signal (Figure 4A). Under microscopy, fluorescence of GFP-COLD1 overlapped with that of BiP-RFP at the ER (Figures 4B, 4C, and S4D) and with that of PIP2-mCherry, a marker for the plasma membrane and ER (Lee et al., 2009), at the plasma membrane (Figure S4E). Similarly, the signal of COLD1-GFP co-localized with that of PIP2-mCherry at ER with a reticular pattern and at the plasma membrane (Figures S4F, S4G, and 4D). The plasma membrane localization was independent on the myristoylation of G2 in the *N*-terminal motif M1-G2-W3 of COLD1 (Figures S4H and S4I) (Batistic et al., 2008; Yamauchi et al., 2010). These results suggest that COLD1 is mainly localized to the ER and plasma membrane.

COLD1 Interacts with G-Protein α Subunit

Based on hidden Markov model (Krogh et al., 2001) predictions, COLD1 contains nine transmembrane domains with a preferred orientation of an extracellular N terminus and an intracellular terminus, similar to the pattern of its *Arabidopsis* orthologs (Figures S4 and S5), GTG1/2, which interact with G-protein α subunit. We confirmed the interaction between COLD1 and the rice G-protein α subunit 1 (RGA1) (Ludewig et al., 2003; Stagljar et al., 1998) in vitro and in vivo. Yeast cells co-transformed either with $COLD1^{jap}$ or $COLD1^{ind}$ or $COLD1^{\Delta jap}$ and *RGA1* grew well on medium lacking His and Ade and showed X-gal staining, in contrast to the negative controls (Figure S5). In co-immunoprecipitation (Co-IP) assays, GFP-COLD1 was detected in complexes immunoprecipitated with the anti-FLAG antibody from leaves of transgenic plants expressing GFP-COLD1 and FLAG-RGA1 (Figure 5A). Bimolecular fluorescence complementation (BiFC) assays revealed reconstituted YFP fluorescence in the plasma membrane of transgenic lines harboring COLD1-YFP^C and RGA1-YFP^N (Figure 5B). By contrast, no fluorescence was detected in the negative controls OsBAK1-YFP^C and RGA1-YFP^N. These data demonstrate that COLD1 can physically interact with RGA1 in plant cells.

COLD1 Functions as a GTPase-Accelerating Factor on RGA1

Biochemical activity assays confirmed that RGA1 instead of COLD1 alone had GTPase activity, dependent on Mg^{2+} concentration in the reaction (Figures 5C, 5D, and S5D). RGA1 GTPase activity was accelerated in the presence of $COLD1^{jap}$ ($SNP^{jap(A)}$). By contrast, $COLD1^{ind}$ ($SNP^{ind(A)}$), as well as the truncated protein $COLD1^{\Delta jap}$ from *cold1-1*, suppressed RGA1 GTPase activity over the course of the assay (Figure 5C). The $COLD1^{jap}$-induced acceleration of RGA1 GTPase activity was impaired by inclusion of $COLD1^{ind}$ in the reaction (Figure 5D), which may explain the tolerance differences between $COLD1^{ind}$ and $COLD1^{jap}$ transgenic lines on the *japonica* background, as well decreased tolerance of *cold1-1* (Figure S3). A time-course assay for the tolerance showed that the *RGA1* mutant *d1* was significantly more sensitive to chilling for survival compared with wild-type Shiokari (Figure 5E). This is consistent with that the COLD1 and RGA1 complex is required for the tolerance.

We used an electrode voltage clamp approach to record the currents of oocytes co-expressing *COLD1* and *RGA1* (Figure 5F). Upon cold treatment, an inward current was significantly activated in the cells co-expressing $COLD1^{jap}$ and *RGA1* compared with expression alone, which was in contrast to their patterns showing no response to heat stimulation (40°C) (Figure S5) (Finka et al., 2012). The cold-activated response lagged by several seconds and returned rapidly to baseline levels after removal of cold stimulation. The cold-stimulated inward current was 588 ± 90 nA. By contrast, control cells and oocytes co-expressing $COLD1^{ind}$ and *RGA1* generated background currents of 373 ± 36 and 246 ± 41 nA, respectively. Co-expression of the truncated gene $COLD1^{\Delta jap}$ and *RGA1* led to a weaker inward current in response to cold stimulation than that of $COLD1^{jap}$. This suggests that the cold-stimulated inward current signal is dependent on interaction between COLD1 and RGA1 in the present of Ca^{2+}. Probably, a complex of COLD1 that has a GTPase-accelerating on RGA1 may affect influx of cations (such as Ca^{2+}) to cause changes of the membrane currents in oocyte cells. The *japonica* allele $COLD1^{jap}$ showed a stronger response with RGA1 on the cold-stimulated inward current signal than did the *indica* allele $COLD1^{ind}$.

