New Phytologist

Research

Natural variation of *C-repeat-binding factor* (*CBFs*) genes is a major cause of divergence in freezing tolerance among a group of *Arabidopsis thaliana* populations along the Yangtze River in China

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Received: *8 March 2013*
Accepted: *18 April 2013*

New Phytologist (2013) **199:** 1069–1080
doi: 10.1111/nph.12335

Key words: *Arabidopsis thaliana*, *C-repeat-binding factor* (*CBF*), freezing tolerance, local adaptation, natural variation, Yangtze River.

Summary

- We used a monophyletic group of four natural populations of *Arabidopsis thaliana* expanded from a single ancestor along the Yangtze River *c*. 90 000 yr ago to study the molecular mechanism of the divergence in their freezing tolerance, in order to gain an insight into the genetic basis of their local adaption to low temperatures.
- Freezing tolerance assays, measurements of metabolites in the raffinose biosynthesis pathway and transactivation-activity assays of variation in forms of cold-responsive transcription factors were conducted on the four populations. Quantitative trait locus mapping was adopted with F_2 populations of the most- and least freezing-tolerant populations.
- The degree of freezing tolerance among the four populations was negatively correlated with the lowest monthly average temperature of January in their native habitats, and positively correlated to the expression level of some cold-regulated genes. We identified a major locus harboring three cold-responsive transcription factor genes *CBF1–3*, and found a nucleotide insertion in *CBF2* in all populations except SXcgx, which generated a dysfunctional CBF2 protein.
- The *CBF2* in SXcgx experienced a stronger natural selection in the cooler environment after *CBF3* lost its response to low temperature, which possibly reflects a local adaptation of these populations during the expansion from a common ancestor.

Introduction

Temperature is one of the major environmental factors affecting the geographic distribution of plants and agricultural range of crops. Low temperature is an abiotic stress to most plant species. In a long history of evolution, plants have evolved in various ways to adapt to low temperature. Studying the molecular mechanisms regulating the cold responses of plants will help us to understand plant adaptation to local environment at the molecular level, and eventually help for molecular marker-assisted crop breeding. *Arabidopsis thaliana* is found across a wide range of geographic regions with different climatic conditions (Koornneef *et al.*, 2004; Weigel, 2012). Such natural populations of *Arabidopsis thaliana* vary significantly in their cold responses (Hannah *et al.*, 2006; Hasdai *et al.*, 2006; Zhen & Ungerer, 2008a; Gery *et al.*, 2011; Zuther *et al.*, 2012), providing a suitable model for studying natural variation of adaptive traits (Alonso-Blanco *et al.*, 2009; Trontin *et al.*, 2011). Although there have been several reports demonstrating a highly significant correlation between freezing tolerance and the average minimum habitat temperature or the latitude of the origin of different accessions (Hannah *et al.*, 2006; Zhen & Ungerer, 2008a), a detailed dissection of the genetic, biochemical and molecular basis of the correlation in natural populations of a monophyletic origin has not been reported.

It is well established that cold acclimation, a complicated process involving many physiological and biochemical changes in plants after being exposed to low but nonfreezing temperatures, improves the freezing tolerance of many plant species (Levitt, 1980; Thomashow, 1999). Extensive studies on the mechanisms underlying cold acclimation have been conducted on *Arabidopsis thaliana* (Chinnusamy *et al.*, 2007; Zhu *et al.*, 2007; Guy *et al.*, 2008; Wang & Hua, 2009; Thomashow, 2010). Based on the sequence analysis on the promoters of the cold-induced genes, such as cold regulated (*COR*) genes, the C-repeat/dehydration-responsive elements (CRT/DRE) were identified (Gilmour & Thomashow, 1991), and a family of transcription factors

known as C-repeat-binding factor (CBF) (Stockinger *et al.*, 1997; Thomashow, 1999) or dehydration-responsive element-binding factor (DREB) (Liu *et al.*, 1998) was reported to work specifically in response of plants to low temperature (Shinwari *et al.*, 1998). *CBF1*, *CBF2* and *CBF3* (or *DREB1B*, *DREB1C* and *DREB1A*, respectively), which are rapidly induced by cold stress, are considered as the main components involved in cold/freezing tolerance of plants, by activating many downstream genes known as CBF regulon (Gilmour *et al.*, 2004; Vogel *et al.*, 2005). Several studies have also revealed the crosstalk between *CBF*s and plant circadian and phytohormones in the cold response (Fowler *et al.*, 2005; Achard *et al.*, 2008; Shi *et al.*, 2012). The CBF-mediated pathway is considered as one of the major signaling pathways involved in plant cold acclimation (Medina *et al.*, 2011).

In *A. thaliana*, *CBF1*, *CBF2* and *CBF3* share high sequence identity, and are linearly clustered within an 8.7-kb region in chromosome 4. It was reported that this region was subjected to different selective forces with different demographic histories (Alonso-Blanco *et al.*, 2005; Lin *et al.*, 2008; McKhann *et al.*, 2008; Zhen & Ungerer, 2008b). For example, a 1.6-kb deletion in the promoter region of *CBF2* in accession Cvi caused the loss of *CBF2* expression and thus reduction in freezing tolerance (Alonso-Blanco *et al.*, 2005). Relaxed selection on *CBFs* was reported to be associated with decreased freezing tolerance in natural populations of *A. thaliana* in southern Europe (Zhen & Ungerer, 2008b). Molecular and population genetic evidence showed that the major divergence of CBF proteins was the result of selection relaxation in two regions of the transcriptional activation domain which was under positive selection after duplication (Lin *et al.*, 2008). Therefore, it is most likely that the divergences of this small gene family among populations played an important role in the adaptive variation of low-temperature tolerance of *A. thaliana* in different geographic regions.

Metabolic changes occur during cold acclimation of plants, for instance, soluble carbohydrates such as raffinose family oligosaccharides (RFOs) accumulate (Kaplan *et al.*, 2004, 2007; Maruyama *et al.*, 2009). The expression of *AtGolS3* which encodes a galactinol synthase catalyzing the first committed step in the biosynthesis of RFOs was regulated by CBF3 at low temperature, resulting in an accumulation of a large amount of galactinol and raffinose which may function as cold protectants for plants (Taji *et al.*, 2002).

