INTRODUCTION

Cold stress is a major environmental factor that limits the geographical distributions of plant species and has serious impacts on the growth and development of plants adapted to warm climates (Boyer, 1982). Rice (Oryza sativa L.) originated in tropical or subtropical areas and is a naturally warm-adapted species that is sensitive to low temperatures; the optimum temperature for rice growth is 15–35°C. Temperatures below 15°C retard rice growth and development and can cause serious physiological damage (Andaya & Tai, 2006; Fujino et al., 2004). Currently, the area of rice cultivation in China is gradually expanding from the south into northern temperate regions, where varieties that are adapted to warmer climates may suffer from low-temperature stress, affecting crop establishment and reproductive stages (Wang et al., 2014). Low-temperature stress causes 3–5 million tonnes of rice yield losses in China every year (Zhang, Jiang, & Wang, 2013), and rice grown in cool temperate regions must have a requisite level of cold tolerance to be productive in these climates.

Concomitant with the spread of rice cultivation into northern climates, weedy rice (Oryza sativa f. spontanea) has rapidly adapted to the same cool climates. Weedy rice is a major agricultural weed of rice production fields worldwide that is morphologically and physiologically similar to rice, threatening the yield and quality of rice harvests. The mechanisms by which weedy rice (Oryza sativa f. spontanea) has adapted to endure low-temperature stress in northern latitudes remain unresolved. In this study, we assessed cold tolerance of 100 rice varieties and 100 co-occurring weedy rice populations, which were sampled across a broad range of climates in China. A parallel pattern of latitude-dependent variation in cold tolerance was detected in cultivated rice and weedy rice. At the molecular level, differential cold tolerance was strongly correlated with relative expression levels of CBF cold response pathway genes and with methylation levels in the promoter region of OsICE1, a regulator of this pathway. Among all methylated cytosine sites of the OsICE1 promoter, levels of CHG and CHH methylation were found to be significantly correlated with cold tolerance among accessions. Furthermore, within many of the collection locales, weedy rice shared identical or near-identical OsICE1 methylation patterns with co-occurring cultivated rice. These findings provide new insights on the possible roles that methylation variation in the OsICE1 promoter may play in cold tolerance, and they suggest that weedy rice can rapidly acquire cold tolerance via methylation patterns that are shared with co-occurring rice cultivars.

KEYWORDS
CBF pathway, climatic adaptation, cold tolerance, DNA methylation, OsICE1, weedy rice
Substantial evidence indicates that many weedy rice ecotypes are directly descended from cultivated rice (especially from early landraces that predate modern cultivars) and are closely related to the crop (He, Kim, & Park, 2017; Ishikawa et al., 2005; Kane & Baack, 2007; Li, Li, Jia, Caicedo, & Olsen, 2017; Qiu et al., 2017, 2014; Reagon et al., 2010; Song, Chuah, Tam, & Olsen, 2014; Sun et al., 2013; Zhang et al., 2015, 2018). Almost all rice cultivation areas globally are affected by weedy rice, which suggests that this weed can adapt quickly to a range of different local environmental conditions. In the case of cold adaptation, the temperature required for weedy rice seed germination has been found to be lower for populations originating from high latitudes than those from lower latitudes. This allows weedy rice seeds to avoid growing in inappropriate environments via changes in critical temperature cues for seed germination (Xia, Xia, Ellestrand, Yang, & Lu, 2011). While both cultivated and weedy rice are primarily self-fertilizing, outcrossing can occur, and genetic introgression from local cultivated rice into co-occurring weed populations could potentially promote the weed’s rapid adaptation across a range of climates (Ellestrand & Schierenbeck, 2006; Langevin, Clay, & Grace, 1990). Elucidating the mechanisms by which weedy rice and cultivated rice adapt to low temperatures may provide clues for weedy rice control and the cultivation of high-quality, cold-tolerant rice varieties.