Figure 4. COLD1 Localization

(A) Immunoblotting assay showing GFP antibody recognized GFP-tagged COLD1 in the membrane protein fraction from transgenic tobacco. H^+-ATPase, membrane protein control; BiP, ER marker control; GAPDH, glyceraldehydes- 3-phosphate hydrogenase soluble protein control.

(B) ER localization of COLD1 in *Arabidopsis* protoplast cells. The b1 images (lower) show enlargements of the regions framed in white (upper).

(C) Co-localization of COLD1 with ER marker. GFP-COLD1 signal was merged with that of the RFP-tagged BiP marker in *Arabidopsis* mesophyll protoplasts. The images with labels c1, c2, and c3 (lower) are enlargements of the regions framed in white (upper). Scale bars, 10 μm.

(D) Plasma membrane localization of COLD1 in cells. COLD1-GFP signal was merged with that of the PIP2-mCherry (an intrinsic plasma membrane protein) marker in Tobacco mesophyll protoplasts. The fluorescence intensity was scanned with the ImageJ plot profile tool (ImageJ v.1.47; http://rsbweb.nih.gov/ij/download.html). y axes are relative pixel intensity. Scale bar, 10 μm.

All experiments were performed with at least three biological replicates. See also Figure S4.

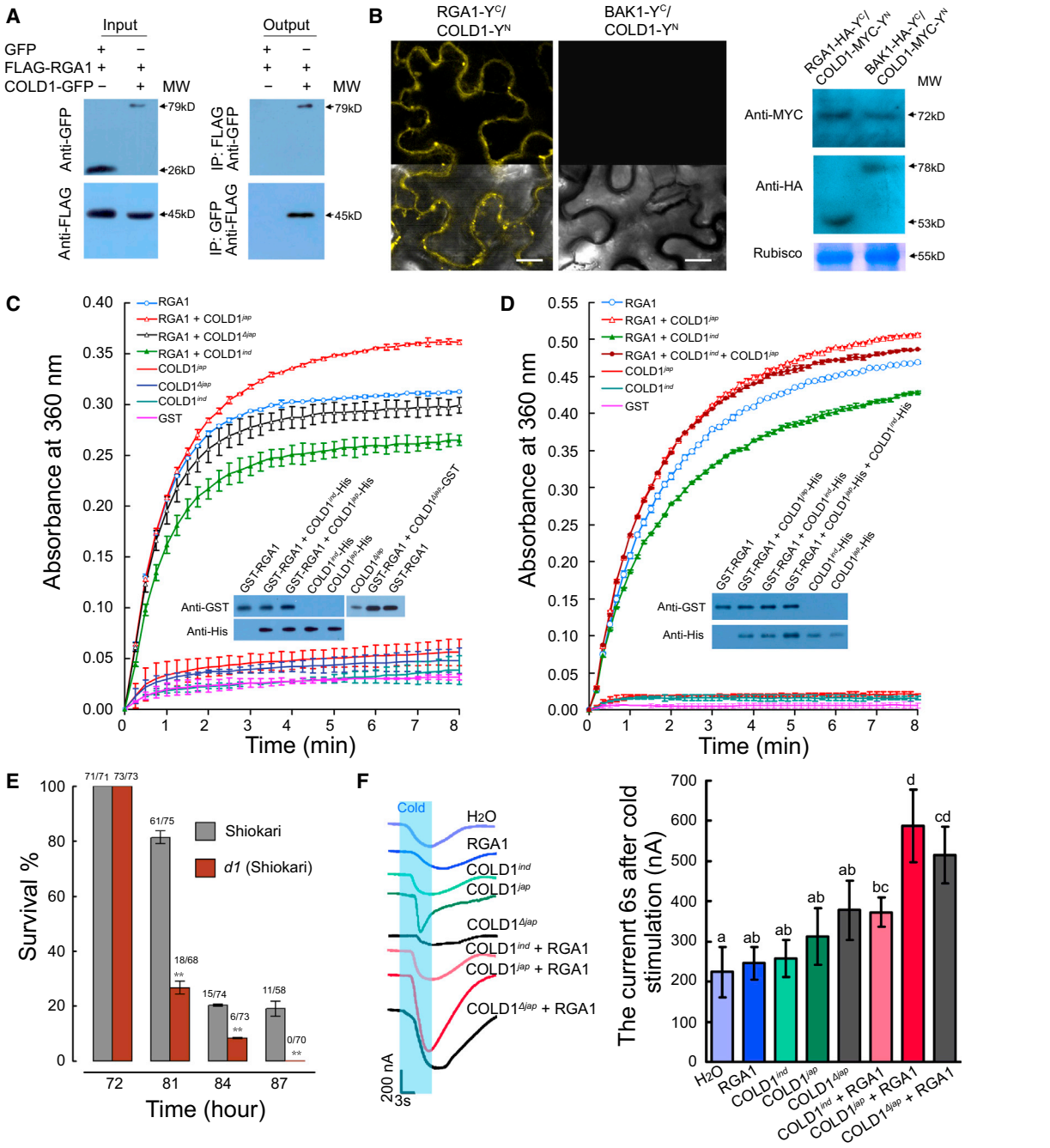

Figure 5. COLD1 Interacts with RGA1

(A) Co-immunoprecipitation assays confirming the interaction between COLD1 and RGA1. Co-expressed FLAG-RGA1 and COLD1-GFP in tobacco leaves were immunoprecipitated by anti-FLAG or -GFP. Blots were probed with by anti-GFP or -FLAG.