In those studies using worldwide populations of *A. thaliana*, the samples from East Asia, especially from China, were rarely included (Weigel, 2012). A survey on the genetic diversity of natural populations of *A. thaliana* in China using ISSR and RAPD markers revealed a significant correlation between geographic distance and genetic distance (He *et al.*, 2007). A study on the origin of *A. thaliana* in China using chloroplast DNA markers demonstrated that populations along the Yangtze River originated from a recent common ancestor, and went through a rapid demographic expansion *c.* 90 000 yr ago (Yin *et al.*, 2010). These populations are along the most southeastern range of the natural distribution of *A. thaliana*. An analysis on the gene expression profile of these Chinese populations at 4°C found that *CBF3* in four populations along the Yangtze River could not respond to low temperature due to a fragmental insertion at its promoter region (He *et al.*, 2008). In the present study, we try to address the following three questions. Has the freezing tolerance differentiated among the populations along the Yangtze River in *c.* 90 000 yr? What are the biochemical and molecular mechanisms behind the differences, if any? Would the differences have any adaptive significance? We analyzed four populations along the Yangtze River together with Col, and found that the freezing tolerance, gene expression and metabolite profiles were diverged among the populations under cold treatment. We identified one major quantitative trait locus (QTL) corresponding to the divergence of freezing tolerance, in which *CBF* genes were harbored. We demonstrated that a point mutation in coding region resulted in the loss of transactivation activity of CBF2 in the populations in relative warm habitats, while an intact and fully functional *CBF2* was found in a population located in the cooler habitat. The differential fixation of mutations in *CBF* genes may reflect an adaptation of a monophyletic group of *A. thaliana* to different habitats during their expansion along the Yangtze River on a limited timescale.

Materials and Methods

Plant materials and growth condition

The four populations of *Arabidopsis thaliana* (L.) Heynh along the Yangtze River used in this study were collected from: Tongliang, Chongqing City (CQtlx); Jiujiang, Jiangxi Province (JXjjs); Qianshan, Anhui Province (AHqsx); and Chenggu, Shannxi Province (SXcgx). Other populations or accessions used in this study were either collected from their native habitats in China or obtained from the Nottingham *Arabidopsis* Stock Centre (NASC; University of Nottingham, UK) or the *Arabidopsis* Biological Resource Center (ABRC; Ohio State University, USA) (Supporting Information Table S1). The Columbia ecotype (Col) was used as a reference. The seeds were sown and seedlings were grown in the growth facility at Peking University as described by He *et al.* (2007). Normal growth conditions were set at 16-h photoperiod with a minimum illumination of 120 μmol m^{-2} s^{-1} and a day : night temperature of 22 ± 2°C, and cold acclimation set at 4°C within a growth chamber (Percival Intellus Environmental Controller, Perry, IA, USA) for 72 h with the same photoperiod and illumination. Sample collections for the gene expression analysis were conducted at a fixed time each day, that is, at 17:00 h after 9 h of light, to avoid the influence of the circadian rhythm.

Freezing tolerance assay

The freezing tolerance assay was performed as described by Verslues *et al.* (2006) with some modifications to check the basal and acclimated tolerance. Three-week-old plants in Petri dish with or without cold acclimation (abbreviated as CA and NCA, respectively) were transferred into a low-temperature growth chamber (Percival Intellus Environmental Controller) at −1 ± 0.1°C (dark). After the temperature of the whole plate fell down to −1°C,the plates were sprinkled with finely crushed ice and incubated at −1°C at least for 16 h. Then the temperature in the chamber was decreased by 1°C h^{-1} to the desired temperatures

(−6 or −8°C, which were set based on preliminary experiments exploring the full range of tolerance). After being kept at the desired temperature for 1 h, the plates were removed from the chamber and incubated at 4°C for 24 h in dark and then put under normal growth conditions. The plants that kept two or more newest rosettevleaves green after the freezing treatment and a recovery growth under normal condition for 2 d were identified as survivors. The survival rate was calculated for each population/accession. Each assay was conducted on at least three plates as replicates at a time with *c.* 25–30 individuals in each plate.

Gene expression analysis

The seedlings were harvested for total RNAs extraction after they were treated at 4°C for 0.25, 0.5, 1, 2, 3, 12, 24 h and 7 d. The plants growing under normal conditions were used as control. Total RNA was extracted using TRIzol reagent (Invitrogen), and then treated with RNase-free DNase (TaKaRa Biotechnology, Dalian, China) to eliminate genomic DNA. Total RNA was reverse-transcribed using the Superscript II RT kit (Invitrogen), and the cDNA was used as the template for PCR amplification.

Real-time quantitative reverse transcription-PCR (qRT-PCR) was conducted using an ABI PRISM 7700 Sequence detector, using DyNAmo STBR Green qPCR kit (Finnzymmes, Finland). All primer sequences for *CBF*s and downstream genes were designed with the program Primer3 (http://frodo.wi.mit.edu/primer3/) and listed in Table S2. Real-time qRT-PCR amplifications were performed in a total volume of 20 μl, including 2 μl of gene specific primers (5 μM) for 5′ and 3′ respectively, 1 μl of cDNA, 10 μl reaction mixtures of enzymes and fluorescent dyes, and 5 μl of double-distilled water. PCR-cycling conditions comprised an initial polymerase activation step at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 59°C for 30 s, and 68°C for 30 s. The data were collected after each extension step (30 s at 68°C). For a negative control, 1 μl cDNA was replaced by the equal amount of double-distilled water. Relative gene expression values were standardized to the expression level of *EF1α* gene (translation elongation factor EFTu/EF1A, *At5 g60390*), which was demonstrated to be not influenced by cold stress in all populations. For each sample, at least three replications were carried out each time.

Metabolic pathway analysis

The expression profile data under cold treatment generated by He *et al.* (2008) on the same populations was re-analyzed on the genes with reported cold-inducible expression and with higher expression (by at least two-fold) in SXcgx than in AHqsx, CQtlx and JXjjs. We identified 59 and 282 genes, respectively. The gene set enrichment analysis (Subramanian *et al.*, 2005) was used on the 282 genes to identify possible metabolic pathways involved using AraCyc Pathway (Zhang *et al.*, 2005; http://arabidopsis.org/biocyc/index.jsp).

Measurement of the content of galactinol and raffinose

The contents of galactinol (galIno) and raffinose (raf) were measured as described by Horbowicz & Obendor (1994) with some modifications as follows. Three-week-old seedlings grown in a glasshouse were moved into the growth chamber of 4°C for 14 or 30 d before 100 mg leaf tissues of each sample were collected. The leaf samples were homogenized in a mortar with 0.6 ml of ethanol:water (1 : 1, v/v) containing 100 μg of phenyl-α-D-glucoside as the internal standard. The samples were centrifuged at 10 265 ***g*** for 20 min after being heated at 80°C for 45 min, extracted twice and the supernatants were mixed. The combined supernatant was passed through a 10 000 M_r cutoff filter and the aliquots of the filtrate were dried and derivatized with 200 μl of silylation mixture (trimethylsilylimidazole : pyridine, 1 : 1, v/v) in tightly capped silylation vials (Supelco, Bellefonte, PA, USA) at 70°C in a heat block for 30 min, and then cooled to 22°C. One microliter of the trimethylsilyl (TMS) derivatized soluble carbohydrates was injected into a split-mode injector of a gas chromatograph equipped with a flame ionization detector and integrator. Soluble carbohydrates were analyzed by gas chromatograph mass spectrometer (Agilent GC 7890A /MS 5975C) using a DB-1 capillary column (J&W Scientific, Folsom, CA, USA). All standards were dissolved in ethanol : water (1 : 1, v/v) before analysis. For each sample at least three replications were carried out each time.