Rice is one of the most globally important staple crops, and numerous studies have been conducted on cold tolerance in this species (Andaya & Mackill, 2003; Cruz Damilach, Kothefederizzi, & Carlos, 2006; Hahn & Walbot, 1989; Ma et al., 2015; Mukhopadhyay, Vij, & Tyagi, 2004; Nakamura et al., 2011; Oh et al., 2004; Saijo, Hata, Kyozuka, Shimamoto, & Izu, 2000; Sakamoto & Murata, 1998; Tao et al., 2011; Wang et al., 2013). Different rice varieties show markedly different levels of cold tolerance, with differential plant survival rates, electrolyte leakage and \( \text{H}_2\text{O}_2 \) contents under low-temperature treatment (Guo Zf, Sy, & Zhong, 2006; Lv et al., 2015; Ma et al., 2015). The cold tolerance of rice populations has been found to be correlated with both latitude and subspecies, with members of the japonica subspecies characterized by greater cold tolerance and a wider latitudinal distribution than indica varieties (Lv et al., 2015). This indica-japonica rice cold tolerance differentiation is also correlated with QTLs and single nucleotide polymorphisms (SNPs) in rice cold-responsive genes. The abiotic stress response gene OsMYB2, two nonsynonymous SNPs in exon 2 (one causing a Cys\(^{57} \) to Tyr\(^{57} \) change, and the other causing a Trp\(^{57} \) to Arg\(^{57} \) change) may be involved in indica-japonica differences in cold tolerance: japonica rice has Cys/Trp, while indica accessions have Tyr/Arg at the two sites (Lv et al., 2015). In the fourth exon of CHILLING TOLERANCE DIVERGENCE (COLD1), a T/C versus A SNP underlies differences of Met\(^{187} / \)Thr\(^{187} \) in indica compared to Lys\(^{187} \) in japonica varieties; this leads to japonica varieties having a greater cold tolerance than indica varieties (Ma et al., 2015). More broadly, studies involving quantitative trait locus (QTL) analysis support the idea that cold tolerance is a polygenic trait and that the accumulation of major-effect cold tolerance QTLs can be of considerable value for increasing rice cold tolerance and adaption to high-latitude regions (Andaya & Tai, 2006; Liu et al., 2013; Mao et al., 2015).

Rice responses to low temperatures include cold sensing, transcriptional regulation and post-transcriptional processing. The important roles of calcium, reactive oxygen species (ROS) and abscisic acid (ABA) in cold sensing and signalling have also been revealed. The \( \text{Ca}^{2+} \)-regulated calcium-regulated protein kinase (CDPK) pathway, ABA-dependent ABRE-binding factor (ABF/AREB) pathway and ROS-dependent mitogen-activated protein kinase (MAPK) pathway all play important roles in signalling transcription in response to low temperatures in rice. Transcriptional regulation includes the dehydration-responsive element-binding proteins-C-repeat/dehydration-responsive elements (CBF) pathway and the NAM-ATAF-CUC (OsNAC) transcription factor (Zhang et al., 2013). Post-transcriptional processing mainly involves factors that affect the processing of mRNAs, such as miR-171 and miR-444a (Zhang et al., 2013).

The CBF pathway, composed of members of the DREB (dehydration-responsive element-binding) gene family and their regulators, is the best documented and most extensively characterized cold response pathway to date (Agarwal, Agarwal, Reddy, & Sopory, 2006; Dubouzet et al., 2003; Ito et al., 2009; Qin et al., 2007; Su et al., 2010; Thomashow, 1999, 2010; Yang, Dai, & Zhang, 2012; Zhang, Chen, Wang, Hong, & Wang, 2014). These TFs can bind to GCC-box and C-repeat/dehydration-responsive elements (CRT/DRE) to promote the expression of cold-responsive genes (Zhang et al., 2013). CBF1 and CBF3 play important roles in increasing plants’ cold tolerance. CBF2 functions in a complementary role as a negative regulator of CBF1 and CBF3, ensuring that their expression is transient and tightly controlled, which in turn guarantees the proper induction of downstream genes (Novillo, Alonso, Ecker, & Salinas, 2004). OsMYB2 encodes a stress-responsive MYB transcription factor that promotes the expression of CBFs (Zhang et al., 2014). Inducer of CBF expression 1 (ICE1) is another important regulator of CBF pathway genes that promotes their expression by binding to MYC recognition sites in the CBF promoter regions. Levels of expression of CBF pathway genes are known to be correlated with cold tolerance, with significantly higher relative expression found in cold-tolerant cultivated rice and weedy rice than in cold-sensitive populations (Bevilacqua et al., 2015). However, the mechanisms underlying the differential expression of CBF pathway genes have not yet been fully elucidated. A recent study has suggested that cold tolerance variation in rice may be related to methylation of the CBF regulator OsICE1 (Han, Li, Xie, Dai, & Qiang, 2017). Thus, methylation-mediated epigenetic control of gene expression could be an important mechanism underlying cold response variation in cultivated rice and co-occurring weedy rice.