(B) BiFC assays showing that the proteins interact in vivo. The bottom ones are the merged images. Immunoblots (right) confirmed the expression of the interaction proteins in the transgenic leaf tissues used in the BiFC assay. Y^N, YN173; Y^C, YCM. Scale bars, 20 μm.

(C) Intrinsic GTPase activity of RGA1 was accelerated by COLD1^{jap} but impaired by COLD1^{ind} or COLD1^{Δjap}. The molar ratio of RGA1/COLD1 was 4.8. Values are expressed as mean ± SD, n = 3. The immunoblots show amount of proteins in the reaction.

(D) Acceleration of RGA1 GTPase activity by COLD1^{jap} was inhibited by addition of COLD1^{ind} in vitro. The molar ratio of RGA1/COLD1 was 4.8. Values are expressed as mean ± SD, n = 3. The immunoblots show amount of proteins in the reaction.

(E) Time course of chilling tolerance showing that the *d1* mutant is sensitive to cold treatment. The numbers above the bars are alive and total plants. Values are expressed as mean ± SD, n = 3; **p < 0.01.

(F) Electrophysiological characterization of *Xenopus* oocytes co-expressing COLD1 and RGA1, as well as the control RGA1 only. The blue background represents a duration for cold treatment in solution. The holding potential was −110 mV.

Values are expressed as means ± SD, n = 7. Statistically different values (p < 0.05) are indicated by different letters. See also Figure S5.

(legend continued on next page)