Quantitative trait locus (QTL) mapping

An interpopulation F_2 mapping population was obtained starting from a cross between SXcgx (with lowest monthly average temperature in January) and CQtlx (with the highest average monthly temperature in January). The individuals of F_2 generated by selfing of F_1 were used for mapping. The expression level of *AtGolS3* of 320 individuals from the F_2 population was screened using real-time qRT-PCR both as a marker to look for its possible regulators and as an indicator of freezing tolerance. In total, 243 pairs of insertion/deletion (indel) markers were selected from the results of whole genome re-sequencing on SXcgx and CQtlx (data not shown) to screen for polymorphism between them. As a result, 78 F_2 individuals with different expression level of *AtGolS3* were genotyped with 58 indel markers (Table S3). Genetic linkage maps for the F_2 population were constructed by using Map Manager QTX17 (Manly *et al.*, 2001) with a *P*-value threshold of 0.01 for search linkage criterion. The Kosambi mapping function was used for calculating map distances. The QTL marker regression and interval mapping of the expression level of *AtGolS3* were defined on this F_2 genetic linkage map. The criterion of *P*-value was 0.05 for maker regression (free regression model), while in the interval mapping, the threshold values of the LRS (likelihood ratio statistic) for significant and highly significant were 8.8 and 21.3 (LOD score 2.07 and 5, respectively) based on performing 1000 permutations of the data (free regression model and with a window size of 1 cM) with a significance level of $P \leq 0.05$.

Transactivation activity assays

The full-length coding region of *CBF1–3* and truncated forms of *CBF2* from both populations CQtlx and SXcgx were amplified using PCR. The products were cloned in frame to the sequences

encoding the GAL4 DNA-binding domain into *Eco*R I/*Not* I sites of pYF503. All the constructs were confirmed by sequencing. Yeast strain *EGY48* and the reporter vector pG221, which harbors the *CYC1* core promoter and β-galactosidase (*LacZ*) reporter gene, were used for this assay. Empty pYF503 vector was used as the negative control and pYF504, which harbors the full length *GAL4* gene, was used as the positive control (Ye *et al.*, 2004). Yeast LiAc-mediated transformations and β-galactosidase filter assays using X-gal as substrate were performed as described Li *et al.* (2006). Yeast transformants were screened on the synthetic drop-out SD/-Ura and SD/-Ura/-Trp medium. At least three independent strains were tested for each construct as replicates.

β-galactosidase activity was quantified using an ONPG (O-nitrophenyl-β-D-galactopyranoside) liquid assay. Briefly, 0.5 ml samples of yeast cells in log phase liquid culture were collected by centrifugation, dissolved in 100 µl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$; pH 7.0) and disrupted by the freeze/thaw procedure in liquid nitrogen at least three times. After thawing, 700 ml of Z-buffer and 160 µl of ONPG (freshly prepared, 4 mg ml^{-1} in Z-buffer) were added to the reaction mixture and incubated at 30°C. The reaction was stopped by the addition of 400 ml of 1 M Na_2CO_2 and centrifuged for 10 min at 14 462 ***g*** in a micro-centrifuge. β-galactosidase activity (Miller unit) was calculated using the equation $1000 \times (OD_{420}/OD_{600} \times V \times t)$, where V is the culture volume and t is the reaction time (Miller, 1972; Sambrook & Russell, 2001). Here, again, experiments were performed with three independent yeast strains and three replicates for each strain.

Results

Freezing tolerance differentiated the natural populations along the Yangtze River

The four populations used in this study were collected along the Yangtze River and selected in such a way that their habitats covered those areas with the coldest temperature at Chenggu-xian, Shaanxi Province (SXcgx), the warmest temperature at Tongliang-xian, Chongqing City (CQtlx), and intermediate temperatures at Qianshan-xian, Anhui Province (AHqsx) and Jiujiang City, Jiangxi Province (JXjjs). Population SXcgx is located northernmost among the four populations with the lowest monthly average temperature in January, while CQtlx has the highest monthly average temperature in January. The distribution map, and locality and detailed habitat information are shown in Fig. 1 and Table 1, respectively. Freezing tolerance assays were conducted with CA and NCA at −6°C. As a result, the survival rate of the four populations ranged from 37.6% for CQtlx to 79.1% for SXcgx with CA, and varied from 25% for CQtlx to 55.7% for SXcgx with NCA (Fig. 2a). The survival rates both with CA and NCA were negatively correlated with the monthly average temperatures of January in the local habitats ($P < 0.01$, Fig. 2b). When temperature dropped to −8°C, the survival rate varied

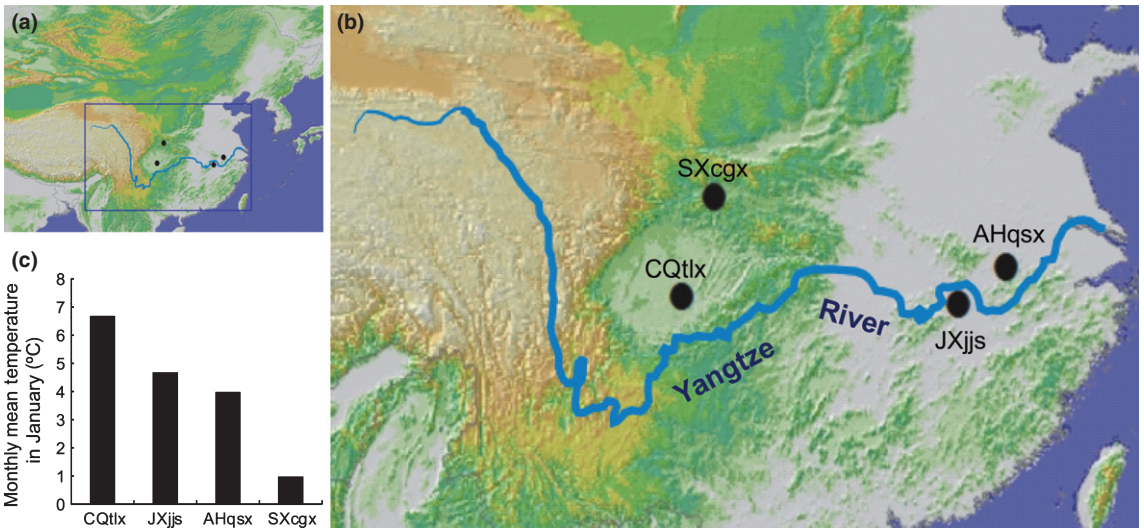

Fig. 1 Distribution of four natural populations of *Arabidopsis thaliana* and January monthly average temperature in the habitats. (a) Distribution map of four natural populations along the Yangtze River. (b) The enlarged portion of (a). (c) January monthly average temperature at the localities where the populations were collected.