DNA methylation, an important form of epigenetic regulation, is broadly involved in gene expression regulation and stress response during growth and development (Chinnusamy, 2009; Kawakatsu et al., 2016; Sanchez & Paszkowski, 2014; Urano, Kurihara, Seki, & Shinozaki, 2010; Zilberman, Gehring, Tran, Ballinger, & Henikoff, 2007). In plants, DNA methylation occurs...
at three distinct site classes: CG, CHG and CHH (where H is A, C or T). Cytosine methylation in the promoter region tends to negatively regulate gene expression (Smith & Meissner, 2013; Zhang et al., 2006). Methylocnic studies have shown that the methylation level of CG sites is positively correlated with gene expression levels (Wang et al., 2015); however, other studies have not observed a correlation with gene expression (Jones, 2012). Epigenetic studies in Arabidopsis thaliana have suggested that CHH methylation increases with growth temperatures while CG methylation is instead correlated with the climate of origin (Dubin et al., 2015). However, most current studies characterizing DNA methylation are based on DNA methylocnic analyses (Kawakatsu et al., 2016; Zilberman et al., 2007), and few studies have directly examined the methylation of individual genes.

In a previous study, we found that methylation of the ICE1 coding region, including CG, CHG and CHH sites, is correlated with cold tolerance in crofton weed (Ageratina adenophora), which could potentially contribute to its rapid invasion northward in China (Xie et al., 2015). Few studies have been conducted on the role of DNA methylation in rice cold tolerance (Dai et al., 2015; Pan et al., 2011). Moreover, the DNA methylation of the CBF pathway genes has not been examined in relation to the cold tolerance of weedy rice. Since weedy rice is physiologically and morphologically similar to rice, assessing CBF gene methylation in relation to cold tolerance variation of different geographical populations of weedy rice and rice may help to reveal the mechanisms underlying their parallel adaptation to local climates. Studying the expression of CBF pathway genes of different cold-tolerant weedy rice and rice populations can further elucidate the molecular mechanism underlying cold tolerance codifferentiation. To address these topics, we assessed the cold tolerance of 100 weedy rice populations and co-occurring rice varieties across China and investigated whether DNA methylation of the OsICE1 gene is involved in cold tolerance regulation.

2 | MATERIALS AND METHODS

2.1 | Sampling and geographical information collection

A total of 100 weedy rice and 100 associated rice populations were collected in 100 sites across 23 provinces in China, including Heilongjiang, Ningxia, Jiangsu, Guangdong and Hainan provinces, between 2006 and 2016 (Table S1). Seeds of more than 20 weedy rice individuals were collected at each sampling site, with sampling spaced at 20-m intervals. Since cultivated rice plants were typically a single variety within each sampling site, seeds of only one rice individual were collected per site. Seeds of each sampled individual were stored in paper bags, air-dried at ambient temperature and then stored at −20°C. After the weedy rice and rice seeds were collected from the field, the seeds were grown in a common garden environment in Nanjing, China (118°37′E, 32°02′N). The seeds produced in the common environment were used as the materials for the experiment. The latitude, longitude and altitude of each sampling location were recorded using the GPS recorder etrex (Garmin International 1200 E. 151 St. Dock Door #24. Olathe, KS 66062). Climatic information for each sampling site was calculated using data between 2006 and 2016 from the China Meteorological Network (http://data.cma.cn/site/index.html).

2.2 | Cold tolerance determination of weedy rice and rice populations

Seeds of weedy rice and rice were heat-treated at 50°C for 48 hr to eliminate seed dormancy. Then, the seed surfaces were sterilized with 70% ethanol for 30 s and washed with sterile water five times. The seeds were soaked in water overnight and germinated in the dark for 4 days before being transplanted to 10-cm-diameter pots. Pots were maintained at 28°C in a growth chamber with a 12/12-hr photoperiod, 300 µmol m⁻² s⁻¹ fluorescent lighting and 70%–80% relative humidity. For each sampling site, seeds from five weedy rice individuals and the cultivated rice individual were planted; four pots with three seeds per pot were planted for each individual. At the four-leaf stage, three of the pots of seedlings of each accession were placed at 5°C for 3 days and then returned to a 28°C growth chamber for another 3 days (300 µmol m⁻² s⁻¹, 12/12-hr photoperiod, 70%–80% relative humidity). The fourth pot of seedlings was placed at 28°C for 6 days as a control. The cold injury indices of the seedlings were determined as previously described using the 1-cold injury index (Li, Qiang, & Qian, 2008). After seedlings were treated at 5°C for 3 days, photosynthetic parameters of the weedy rice and rice populations were determined using an Imaging-PAM fluorometer (Heinz Walz GmbH; Ehlert & Hincha, 2008). Seeds of five weedy rice individuals and one cultivated rice individual were also used for photosynthetic parameter determination. Each individual, represented by three leaves from three different seedlings, was assessed for photosynthetic parameters including the effective quantum yield of photosystem II (Y(II)), electron transport rate (ETR1) and photochemical quenching (qP); the experiment was conducted in triplicate for each individual. The plants were dark-adjusted for 30 min before measurement, and the distance between the leaf and charge-coupled device (CCD) camera was set to 18 cm. The instrument settings and parameters were set as described previously (Schreiber, Walz, & Kolbowski, 2003), and the saturation pulse intensity was set to 13.