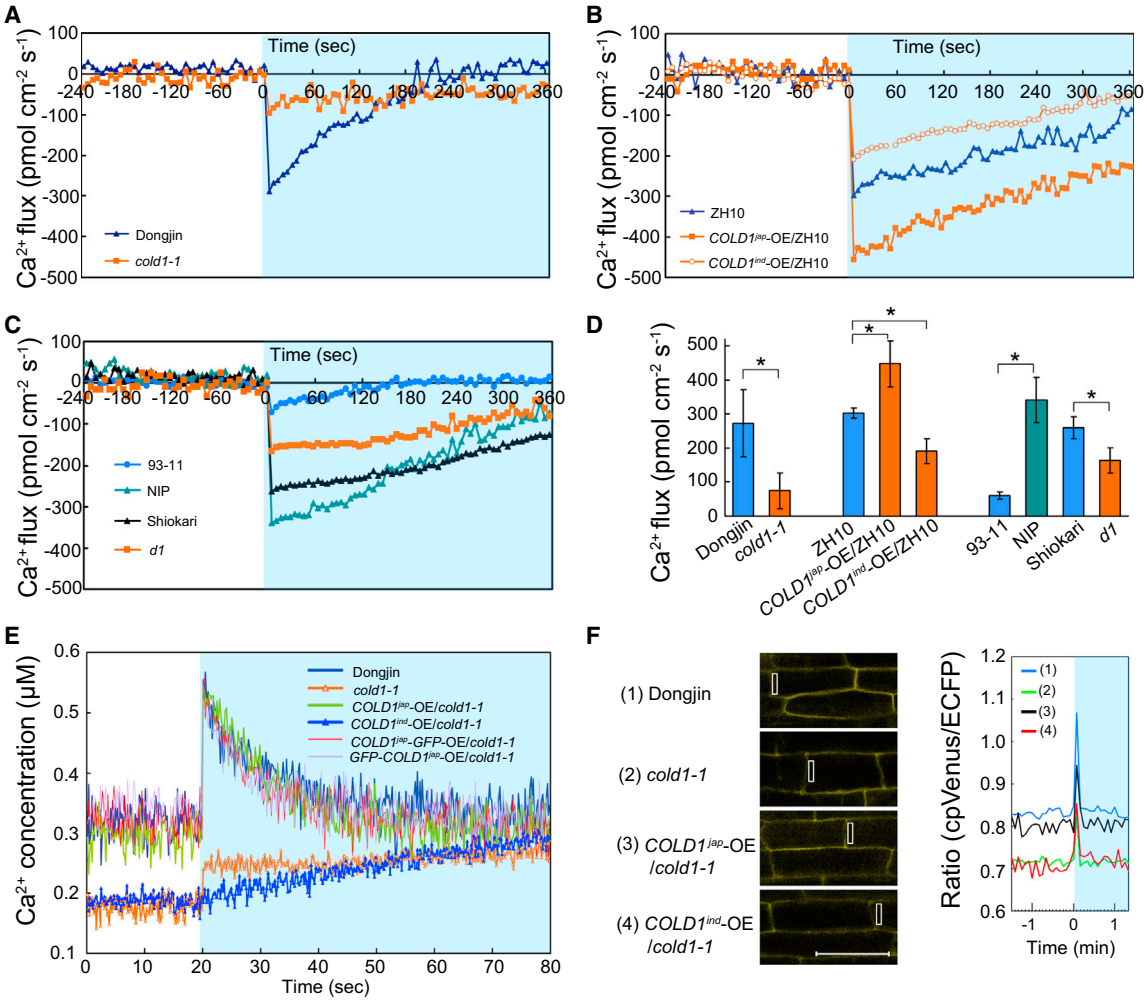

Figure 6. Ca^{2+} Signaling upon Cold Shock in Rice Plants

(A–C) SIET measurements show extracellular Ca^{2+} influx upon on cold shock in live roots of various genetic backgrounds (n > 6).

(D) Significance testing of the mean maximal Ca^{2+} influxes. Values are expressed as mean ± SD, n > 6, Student's t test, *p < 0.05.

(E) $[Ca^{2+}]_{cyt}$ monitored with aequorin in response to cold shock in wild-type Dongjin and the *cold1-1* mutant (n > 6).

(F) Cold response of $[Ca^{2+}]_{cyt}$ in live root cells using Yellow Cameleon (NES-YC3.6). Scale bars, 50 μm. The rectangles represent regions of interest (ROIs) considered for ratiometric measurements. The numbers used for ratiometric measurements are indicated in the boxes. The experiments were replicated at least three times. The blue background represents a duration for cold treatment.

See also Figure S6.

COLD1 Is Essential for Changes in Ca^{2+} Influx upon Cold Treatment

To examine Ca^{2+} flux in response to cold shock, we used the scanning ion-selective electrode technique (SIET) on rice roots (Ludewig et al., 2003). Upon cold stimulation, there was a significant influx of extracellular Ca^{2+} with a minus peak in wild-type Dongjin roots (Figures 6A and S6). By contrast, *cold1-1* showed no remarkable changes in SIET signals under the same conditions. Compared with wild-type ZH10, the *COLD1^{jap}* transgenic line exhibited more Ca^{2+} influx in response to cold treatment, but the *COLD1^{ind}* transgenic line displayed less (Figure 6B). Nipponbare, *japonica* rice, showed a stronger response than did *indica* 93-11 (Figure 6C). In addition, the *d1* mutant of *RGA1* showed less Ca^{2+} influx than did wild-type Shiokari. The mean maximal influxes of cold shock between *cold1-1* or transgenic lines and wild-type were significantly different (Figure 6D). In response to salt stress, by contrast, the overlapped SIET patterns between *cold1-1* and DJ indicated that salt stimulation signaling may be independent to *COLD1* (Figure S6). The extracellular Ca^{2+} influx peaks in response to cold shock hint that the net cytoplasm $[Ca^{2+}]_{cyt}$ derived from bulk extracellular Ca^{2+} might be substantially increased.