Table 1 Habitat information for the four natural populations of *Arabidopsis thaliana*

	Location	Latitude & longitude	Altitude, m	Monthly mean temperature in January (°C)	Minimum temperature in January (°C)
CQtlx	Tongliang, Chongqing	29°49.40N 106°03.38E	263	6.7	5.3
JXjjs	Jiujiang, Jiangxi	29°35.68N 115°54.74E	80	4.7	1.2
AHqsx	Qianshan, Anhui	30°44.77N 117°37.43E	150	4.0	0.5
SXcgx	Chenggu, Shaanxi	32°55.93N 107°12.65E	607	1.0	−3.6

from 13.9% of CQtlx to 42.4% of SXcgx with NCA (Fig. 2c), and the correlation was still significantly negative ($P < 0.01$, Fig. 2d). These results suggest that freezing tolerance is increased, as expected, in all populations after cold acclimation, and that, although these four populations were derived from a common ancestor not long time ago, the freezing tolerance in CQtlx, JXjjs and AHqsx were significantly lower than in SXcgx at −6°C both with CA and NCA, and at −8°C with NCA ($P < 0.05$).

The differentiation of expression profile under cold treatment was consistent with the divergence of freezing tolerance among populations in general

We examined the expression level of 59 reported cold-responsive genes based on the expression profile data from the same populations (He *et al.*, 2008), and found that about a half of them (31 out of 59; Table S4) expressed higher in SXcgx than in CQtlx, JXjjs and AHqsx. This was confirmed by real-time qRT-PCR analysis, as indicated by two representative genes *COR15A* and *RD29A* (Fig. 3). These results suggest that the higher expression of some cold-responsive genes might be responsible for the higher freezing tolerance in SXcgx.

The gene expression and contents of galactinol and raffinose in the raffinose family oligosaccharides (RFOs) biosynthesis pathway were related to freezing tolerance

In order to investigate which metabolic pathway was significantly involved in the higher freezing tolerance in SXcgx population, GSEA (gene set enrichment analysis; Subramanian *et al.*, 2005; Zhang *et al.*, 2005) was applied on 282 genes more highly expressed (by at least two-fold) in SXcgx than in AHqsx, CQtlx and JXjjs under cold treatment based on the data generated by He *et al.*

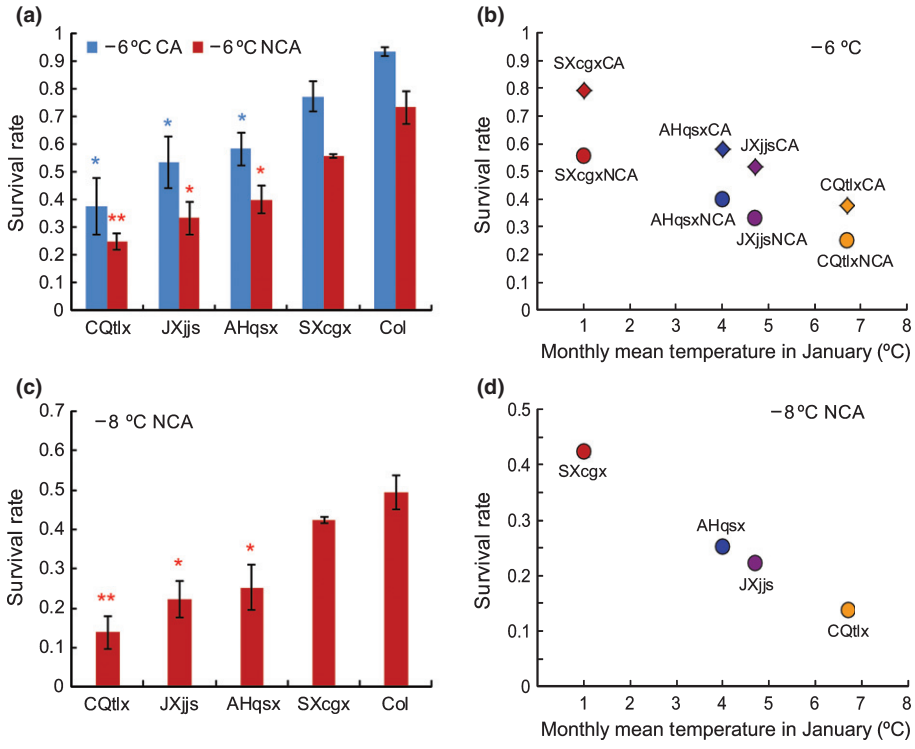

Fig. 2 Freezing tolerance assay on four natural populations and the Col ecotype of *Arabidopsis thaliana*. (a) Survival rate with acclimation (CA, blue solid column) or without CA (NCA, red solid column) at −6°C. (b) Correlation test between survival rate at −6°C and monthly average temperature in January in habitats: Pearson $r_{CA} = -0.999$ ($t = -38.4827$, $P = 0.0007$; $R^2 = 0.9987$); Pearson $r_{NCA} = -0.995$ ($t = -13.9433$, $P = 0.0051$, $R^2 = 0.9898$). (c) Survival rate with NCA at −8°C. (d) Correlation test between survival rate at −8°C and monthly average temperature of January in habitats: Pearson $r = -0.996$ ($t = -15.0573$, $P = 0.0044$, $R^2 = 0.9913$). Colored diamonds: survival rate with CA; circles: survival rate with NCA. Different colors represent different populations. * significant ($P < 0.05$) and **highly significant ($P < 0.01$) difference compared to Col. Each treatment and measurement had at least three replicates, and error bars represent ± SD.

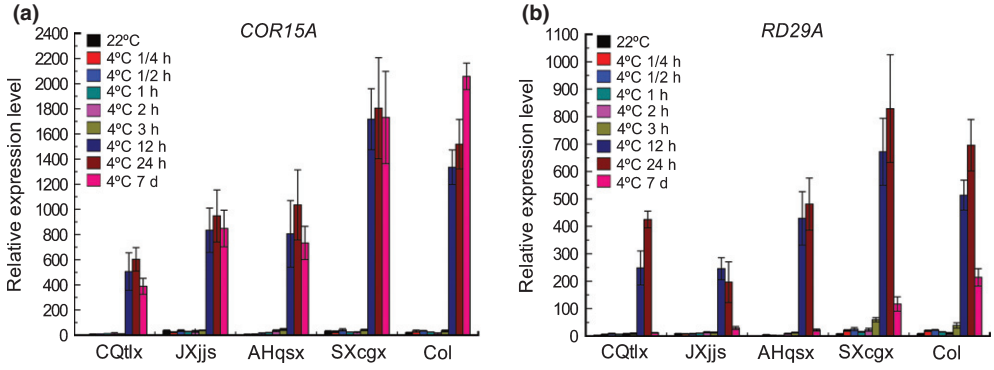

Fig. 3 The expression level of two downstream cold-response genes in four natural populations and the Col ecotype of *Arabidopsis thaliana* under cold treatment. The expression level of (a) *COR15A* and (b) *RD29A* under cold treatment. The relative expression values of each gene were normalized to the expression level of *EF1α* gene and multiplied by 1000. Each treatment and measurement had at least three replicates, and error bars represent ± SD.