2.3 | Quantitative real-time PCR (qPCR) analysis of CBF pathway genes

The expression levels of five CBF pathway genes were determined by qPCR analysis of three weedy rice populations (WRLN004, WRJS023 and WRGD008) and their associated rice populations.
that together represent the phenotypic range of cold tolerance observed in the study. The five targeted genes were OsICE1, OsCBF1, OsCBF2, OsCBF3 and OsMYB2. As described above, five weedy rice individuals and one cultivated rice individual were used for qPCR analysis. Eight seedlings of each individual were treated at 5°C for 0, 0.5, 1, 2, 4, 8, 12 and 24 hr, and 0.1 g of leaves from each treatment was used for RNA isolation using RNAiso for Polysaccharide-rich Plant Tissue (TaKaRa Bio Inc. Setu 3-4-1, Otsu, Shiga 520-2193, Japan) according to the manufacturer’s protocol. The cDNA was prepared using PrimeScript™ RT Reagent Kit with gDNA Erase (TaKaRa). The reaction mixture (20 µl) contained 10 µl of SYBR® Premix Ex Taq™ (2×; TaKaRa), 2 µl of primers (Table S2), 1 µl of cDNA and 7 µl of ddH2O. The reaction conditions for qPCR were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The fold changes in the CBF pathway genes, which were normalized to beta-actin, were calculated for each population using the 2−ΔΔCt method (Livak & Schmittgen, 2001). Three biological replicates were conducted for each gene, and three different seedlings were used for qPCR analysis in each treatment.

2.4 Methylation determination

After seedlings of the five weedy rice individuals and one rice individual were treated at 5°C for 3 days and cultured at 28°C for another 3 days, 0.1 g of leaves from each individual was used for DNA isolation using a Plant Genomic DNA Kit (TIANGEN Biotech (BEIJING) CO, LTD. Building 1-604, Hangxing, No. 27 Long 258 Caqix Road Xuhui District, Shanghai, China). The methylation sites in the OsICE1 gene of three weedy rice populations (WRLN004, WRJS023 and WRGD008) and their associated rice populations (WRLN004R, WRJS023R and WRGD008R) were determined using bisulphite modification. The OsICE1 promoter (1,000 bp upstream of ATG) of 100 weedy rice populations and their associated rice populations was also determined. Up to 450 ng of genomic DNA was treated using an EZ DNA Methylation-Gold™ Kit (ZYMOL Research) following the manufacturer’s guidelines. Then, PCR amplification was performed using Epitaq™ HS (for bisulphite-treated DNA; TaKaRa) under the following conditions: 10 s at 98°C; 35 cycles of 10 s at 98°C, 1 min at 52°C and 1 min at 72°C; and a final extension for 10 min at 72°C. Each reaction mixture contained 5 µl of 10× Epitaq PCR Buffer (Mg2+ free), 6 µl of a dNTP mixture, 5 µl of MgCl2 (25 mM), 2.0 µl of primers (10 µM; Table S3), 1.25 U of Epitaq HS, 100 ng of DNA and sterilized distilled water (to a final volume of 50 µl). The PCR product was ligated to the pMD™19-T vector (pMD™19-T Vector Cloning Kit, TaKaRa), and 10 clones were sequenced for each sample. The obtained sequences (GenScript USA Inc.) of each sample were compared with the original OsICE1 sequence, and a converted cytosine indicated an unmethylated site. The thresholds for labelling a cytosine as methylated in the CG, CHG or CHH contexts (where H is A, C or T) were set to 80%, 50% and 50%, respectively (Cokus et al., 2008). OsICE1 methylation determination was conducted three times for each individual.

2.5 Data analysis

Correlation analysis was performed to test for associations between the geographical and climate information (latitude, altitude, annual average temperature, maximum temperature, minimum temperature, June average temperature), cold tolerance data, single nucleotide polymorphisms (SNPs) of the OsICE1 promoter and OsICE1 methylation patterns of the different weedy rice and rice populations; analyses were conducted using SPSS 20 software (IBM SPSS Statistics 20), with the Pearson index as the correlation coefficient. Differences in the relative expression levels of the five CBF pathway genes for weedy and cultivated rice populations were analysed by one-way analysis of variance (ANOVA; IBM SPSS Statistics 20) with the least significant difference (LSD) multiple comparison method. These data were plotted by Origin 9 (OriginLab Corporation). The geographical distributions of the cold tolerances and OsICE1 methylation states of the different weedy rice and rice populations were plotted by ArcMap 10 (Environment Systems Research Institute). Differences in OsICE1 methylation levels of CHG and CHH at different latitudes were compared by independent sample t tests (SPSS 20). The boxplots were plotted by Origin 9.