We also monitored Ca^{2+} concentration in the cytoplasm ($[Ca^{2+}]_{cyt}$) using cytosolic aequorin. Immediately upon the onset of cold treatment, Dongjin showed a significant $[Ca^{2+}]_{cyt}$ peak up to 0.554 ± 0.013 μM from 0.319 ± 0.029 μM (n = 7), which then decreased (Figure 6E). By contrast, *cold1-1* showed a much smaller increase in $[Ca^{2+}]_{cyt}$ from 0.177 ± 0.014 to

0.240 ± 0.040 μM (n = 9) and subsequently maintained a nearly stable level under the same conditions (Figure 6E). With regard to calcium level, the cold shock pattern of $[Ca^{2+}]_{cyt}$ in the *COLD1*^{jap}-complemented lines (harboring either *COLD1*^{jap}-*GFP* or *GFP*-*COLD1*^{jap}) (0.545 ± 0.042 μM [n = 6]) nearly overlapped with that of wild-type, whereas the *COLD1*^{ind} transgenic line on *cold1-1* (0.186 ± 0.011 μM [n = 6]) showed similar pattern as *cold1-1* (Figure 6E).

We used the Cameleon technique to further confirm the genetic complementation effect on Ca^{2+} elevation (Krebs et al., 2012). The root cells of DJ showed a remarkable cytoplasm Ca^{2+} peak after cold treatment, while *cold1-1* had a weaker peak, as well as a relatively low basal level (Figure 6F). The complemented lines of *COLD1*^{jap} almost completely rescued the cold-stimulated Ca^{2+} elevation in the *cold1-1* background. It is also notable that the recovered Ca^{2+} patterns of *COLD1*^{jap} included the basal elevation compared to *cold1-1*. By contrast, overexpression *COLD1*^{ind} in *cold1-1* did not rescue Ca^{2+} response in either the peak or basal level. In addition, the trends on fluorescence dyeing data for $[Ca^{2+}]_{cyt}$ cold responses were in accord with these results (Figure S6).

In addition, the genetic complementation lines of *COLD1*^{jap} in *cold1-1* background showed more remarkable cold-induced expression patterns for the stress-specific downstream genes, such as *OsAP2*, *OsDREB1A*, *OsDREB1B*, and *OsDREB1C* than did the overexpression of *COLD1*^{ind} line (Figure S6). Thus, the findings on both the extracellular Ca^{2+} influx and the net cytoplasm $[Ca^{2+}]_{cyt}$ signaling are consistent with the idea that *COLD1* is essential for cold shock-dependent intracellular Ca^{2+} changes in rice.

DISCUSSION

In this work, we identified the QTL *COLD1*, which is required for chilling tolerance in *japonica* rice during the seedling stage. The *COLD1* locus enhanced chilling tolerance in near-isogenic lines NIL4-1 and NIL4-6 from the background cultivar *indica* 93-11 (Figure S1). It is worth noting that mature rice plants of both NILs with chilling tolerance displayed increased seed number per panicle and maintained grain yield per plant compared with 93-11, which is one of the desirable parental lines of the Chinese super hybrid rice. Thus, these NILs could potentially be used as parents of super hybrid rice, conferring chilling tolerance without negative effects on grain yield. This finding, along with the enhanced tolerance of the *COLD1*^{jap} overexpression lines, emphasizes the potential of either genetic or transgenic approaches to improve chilling tolerance for rice breeding.

Chilling tolerance, i.e., the capacity to reestablish differentiation and growth under normal conditions after cold exposure, is a complex trait in seedlings that is controlled by multiple QTLs. Most of the QTLs genetically interacted with each other, resulting in a higher genetic contribution to chilling tolerance in the population. For instance, the *COLD2* QTL interacted genetically either with *COLD4* or *COLD5* resulting in an overall contribution to chilling tolerance of more than 16.8% (Table S1). By contrast, *COLD1* did not genetically interact with other QTLs and already alone contributed 7.23% to overall chilling tolerance. Nucleotide diversity analysis suggested that there was strong artificial selection on the *COLD1* locus during *japonica* domestication (Tables S2 and S3).