(2008). Several metabolic pathways were identified, in which RFOs biosynthesis pathway is one of the most significant ones. In this pathway, galactinol synthase (GolS) catalyzes the first committed step of the biosynthesis of raffinose (Fig. 4a) and plays a key regulatory role in carbon partitioning between sucrose and RFOs (Saravitz *et al.*, 1987). GolS is encoded by a multi-gene family in *A. thaliana*, and *AtGolS3* was reported to be specifically induced by cold (Taji *et al.*, 2002). Our real-time qRT-PCR analysis showed that *AtGolS3* was induced to a much higher level at 12 h after cold treatment in SXcgx than in CQtlx, JXjjs or AHqsx (Fig. 4b), consistent with the microarray data (He *et al.*, 2008). We found that the expression level of *AtGolS3* was positively correlated with the degree of freezing tolerance of the four populations and Col ($P < 0.05$). Meanwhile, the contents of galactinol (galIno) and raffinose (raf) were significantly higher in SXcgx than in the other three populations (Fig. 4c,d), and there was a significantly positive correlation between the contents of galactinol and raffinose after growing at 4°C for 30 d and freezing tolerance at −6 or −8°C ($P < 0.05$). Sequence analysis showed that the promoter and coding region of *AtGolS3* were conserved among the four populations (Fig. S1), suggesting that higher expression of *AtGolS3* in SXcgx is likely attributed to the higher activity of several regulatory gene(s).

Quantitative trait locus (QTL) mapping revealed *CBF*s on the major locus

In order to characterize the genetic factors responsible for the differential *AtGolS3* expression and freezing tolerance we conducted QTL mapping analysis. An F_2 mapping population was developed from a cross between two populations which had the highest (SXcgx) and lowest (CQtlx) freezing tolerance. Based on the analysis on expression level of *AtGolS3* of 320 F_2 individuals, a genetic linkage map was constructed by genotyping 78 F_2 individuals with different expression of *AtGolS3* using 58 polymorphic indel markers (Table S3). This map was the basis for all subsequent marker regression and interval mapping analyses. Six markers –four located in the long arm of chromosome 4 (M406.70, M408.98, M413.02 and M415.34) and two in the long arm of chromosome 5 (M519.71 and M522.37) – were statistically significant in marker regression ($P < 0.05$). In interval mapping we detected two significant QTLs, *qDEPL1* (*differential expression loci 1*) and *qDEPL2* (*differential expression loci 2*), on chromosomes 4 and 5, respectively (Fig. 5a). *QDEPL1* was on the long arm of chromosome 4, and comprised an interval of *c.* 2.3 Mb covered by two molecular markers, M413.02 and M415.34. The peak LOD score of *qDEPL1* was 6.50 (LRS value 27.7) and could explain 30% ($P < 0.001$) of the variation in *AtGolS3* expression level. Moreover, *qDEPL2* was on the long arm of chromosome 5, comprising an interval of *c.* 2.5 Mb covered by M519.71 and M522.37. The peak LOD score of *qDEPL2* was 2.36 (LRS value 9.9) and could explain 12% ($P < 0.01$) of the variation in *AtGolS3* expression level. Further analysis of *qDEPL1* revealed that three key cold response transcription factors, *CBF1*, *CBF2* and *CBF3*, were located within this interval (Fig. 5b); therefore, we focused our attention on this region.

Both the promoter and coding regions of *CBF*s were altered in the four populations compared to those in Col

We examined the expression of *CBF1*, *CBF2* and *CBF3* in the plants of the four populations and Col under cold treatments,

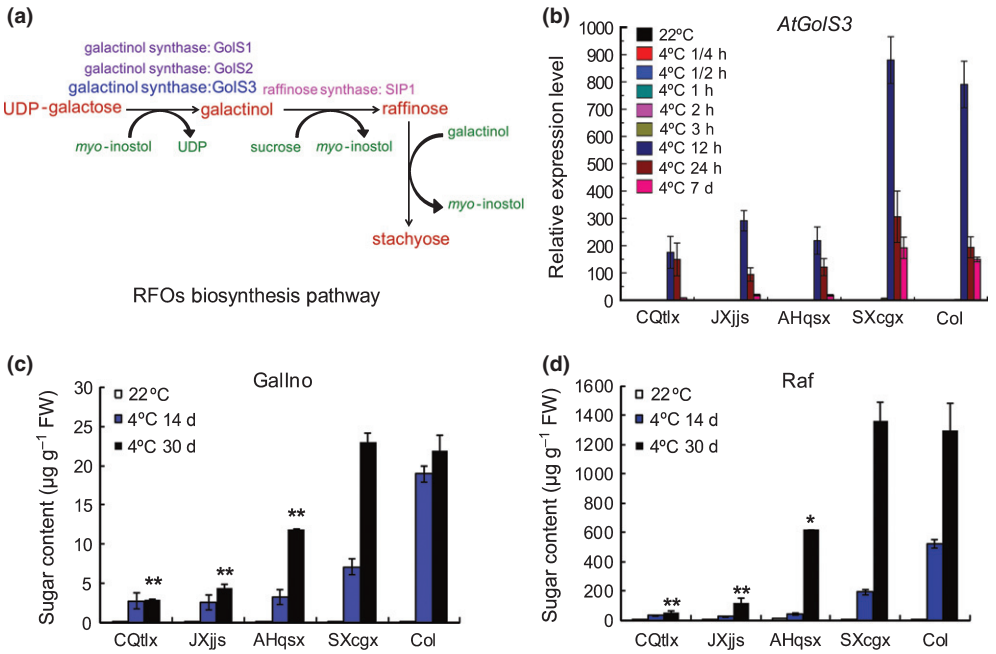

Fig. 4 The biosynthesis pathway and content of galactinol and raffinose in four natural populations and the Col ecotype of *Arabidopsis thaliana*. (a) The RFOs biosynthesis pathway. Cold-specifically responsive *GolS3* is marked blue; (b) Time course of *AtGolS3* expression under cold treatment. The relative expression values of each gene were normalized to the expression level of the *EF1α* gene and multiplied by 1000. (c, d) The content of galactinol (GalIno, c), and raffinose (Raf, d) under cold treatment. * significant ($P < 0.05$) and **highly significant ($P < 0.01$) difference compared to SXcgx. Each treatment and measurement had at least three replicates, and error bars represent ± SD.