3 RESULTS

3.1 Cold tolerance variation in weedy and cultivated rice populations

Sampled weedy rice and cultivated rice accessions varied widely in their response to the cold treatment (5°C for 3 days; see Section 2), and accessions were classified into three categories according to their cold tolerance abilities: cold-tolerant populations (cold tolerance ≥ 0.58), intermediately cold-tolerant populations (0.20 ≤ cold tolerance < 0.58) and cold-sensitive populations (cold tolerance < 0.20). Representative weedy rice and cultivated rice phenotypes for the three cold tolerance categories are shown in Figure 1. Cold-sensitive plants were nearly dead after cold treatment (Figure 1a,b). Intermediately cold-tolerant plants lost greenness in their leaves, and their growth was significantly inhibited (Figure 1c,d). Cold-tolerant plants showed minimal visible signs of damage, with only a small portion of the leaves curled after cold treatment and no significant differences evident from the untreated control seedlings (Figure 1e,f).

For all plants tested, the cold treatment significantly decreased photosynthetic parameters, including the effective quantum yield of photosystem II (YII), electron transport rate (ETR1) and photochemical quenching (qP). However, as with the cold injury index,
the level of decrease varied widely among samples from different climates (Figure 1g,h). The Y(II), ETR1 and qP values of the cold-tolerant weedy rice populations decreased by 48%–56%, 30%–48% and 35%–60%, respectively, after cold treatment for 3 days. For the intermediate cold-tolerant weedy rice populations, these photosynthetic parameters were decreased by 49%–92%, 45%–72% and 39%–84%, respectively. For the cold-sensitive weedy rice populations, the photosynthetic parameters were decreased by 94%–97%, 81%–100% and 90%–100%, respectively. The cold-sensitive populations showed the most significant decrease in fluorescence parameters compared with those of the intermediately cold-tolerant and cold-tolerant populations, indicating that cold temperatures caused more serious damage to the photosynthetic reaction centre than that exhibited by the other populations.
3.2 | Cold tolerance is correlated with sampling site locations

For both weedy rice and co-occurring cultivated rice varieties, cold tolerance levels were strongly correlated with geographical and climatic data for the sampling sites. For both groups, there were significant positive correlations between cold tolerance and latitude (Figure 2a) and significantly negative correlations with average annual minimum temperature (Figure 2b), average annual temperature (Figure 2c) and June average temperature (Figure 2d). Interestingly, while the weedy rice and cultivated rice populations from different collection sites showed significant cold tolerance variation, weedy and cultivated individuals from a given sampling site showed no significant differences from each other (p < .05; Figure 1g,h). Thus, the weedy rice and associated cultivated rice showed parallel patterns of cold tolerance across the different sampled populations. For both weedy rice and cultivated rice, cold tolerance was not correlated with altitude (Figure 2e) or annual maximum temperatures (Figure 2f) of the sampling sites.

Cold tolerance was broadly correlated with geographical regions of rice cultivation across China. The high-latitude populations, represented by 26 weedy rice populations and 27 cultivated rice varieties, were all in the cold-tolerant category. These populations were located in Heilongjiang, Liaoning, Xinjiang, Ningxia, Gansu and Shanxi provinces at latitudes ranging from 37° to 48°. These northern, cold-tolerant populations occur in regions with low annual average temperatures, ranging from 1 to 12°C. The high-latitude locales are also characterized by low annual minimum temperatures (−37 to −23°C) and cool June average temperatures (16 to 19°C). The intermediately cold-tolerant populations, comprising 63 weedy rice populations and 62 rice populations, were located in central and central-southwest China. These mid-latitude regions (latitudes ranging from 21° to 39°) serve as the main area for rice cultivation in China and include the Hebei, Shandong, Jiangsu, Shanghai, Anhui, Henan, Sichuan, Guizhou, Yunnan and Guangxi provinces. These areas have subtropical or warm temperate climates and relatively higher annual average temperatures, ranging from 11 to 23°C, with minimum temperatures in the range of −20 to 1°C and June average temperatures ranging from 19 to 25°C. The low-latitude populations (latitudes ranging from 18° to 21°), which were represented by 11 weedy rice and co-occurring cultivated rice populations, were all cold-sensitive. These regions have tropical or southern subtropical monsoon climates and high annual average temperatures ranging from 20 to 27°C, the warmest annual minimum temperatures (−6 to 9°C) and the warmest June average temperatures (25 to 28°C).