COLD1's topology, localization and interaction with RGA1, as well as its regulatory effects on RGA1 GTPase activity, support the idea that COLD1 is a RGS with GTPase-accelerating activity, similar to AtRGS1 (Chen et al., 2003; Johnston et al., 2007; Shabala and Newman., 2000; Stagljar et al., 1998; Urano et al., 2012). The subcellular localization pattern of COLD1 on the ER and plasma membrane partially overlaps those of its *Arabidopsis* orthologs GTG1/2 (Johnston et al., 2007; Pandey et al., 2009), but COLD1 is different from those GTG1/2 in intrinsic GTPase activity (Jaffé et al., 2012; Pandey et al., 2009). COLD1 is predicted to contain a Ras GTPase-activating protein domain in the third cytoplasmic loop, and our biochemical data support this. Correspondingly, SNP2$^{jap(A)\ versus\ ind(T/C)}$ in fourth exon would cause an amino acid substitution in the third loop (Dong et al., 2007). Genetic complementation of *COLD1*^{jap} instead of *COLD1*^{ind} in *cold1-1* suggests that SNP2 functions in chilling tolerance (Figure 3B). The specific domain involved (i.e., the loop containing a predicted GTPase-activating protein domain) and its effects on GTPase activity, as well as Ca^{2+} signaling and electrophysiological response, are consistent with a COLD1 biochemical function associated with G-protein signaling. We found that the substitution of Met^{187}/Thr^{187} for Lys^{187} in *japonica* cultivars conferred stronger tolerance to chilling. Overexpression of *COLD1*^{jap} also conferred enhanced tolerance. By contrast, the *COLD1*^{ind} transgenic lines exhibited decreased tolerance, which could be explained by competition between COLD1^{ind} and COLD1^{jap} in interaction with RGA1 for regulation in $[Ca^{2+}]_{cyt}$ level and GTPase activity (Figures 5 and 6).

Our genetic and biochemical analyses of COLD1 revealed several similarities to mammalian cold receptors and plant heat sensors that lead us to hypothesize that COLD1 is involved in sensing cold. (1) COLD1 has broad tissue expression and is plasma- and ER-membrane localized, with nine predicted TM domains. (2) COLD1 acts as a RGS to accelerate RGA1's GTPase activity and has phenotypic effects on chilling tolerance. (3) Cold-induced changes in Ca^{2+} influx and $[Ca^{2+}]_{cyt}$ are mediated by COLD1. (4) Interaction between COLD1 and RGA1 is required for the cold-induced specific electrophysiological response. (5) Differences in chilling tolerance are observed in *cold1-1*, in transgenic lines harboring various alleles from *japonica* and *indica*, and in the *RGA1* mutant, *d1*.

Cold temperature may be sensed through direct alteration of a sensor's structure and membrane fluidity to trigger cations influx for signaling. Notably, changes on Ca^{2+} signal involve both the resting level in the cytoplasm and the temporal elevation. The *cold1-1* showed lower resting levels of Ca^{2+}, which was genetically rescued by *COLD1*^{jap} (Figure 6). This finding may hint that COLD1 itself possibly represents a potential calcium permeable channel or a subunit of such a channel. Consequently, changes of this channel function would affect resting $[Ca^{2+}]_{cyt}$, which would influence the amplitudes of Ca^{2+} signals. The potential function of COLD1 as a cold sensor could be simply explained by the lack of a significant Ca^{2+} gradient in *cold1-1* plants and *COLD1*^{ind}-OE lines in Ca^{2+} resting levels that does not allow the formation of an appropriate Ca^{2+} signal. Therefore, it is appealing to speculate that COLD1 is involved in sensing cold and that changes in

COLD1 protein structure and membrane fluidity in response to cold might initiate signaling through COLD1's physical interaction with RGA1, leading to Ca^{2+} influx into cytoplasm, which would then trigger downstream responses to chilling stress. Subsequently, accelerated GTPase activity of RGA1 by COLD1 might induce a regression shift on equilibrium between GDP- and GTP-bound states of RGA1 (Urano et al., 2012) (Figure S6).