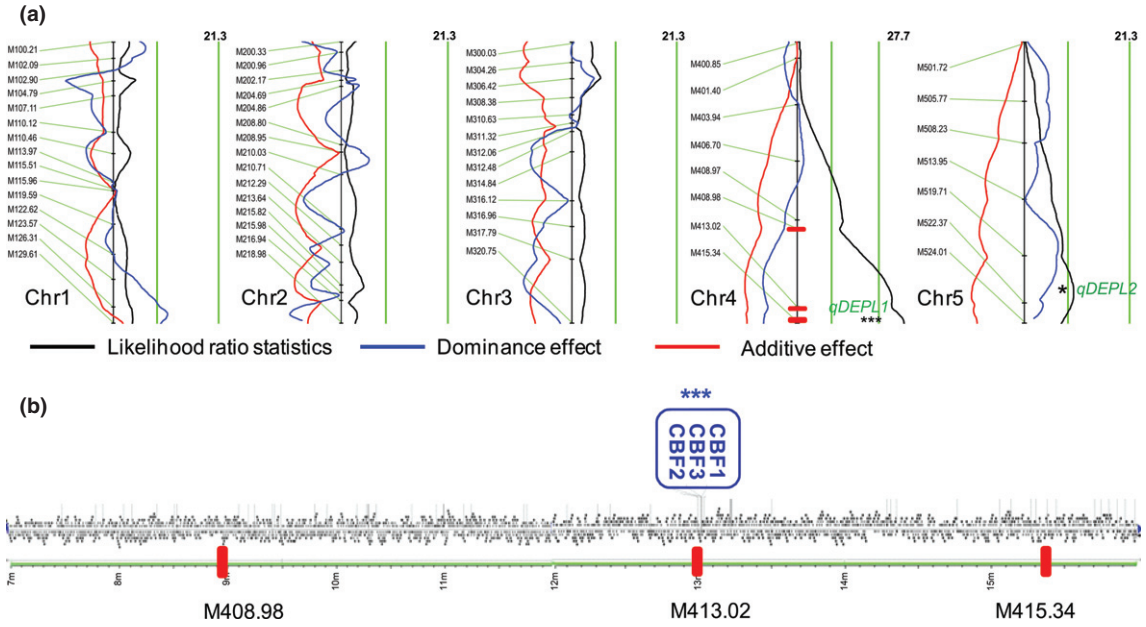

Fig. 5 Quantitative trait loci (QTL) of differential cold-response of *AtGolS3* and genetic architecture of QTLs on chromosome 4 of *Arabidopsis thaliana*. Results of interval mapping on five chromosomes. The two green lines stand for critical values for the likelihood ratio statistic (LRS) indicating minimum values for significant (*), and highly significant (***) results. (b) The genetic architecture of *qDEPL1* on chromosome 4 with *CBF1–3* located between markers 413.02 and 415.34.

and compared the sequences of these three genes from a larger sample of worldwide populations/accessions.

At 4°C, *CBF1* and *CBF2* were quickly induced (as soon as 0.25 h after start of cold treatment). The level of *CBF1* was much lower than that of *CBF2* and *CBF3* in general (Fig. 6a). The level of *CBF2* was higher in the four natural populations than in Col, especially at the beginning of the cold treatment (Fig. 6b). Further sequencing analysis of coding regions revealed that no variation in *CBF1* was found in the four populations. For *CBF2*, populations CQtlx, JXjjs and AHqsx, but not SXcgx, had a single base (adenine) insertion in the transactivation domain, resulting in a frame shift and thus an early stop (Fig. 7a). We then sequenced *CBF2* coding regions of the 57 representative accessions/populations around the world (Table S1) and compared these data with those of 39 accessions from other reports (NCBI database). We found that this single adenine insertion was only detected in the populations along the Yangtze River and in Kas-2 (data not shown). Interestingly, SXcgx was the only population, out of the 19 populations along the Yangtze River, which did not have this insertion in all the individuals tested.

Trancsription factor *CBF3* had a different pattern. In Col, *CBF3* was rapidly induced by cold treatment (after 0.25 h) and reached its highest expression level at 2–3 h. In the four Chinese nature populations, however, the induction of *CBF3* was much lower than that in Col and the expression level of *CBF3* was low throughout the treatment (Fig. 6c). We found that a sequence replacement occurred in the promoter region of *CBF3* in the four populations along the Yangtze River; that is, a new 1725-bp fragment replaced an 854-bp fragment of Col. This 1725-bp fragment includes two transposed fragments of 493- and 138-bp

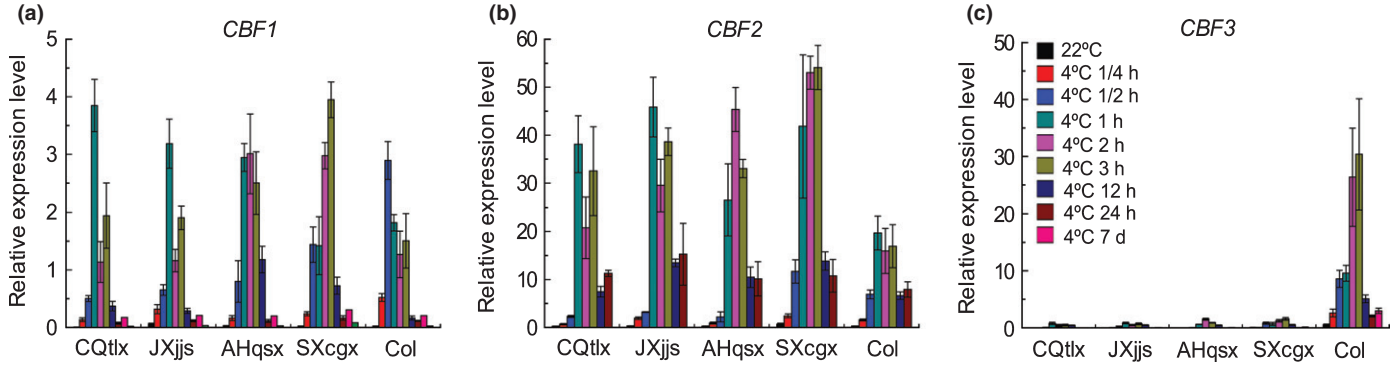

Fig. 6 The time course of expression of the three *CBF* genes in four natural populations and the Col ecotype of *Arabidopsis thaliana* under cold treatment. The relative expression values of *CBF1* (a), *CBF2* (b) and *CBF3* (c) were standardized to the expression level of the *EF1α* gene and multiplied by 1000. Each treatment and measurement had at least three replicates, and error bars represent ± SD.

from chromosome 5 (Fig. S2), suggesting that this structure variation might result from several insertion/deletion (indel) events. We also screened the *CBF3* promoter regions of the 57 accessions/populations by PCR and found that this sequence replacement/indel was only present in the populations along the Yangtze River and Kas-2 (Table S1).

Because *CBF2* had a frame-shift mutation and *CBF3* had six nonsynonymous substitutions in their coding regions in the four populations (Fig. 7a), it is therefore critical to investigate whether these variation forms of CBF2 and CBF3 still have transactivation activity as a transcription factor.

Transactivation activity of CBF2 and CBF3 differentiated between CQtlx and SXcgx

The main function of a transcription factor is to help initiate a program of increased or decreased gene transcription. To determine whether the CBFs in different populations still have transcription activity, we conducted *in vitro* transactivation activity assays. The full length of the coding sequences of *CBF1–3* from CQtlx and SXcgx were cloned into a yeast expression system to test whether different variants of CBFs could initiate the transcription of reporter genes (Ye *et al.*, 2004). Both the qualitative and quantitative results showed that CBF1 and CBF3 from both CQtlx and SXcgx could activate the transcription of the reporter gene significantly (Fig. 7b left column and 7c, $P_{CQtlx\_CBF1} < 0.0001$, $P_{SXcgx\_CBF1} < 0.001$, $P_{CQtlx\_CBF3} < 0.05$, $P_{SXcgx\_CBF3} < 0.001$). These results indicated that the translated products of *CBF1* and *CBF3* still retained transactivation activities even after the induction of *CBF3* in the four populations was impaired by the indels in the promoter region, although the activity of CBF3 from CQtlx was lower than that from SXcgx.