3.3 | The CBF pathway plays a role in the cold tolerance of weedy rice and rice populations

The qPCR analysis showed that the expression levels of OsICE1, OsCBF1, OsCBF2, OsCBF3 and OsMYB2 were correlated with the cold tolerances of the tested weedy rice and associated cultivated rice plants (Figure 3). For all five genes, expression was low for all plants in the control treatment (0 hr), with no significant differences among accessions. However, expression increased over successive time points of the cold treatment, with significant differences emerging between the plants with different levels of cold tolerance. For the CBF regulator OsICE1, no differences in the expression levels were observed among the different populations before 2 hr of cold treatment (Figure 3a). After 4–8 hr of cold treatment, the expression levels in the cold-tolerant WRLN004 and WRLN004R populations were significantly higher than those in the intermediately cold-tolerant and the cold-sensitive populations. After 12–24 hr of cold treatment, WRLN004 and WRLN004R had the highest OsICE1 expression levels, while the cold-sensitive WRGD008 and WRGD008R populations had the lowest OsICE1 expression levels. Throughout the entire cold treatment process, the OsICE1 expression level showed no significant difference between weedy rice and the co-occurring cultivated rice, consistent with their shared levels of cold tolerance.

For OsCBF1, the different cold-tolerant weedy rice and rice populations showed no significant difference in expression levels before 1 hr of cold treatment (Figure 3b). After 1 hr of cold treatment, the different cold-tolerant weedy rice populations varied significantly in OsCBF1 expression levels, while the cultivated rice populations showed no difference. After 4–24 hr of cold treatment, the cold-tolerant WRLN004 and WRLN004R populations exhibited the highest OsCBF1 gene expression levels, while the cold-sensitive WRGD008 and WRGD008R populations exhibited the lowest expression levels. As with OsICE1, the difference among the different cold-tolerant populations increased as the cold treatment process continued.

Expression patterns for OsCBF3 and OsMYB2 were broadly similar to OsICE1 and OsCBF1 over the cold treatment time-course. OsCBF3 expression in the different cold-tolerant populations gradually increased under cold treatment and peaked at 12 hr of cold treatment (Figure 3d). The weedy rice and rice populations continued to exhibit high OsCBF3 gene expression levels after 4 hr of cold treatment, and the cold-tolerant WRLN004 and WRLN004R plants exhibited higher expression levels than the others. For OsMYB2, there was no significant difference or only a small difference in the OsMYB2 gene expression levels among the different weedy rice and rice populations before 4 hr of cold treatment (Figure 3e). After 8 hr of cold treatment, the cold-tolerant WRLN004 and WRLN004R populations showed significantly higher OsMYB2 expression levels than the others. Throughout the entire cold treatment process, weedy rice generally had the same OsMYB2 gene expression level as its associated cultivated rice population.

OsCBF2 encodes a negative regulator of the CBF pathway, and its expression showed a decline from 2–24 hr that was coincident with the increasing expression of the other genes (Figure 3c). At 1–2 hr of cold treatment, the weedy rice and rice populations all had relatively high OsCBF2 gene expression levels. Weedy rice generally had higher expression levels than its associated rice populations. However, there were no significant differences among the different cold-tolerant weedy rice and rice populations. After 4–12 hr of cold treatment, the OsCBF2 gene expression levels decreased gradually, and the
cold-tolerant WRLN004 and WRLN004R populations decreased more rapidly than the others. This led to higher expression levels of this CBF negative regulator in the cold-sensitive WRGD008 and WRGD008R accession than in the cold-tolerant and intermediate cold-tolerant populations. The weedy rice and rice populations all continued to exhibit low OsCBF2 gene expression levels after 24 hr of cold treatment.

In summary, the CBF pathway genes of different cold-tolerant weedy rice and rice populations showed no significant differences at the initial stage of cold treatment, but the difference gradually increased as the cold treatment progressed. Except for the negative regulator, OsCBF2, the expression levels of CBF pathway genes were positively correlated with cold tolerance. In accordance with the similar cold tolerances of weedy rice and its associated rice population, there was no significant difference or only a slight difference in the CBF pathway gene expression levels between the weedy rice samples and associated cultivated rice varieties. These patterns indicate that CBF pathway genes play a role in the shared patterns of cold tolerance in weedy rice and cultivated rice populations.