The strong phenotype of plants with the *COLD1* QTL could result from tight functional interaction of COLD1 with important hormonal pathways. Consequently, an imbalance in COLD1 function likely affects multiple response pathways in this way aggravating the effects of its modulated temperature dependent functionality and thereby leading to significant decreased ability to re-assume growth after chilling stress. In this regard, COLD1 is functionally interconnected with the key gibberellin signaling component D1/RGA1 (Ueguchi-Tanaka et al., 2000) and brassinosteroid signaling, which are involved in regulation of plant height (Hu et al., 2013; Wang et al., 2006). Moreover, D1/RGA1 also affects TUD1, which mediates brassinosteroid signaling to regulate cell proliferation for plant growth and development (Hu et al., 2013; Wang et al., 2006). In addition D1/RGA1 is functionally dependent on SLR of GA signaling pathway for cell elongation (Ueguchi-Tanaka et al., 2000). In fact, our *cold1-1* significantly showed a decrease in plant height compared with wild-type, while plant height of the complemented lines of *cold1-1* with *COLD1* was recovered (Figure S3). Therefore, it is likely that COLD1 exhibits this strong impact on chilling tolerance via the RGA1 by disturbing multiple pathways, such as GA and/or BR signaling pathways (Hu et al., 2013; Wang et al., 2006).

We show here that a SNP of *COLD1* endows *japonica* rice with chilling tolerance, and that the mutation in the coding region of *COLD1* has been fixed in chilling-tolerant *japonica* cultivars. Our phylogenetic and population genetic analyses based on the large number of SNPs identified by resequencing 50 accessions of cultivated and wild rice (Huang et al., 2012; Xu et al., 2012) demonstrate that the chilling-tolerant allele originated from the Chinese *O. rufipogon* populations and was subject to strong human selection during *japonica* domestication, similar to the case of the *SD1* gene for *japonica* domestication (Asano et al., 2011). Therefore, genomic segments bearing agronomic traits can originate in one population and spread across all cultivars through artificial selection (He et al., 2011). Our findings are consistent with archaeological and genetic evidence that *japonica* rice was domesticated in China (Fuller et al., 2009; Huang et al., 2012; Londo et al., 2006; Xu et al., 2012). Importantly, our work demonstrates that the process of rice domestication was associated with fixation and extension of favored alleles or mutations that enhanced chilling tolerance for growth in regions with lower yearly temperatures. The *COLD1* allele and SNPs identified in this work have great potential for improving rice chilling tolerance via molecular breeding techniques.

EXPERIMENTAL PROCEDURES

Genetic Population and Plant Materials

Oryza sativa recombinant inbred lines (RIL) were developed by crossing *japonica* variety Nipponbare (NIP) and *indica* variety 93-11. The F_2 generation from NIP × 93-11 was subjected to more than six rounds of self-pollination to generate the RILs. For QTL genetic assay, the RILs were randomly selected. The near-isogenic lines were generated by backcrossing the NIP × 93-11 lines to 93-11 five times to generate BC_5F_2.

The T-DNA insertion mutant *cold1-1* was obtained from Dr G. An. *O. sativa* ssp. *japonica* cv. ZH10/11 and DJ were used for transformation to create the transgenic lines (Jeong et al., 2002). Mutant *cold1-1* was transformed with *COLD1* for a genetic complementation. The primers used for PCR are listed in Table S4.

Chilling Treatment

To test chilling tolerance, the seedlings were treated at 2°C–4°C for various times based on the genetic background. Subsequently, they were moved to a temperature-controlled greenhouse with 28°C–30°C/25°C day/night cycles for recovery. After 3–7 days, the survival rate was determined as the percentage of the total seedlings that were alive (Ma et al., 2009).

SNP Identification, Phylogenetic Analysis, Genetic Diversity, and Neutrality Tests

Full-length *COLD1* gene was sequenced using the tiling format. The primer sequences are listed in Table S4. The gene sequences from 127 samples were aligned using MEGA 5.0 software. A phylogenetic tree was constructed using the neighbor-joining method in MEGA5 (Tamura et al., 2011).