Different from CBF1 and CBF3, the CBF2 from CQtlx did not have any transactivation activity, while that from SXcgx functioned well (left column of Fig. 7b). Further analysis of different truncated forms of CBF2 with either N-terminal or C-terminal regions deleted showed that none of them had the transactivation activity (right column of Fig. 7b). These results suggest that the frame shift mutation in *CBF2* deprives this transcription factor of transactivation activity, and that both the N-terminal with the DNA-binding domain and the C-terminal with the activation domain are essential for the transactivation activity of CBF2.

Discussion

Tolerance to chilling and freezing is an essential trait for plant survival, and DNA changes (natural variation) of certain key genes play an important role in the survival of plant populations in natural environments with different temperatures. It was reported that chilling and freezing tolerance were negatively correlated with the minimum temperature or the latitude of the habitats in the populations collected around the world (Alonso-Blanco *et al.*, 2005; Hannah *et al.*, 2006; Hasdai *et al.*, 2006; Zhen & Ungerer, 2008a; Gery *et al.*, 2011; Zuther *et al.*, 2012). In this study, we revealed the negative correlation between the freezing tolerance of *Arabidopsis* populations along the Yangtze River and the monthly average temperature in January in the habitats, and this is the first study of this kind to combine the natural variation in sequence of *CBF*s with their biochemical functions and freezing tolerance in a monophyletic group of natural populations of *Arabidopsis thaliana* with a short expansion history. We found a 1.7 kb-long fragment replacement in the promoter region of the important cold-responsive gene *CBF3* in all populations along the Yangtze River, which could serve as a unique marker. The Kas-2 from Kashmir that is part of the same monophyletic group, based on chloroplast DNA (Yin *et al.*, 2010), also has this fragment replacement, suggesting that this insertion/deletion was most likely fixed in the ancestral population(s). Studies on cave stalagmites and ice cores indicated that a warm climate, which could possibly relax the selection pressure on *CBF*s (Zhen & Ungerer, 2008b), occurred in China around 90 000 yr ago (Thompson *et al.*, 1997; Andersen *et al.*, 2004; Yuan *et al.*, 2004). It is coincidental with the time when *A. thaliana* populations expanded along the Yangtze River. Although we do not have the direct evidence yet to support the idea that loss of *CBF3* function increases the fitness of the populations under warmer conditions, the association of fixation of *CBF3* mutation in all populations and warmer climate at their expansion time along the Yangtze River could be an indication of this adaptation.

It was reported that *CBF3* was highly polymorphic in 34 geographically distant ecotypes from Europe, North America, Russia, North Africa, Japan and India, and *c.* 16 nonsynonymous substitutions were identified (Lin *et al.*, 2008). In this study, we also found that the coding region of *CBF3* was most variable among three *CBF*s in the four natural populations. It might result from a relaxed purifying selection on *CBF3* after its expression was impaired by indels at the promoter region.

Another unique variation found in this monophyletic group is the one-nucleotide insertion at the C-terminal of *CBF2*. This insertion caused not only a premature stop of translation, but also the loss of transactivation activity of the protein. Unlike the indels at the promoter of *CBF3*, this insertion has not been fixed in all populations along the Yangtze River. We sequenced *CBF2* of 30 SXcgx individuals, and found none of them had the insertion, whereas in AHqsx, CQtlx, and JXjjs the insertion was detected in all the individuals sequenced (at least five individual plants each population). The plants of *A. thaliana* along the Yangtze River overwinter with rosettes. It would be crucial for their survival if their rosettes could tolerate frequent low temperatures below 0°C in a place such as Chenggu-xian, Shanxi (SXcgx) (Table 1). Because SXcgx has the lowest monthly average temperature in January amongst the four natural populations, it is plausible to conclude that *CBF2* has experienced higher natural selection pressure for freezing tolerance in SXcgx than in the other three populations.

The level of transcription is often used as one indicator of gene function. Our study suggests that the result of transcription analysis should be interpreted with caution and should better be complemented with functional analysis on relevant genes. For example, *CBF3* expression in the four populations was significantly lower, but *CBF2* expression was significantly higher than