3.4 OsICE1 promoter methylation is correlated with cold tolerance in weedy rice and cultivated rice

Whereas the coding regions of OsICE1 were not methylated in three sampled weedy rice populations and their associated rice populations (see data in Dryad archive), methylation was present in the promoter region, and weedy rice shared the same DNA sequence in this promoter region with its associated cultivated rice. There were six SNPs in OsICE1 promoter region (-670C/deletion, -546 C/deletion, -463 deletion/C, -420 deletion/C, -404C/A and -392C/G). However, these SNPs were not correlated with cold tolerance (Table S4). Overall, the total numbers of methylated sites in the OsICE1 promoter were 22–33
and 21–35 for weedy rice and cultivated rice, respectively. Weedy rice had 19–23 CG methylation sites, 1–7 CHG methylation sites and 0–5 CHH methylation sites, while cultivated rice had 15–24 CG methylation sites, 1–7 CHG methylation sites and 0–7 CHH methylation sites (Table S1). Among the 100 geographical locations sampled, 68 were characterized by having the same methylation levels in weedy rice samples as in the co-occurring cultivated rice. For the remaining locales, methylation levels in the weedy rice populations were only slightly different from those in their associated rice populations (Figures 4, S1 and S2). Thus, throughout the sampled geographical range, weedy and cultivated rice from a given locale shared identical or similar OsICE1 promoter methylation patterns.

The OsICE1 promoter methylation levels in the weedy rice and cultivated rice populations were significantly negatively correlated with cold tolerance and the climates of the sampling sites, with plants from colder climates showing low OsICE1 promoter methylation levels and those from warmer climates showing high methylation levels. Consistent with this pattern, the OsICE1 promoter methylation levels in the representative cold-tolerant (WRLN004 and WRLN004R), intermediate cold-tolerant (WRJS023 and WRJS023R) and cold-sensitive (WRGD008 and WRGD008R) weedy rice and rice populations were significantly negatively correlated with OsICE1 gene expression level (Figure 5). Similarly, the promoter methylation levels were also significantly negatively correlated with expression levels of other CBF pathway genes (Figure S3). These patterns held true when considering all OsICE1 promoter methylation sites (Figure 5a) and for CHG and CHH methylation levels specifically (Figure 5b), although not for CG methylation level as the three typical cold-tolerant weedy rice and rice cultivars all had 20 CG methylation sites in the OsICE1 promoter (Table S1). For both weedy rice and cultivated rice, the OsICE1 promoter methylation level was positively correlated with the minimum temperature, annual average temperature and June average temperature of collection locations (Figure 6). These same correlations were also apparent for CHG and CHH methylation levels individually (Figure 7), but not for CG methylation levels (Figure S4).

The CG methylation sites on the OsICE1 promoters of weedy rice and rice populations were highly conserved, as the methylation sites were essentially the same between weedy rice populations and co-occurring rice varieties. The CHG methylation sites were also

FIGURE 3  CBF pathway gene expression levels in different cold-tolerant weedy rice populations and their associated rice populations. The letters indicate significant differences among the different populations, \( p < .05 \)
relatively conserved. Most of the CHG methylation sites between the weedy rice and rice populations were the same and occurred mostly between −385 and −574 bp. Compared with the CG and CHG methylation sites, the diversity of the CHH methylation sites was significantly increased. Only some of the methylation sites were conserved between the weedy rice and rice populations (Figure 8).

3.5 CHG and CHH methylation play different roles in cold tolerance differentiation at different latitudes

At similar latitudes, there were also regional differences in levels of cold tolerance that were correlated with methylation patterns. In the high-latitude regions, where the annual average temperature was approximately 20°C, the weedy rice and cultivated rice populations of the Northeast (Heilongjiang, Jilin and Liaoning) had higher cold tolerance than those of the northwest (Xinjiang, Ningxia, Shanxi and Gansu). These regional differences in cold tolerance were correlated with the CHG methylation levels of the OsICE1 promoter (Figures 9a and S5a), but not with the CHH methylation levels (Figure S5b). Both weedy and cultivated rice populations in Northeast China had only one CHG methylation site on the OsICE1 promoter with few exceptions. The northwest weedy rice and cultivated rice populations had 4–5 and 3–5 CHG methylation sites, respectively.

The mid-latitude regions (including Hebei, Shandong, Henan, Jiangsu, Anhui, Shanghai, Hubei, Jiangxi, Hunan, Zhejiang, Sichuan, Guizhou, Yunnan and Guangxi) serve as the main area for rice cultivation and have an annual average temperature of 24°C. However,
the cold tolerances of weedy rice and rice in these areas also varied, decreasing significantly from the central northeast to the central northwest (Figure S5c,d). Interestingly, in contrast to high-latitude regions, differences in both CHG and CHH methylation levels at the OsICE1 promoter contributed to the cold tolerance differentiation between these mid-latitude regions (Figure 9c,d). The central northwest populations had higher CHG and CHH methylation levels than the central northeast populations. The weedy rice and rice populations in the central northeast, including the Hebei, Shandong, Jiangsu, Anhui, Shanghai and Zhejiang populations, had 4–7 CHG methylation sites and 1–3 and 0–3 CHH methylation sites, respectively. The central northwest weedy rice and rice populations in Henan, Hubei, Jiangxi, Hunan, Sichuan, Guizhou, Yunnan and Guangxi had 4–6 and 3–5 CHG and CHH methylation sites, respectively (Figure S5e,f). Consistent with this observation, the weedy rice and rice populations in these regions were universally cold-sensitive, and little difference in cold tolerance was observed among these populations (Figure S5e,f). Consistent with this observation, the weedy rice and cultivated rice populations all had similar CHG and CHH methylation levels, with 6–7 CHG methylation sites and 3–5 and 3–7 CHH methylation sites, respectively (Figure 9e,f). Compared with the high-latitude and mid-latitude populations, the relatively high CHG and CHH methylation levels in the low-latitude populations is consistent with the low cold tolerance observed in this subtropical to tropical region.