Estimates of nucleotide diversity and population genetic analyses were performed for each group using DnaSP 5.1 (Librado and Rozas, 2009). Tajima's D (Tajima, 1989) and maximum likelihood Hudson-Kreitman-Aguade (MLHKA) (Wright and Charlesworth, 2004) tests were used to examine the departure of *COLD1* polymorphisms from neutrality with a set of known neutral genes, namely, *Adh1*, *GBSSII*, *Ks1*, *Lhs1*, *Os0053*, *SSII1*, *and TFIIAγ-1* (Zhu et al., 2007), as controls. The genome-wide controls with 400-kb regions around *COLD1* in 43 accessions were used for interpret the Tajima's statistics. The coalescent simulation analysis was carried out according to Wu et al. (2013). Details are in Supplemental Information.

Subcellular Localization of COLD1

GFP was fused to COLD1 either at the N or C terminus. Its colocalization assays with marker proteins were carried out in protoplast (*Arabidopsis*, or Tobacco) cells as described previously (Lee et al., 2009). The transformed protoplast cells were examined by a confocal microscopy. See details in Supplemental Information.

Coimmunoprecipitation Assay

Briefly, the recombined plasmids were co-transformed into tobacco leaves according to Liu et al. (2007). The extracts were incubated with anti-FLAG M2 affinity gel (Sigma) or anti-GFP antibody at 4°C overnight. The antigen-antibody complex was collected. Then the sample was separated on SDS/PAGE gels for immunoblots. See details in Supplemental Information.

Bimolecular Fluorescence Complementation

BiFC experiments and gene transformation were performed as described previously (Stagljar et al., 1998; Waadt et al., 2008; Wang et al., 2009). The vectors were from Dr. J. Kudla. See details in Supplemental Information.

Expression and Purification in *Spodoptera frugiperda*

Protein expression and purification of COLD1 in the cells of *Spodoptera frugiperda* (Sf9) were performed as previously described (Wu et al., 2010). Affinity chromatography was used in protein purification. See details in Supplemental Information.

GTPase Activity Assay

The GTPase activity of RGA1 was monitored with the Enzcheck Phosphate Assay Kit as described previously (Dong et al., 2007). The amount of the tested protein (RGA1/COLD1 = 10/1 μg) was measured and confirmed in immunoblots using the FLAG antibodies. Amounts loaded were 1/0.1 μg (RGA1/COLD1) for the blot. Details are in Supplemental Information.

Electrophysiological Assay

For electrophysiological analysis, complementary RNA was prepared using the RNA Capping Kit (Stratagene). *Xenopus* oocytes were injected with

cRNA for *COLD1* and *RGA1*, mixed, and used for voltage-clamp experiments. Details are in Supplemental Information.

Extracellular Ca^{2+} Flux and $[Ca^{2+}]_{cyt}$ Monitoring

The roots of 3-day-old seedlings were used to monitor Ca^{2+} flux with scanning ion-selective electrode technique (SIET) (Ludewig et al., 2003). The solution of 25°C was replaced with that of 0°C for the cold treatment. $[Ca^{2+}]_{cyt}$ in callus was monitored by the cytosolic aequorin method (Saidi et al., 2009). The remaining aequorin was discharged by 1 M $CaCl_2$ and 10% ethanol. Calibration of cytosolic Ca^{2+} concentration was according to Knight et al. (1996).

For monitoring Ca^{2+} elevation using Yellow Cameleon (YC3.6), whole plants were infected rice (GV3101) containing NES-YC3.6. Roots were used to monitor $[Ca^{2+}]_{cyt}$ according to the method described by Krebs et al. (2012). Details are in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.01.046.

AUTHOR CONTRIBUTIONS

Y.M. performed experiments on phenotypic and biochemical assays. X.D. worked on the transgenic lines. Y.X. designed the experiments, analyzed data, and prepared the manuscript. W.L. performed genetic and evolution experiments. In Q.Q.'s lab, Q.Q. and D.Z. created the genetic population. In S.G.'s lab, S.G. and X.Z performed analysis of molecular evolution. In W.W.'s lab, W.W. and X.X. performed SNP analysis. In L.L.'s lab, L.L. and Y.P performed the electrical physiological assay. In J.J.'s lab, J.J and X.L. performed the protein localization. H.L., D.Z., J.X., X.G., and S.X. performed some experiments on calcium and localization. H.Z. joined in calcium analysis. Y.N performed gene transformation. K.C. designed all experiments, analyzed data, and wrote the manuscript.

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