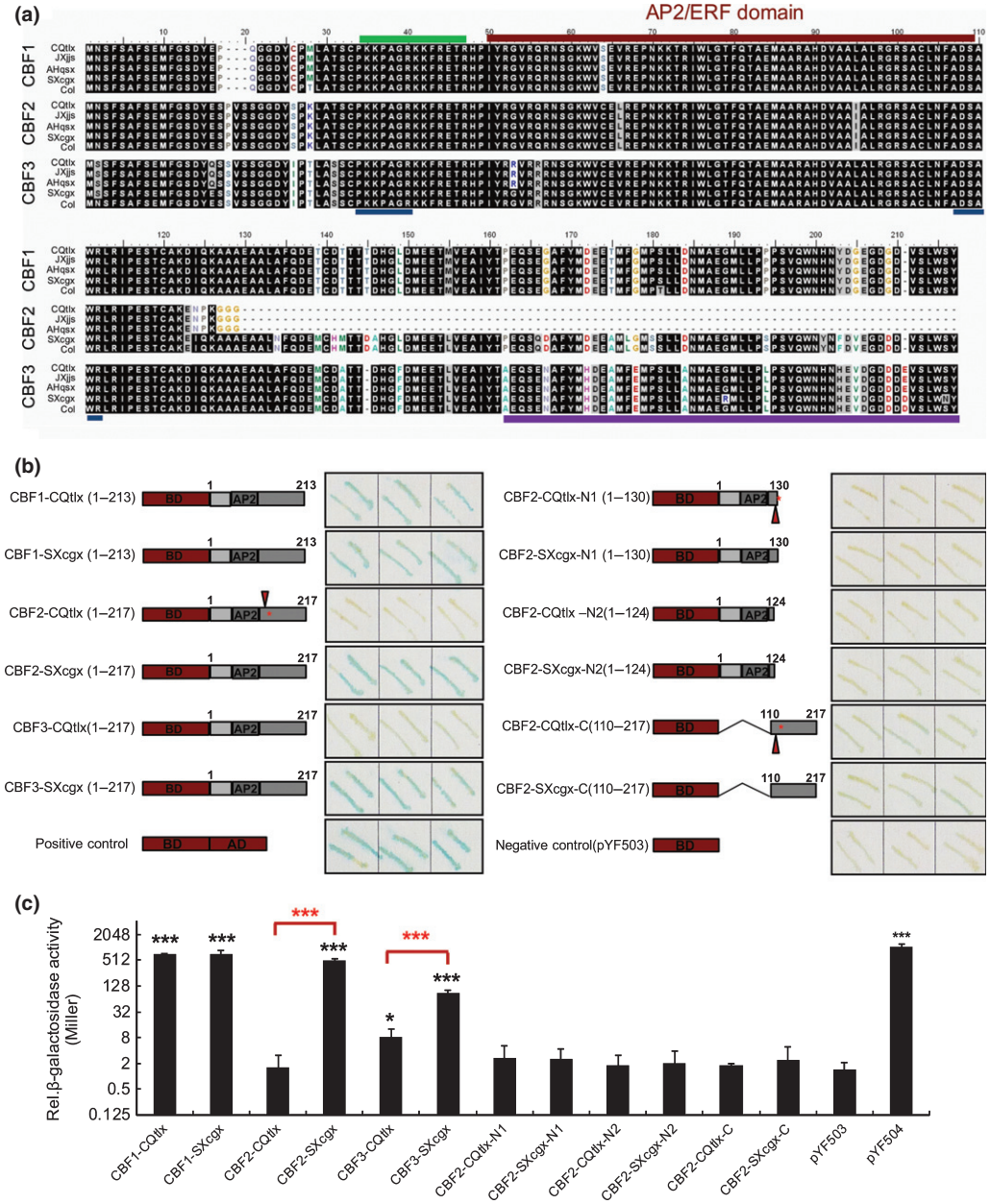

Fig. 7 The polymorphisms of the amino acid sequence in four natural populations and the Col ecotype of *Arabidopsis thaliana* and transactivation activity of CBF1, CBF2 and CBF3 in CQtlx and SXcgx. (a) The polymorphism of the amino acid sequences of CBF1, CBF2 and CBF3 among four natural populations and Col. Red line, AP2/ERF domain; blue lines, signature sequences; green line, nuclear localization signal (NLS); violet line, reported activation domain. (b) β-galactosidase filter assay of yeast carrying a pG221 reporter vector and a pYF503 vector with different *CBF*s fragments from CQtlx and SXcgx fused in-frame with the *GAL4* DNA-binding domain by using X-gal as a substrate. The increasing blue color intensity of yeast cells indicates increasing activity of the transactivation. Yeast strains *EGY48* transformed by an empty pYF503 vector harboring only *GAL4* DNA-binding domain and pYF504 vector harboring full length *GAL4* gene with both DNA-binding (BD) and transactivation domain (AD) were used as negative and positive control, respectively. (c) Transactivation activity of CBFs from CQtlx and SXcgx by the quantitative β-galactosidase assay in yeast liquid culture using ONPG (O-nitrophenyl-β-D-galactopyranoside) as a substrate with the same yeast strains and vectors as the β-galactosidase filter assay. Each measurement had at least three replicates, and error bars represent ± SD. Black * significant ($P < 0.05$) and ***highly significant ($P < 0.001$) difference compared to the negative control (pYF503); red *** highly significant difference ($P < 0.001$) between CQtlx and SXcgx.

those in Col (Fig. 6), not consistent with the corresponding freezing tolerance of the four natural populations and Col. However, some of the CBF-regulated cold-inducible marker genes, such as *COR15A*, *RD29A* and *AtGolS3*, were more highly induced in SXcgx than in the other three natural populations (Fig. 3 and Fig. 4b), which was consistent with their freezing tolerance. Further analysis on coding sequence and biochemical function of *CBF*s found that although high level expression of *CBF2* was detected in all four populations, only the SXcgx plants had a functional *CBF2*. It is therefore reasonable to speculate that the

high expression of *AtGolS3* and downstream cold-responsive genes in SXcgx was upregulated by *CBF2* or *CBF1* and *CBF2*, and that *CBF1* alone was not sufficient to upregulate the downstream genes in the other three populations. This speculation is supported by the result of QTL mapping: the fact that there are multiple binding sites of CBFs in the promoter of *AtGolS3*, and that the *AtGolS3* promoters had no significant sequence variation among the four populations (Fig. S1).

The network of *CBF1–3*-mediated gene regulation in response to low temperature is complex. It was reported that *CBF2* negatively regulated *CBF1* and *CBF3* (Novillo *et al.*, 2004), and that both *CBF1* and *CBF3* were up-regulated and required for the complete development of cold acclimation response (Novillo *et al.*, 2007), whereas the transcription of *CBFs* were feedback-inhibited by CBFs and products of their downstream target genes (Guo *et al.*, 2002). We did find that the level of expression of *CBF2* was much higher in the four natural populations with disfunctional *CBF3* than in Col with a functional *CBF3* under cold treatment (Fig. 6). More work is needed to reveal the molecular interaction among CBF1–3 and their downstream genes or proteins in a cold-responsive regulation network. It is also interesting to note that, although *CBF3* did not express at high levels under cold treatment in four populations, the translated CBF3 protein still possessed the transactivation activity. It is probably due to the fact that the mutations in its promoter region and coding region are too recent (*c.* 90 000 yr ago) to abolish the function of this gene.

That the differential regulation of *AtGolS3* under cold treatment was controlled by multiple loci was suggested by QTL mapping analysis. Although the *qDEPL1* with *CBF*s on chromosome 4 played a major role, another QTL, *qDEPL2*, on chromosome 5 could also have contributed to 12% variation in the expression level of *AtGolS3*, suggesting that there are other genes responsible for the differential expression of *AtGolS3* in those populations along the Yangtze River. It is worth mentioning that we did not observe any significant difference on the effect of acclimation among these four natural populations (Table S5). The function of *CBF2* and *CBF3* in populations CQtlx, JXjjs and AHqsx might be compensated by other pathways involved in acclimation. Therefore, further study is needed to uncover additional genetic factors and mechanisms for the natural differentiation of cold response among these populations.

Acknowledgements

We are very grateful to Professor Jingyun Fang (Peking University), Professor Shuhua Yang (China Agriculture University) and Dr Fei He (University of Münster) for their valuable comments, suggestions, and very informative discussions on this work. We would like to thank Dr Fang Du (Beijing Forestry University) and Dr Wenting Wang (Northwest University for Nationalities) for their help in preparation of distribution map and climate information. Appreciations also go to Ms Guiying Sun for taking care of all plant materials. This study was partially supported by State Key Laboratory for Protein and Plant Gene Research, Peking University, and partially by 111 Project (B06001).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The sequence polymorphism and gene architecture of *AtGolS3* in four natural populations along the Yangtze River and the Col ecotype of *Arabidopsis thaliana*.

Fig. S2 The promoter architecture of *CBF3* and five variation patterns of *CBF3* promoter and the related fragment on chromosome 5 in natural populations/accessions of *Arabidopsis thaliana* from around the world.

Table S1 Fifty-seven natural populations/accessions of *Arabidopsis thaliana* and variation in the promoter of *CBF3* and relevant fragments on chromosome 5

Table S2 Primers for amplifying CBFs and its downstream genes in four natural populations and the Col ecotype of *Arabidopsis thaliana*

Table S3 Fifty-eight indel Markers used in QTL mapping for identifying loci involving in differential expression of AtGolS3 in four natural populations of *Arabidopsis thaliana*

Table S4 Reported cold-responding genes in *Arabidopsis thaliana* and the relative level of their expression in four natural populations and the Col ecotype

Table S5 Statistical test of the differences of the effect of cold acclimation among four natural populations and the Col ecotype of *Arabidopsis thaliana*

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