4 | DISCUSSION

The cold tolerances of different cultivated rice varieties and their associated weedy rice populations differed substantially across China, and cold tolerance was closely correlated with the local climate (Figure 2). Cultivated rice is the product of artificial selection, and only the rice strains that perform well at local temperatures would be expected to be incorporated into the local crop germplasm. Rice frequently encounters low-temperature stress during the sowing period, especially in temperate regions, and the cold tolerance of rice is essential for local adaptation. Increased cold tolerance in rice appears to come at an energetic cost, however, as it reduces rice yield in tropical and southern subtropical regions where low-temperature stress rarely occurs (Mitra & Bhatia, 2008; Morin et al., 2007; Stoks & De Block, 2011). Therefore, cold-tolerant rice strains are unlikely to be selected in these warmer areas.

A notable finding of this study is the very close similarity in levels of cold tolerance between local cultivated rice varieties and their co-occurring weedy rice populations (Figure 2). Unlike cultivated rice, weedy rice has invaded rice production areas despite attempts to keep it out, and it has adapted to local climates without the benefits of deliberate artificial selection. While some weedy rice strains are direct descendants of the modern cultivars whose fields they infest (e.g., some Malaysian weedy rice strains; Song et al., 2014), the majority are not, and recent whole-genome resequencing studies suggest that some contemporary weed strains originated early in the history of rice cultivation (Li et al., 2017). The ability of weedy rice to adapt across such a wide range of climates as were sampled in this study explains why weedy rice can occur in a broad range of environments naturally. This adaptability has undoubtedly played a role in the widespread occurrence of weedy rice in most of the world’s rice production areas, including Asia, Europe, Africa, North America and South America (Wedger & Olsen, 2018).

The cold tolerances of the weedy rice and rice populations were correlated with local temperatures, with the more northeastern populations (corresponding to those at higher latitudes) having higher cold tolerances than the southwestern populations. This pattern corresponds to the lower minimum temperatures in the northeast regions (Table S1) and accounts for the significant correlations between cold tolerance and latitude (Figure 6). This regional variation also corresponds to variation in the predominant rice subspecies that is cultivated locally. In China, japonica rice tends to be grown at higher latitudes while indica rice is grown in warmer, lower-latitude regions. Similarly, japonica-like weedy rice occurs more in cooler, northern regions, and indica-like weeds predominate at lower latitudes. In general, japonica populations have a higher cold tolerance than indica populations (Lv et al., 2015). This provides a basis for the selection of rice varieties in China, and japonica varieties are preferred in high-latitude, low-temperature areas.
FIGURE 6  Correlation analysis of the OsICE1 promoter methylation level and the cold tolerance and geographical information [Colour figure can be viewed at wileyonlinelibrary.com]
FIGURE 7  Correlation analysis of the CHG and CHH methylation levels of the OsICE1 promoter and the cold tolerance and geographical information [Colour figure can be viewed at wileyonlinelibrary.com]
The cold tolerance of weedy rice and rice populations was significantly correlated with CBF pathway gene expression levels, indicating that the CBF pathway plays important roles in weedy rice and rice cold tolerance regulation. However, the differential expression of CBF pathway genes among the different weedy rice and rice populations needs to be further elucidated. Our previous study in invasive crofton weed showed that only ICE1 coding region had varying methylation levels among the different geographical populations. For that species, methylation of the ICE1 coding region, including CG, CHG and CHH sites, was negatively correlated with cold tolerance, which may be responsible for its rapid expansion in China (Xie et al., 2015). Studies in other species have shown that the DNA methylation of promoters negatively regulates gene expression (Smith & Meissner, 2013; Zhang et al., 2006); in contrast, CG methylation of the gene body is significantly associated with Arabidopsis habitat temperatures (Dubin et al., 2015; Kawakatsu et al., 2016). In our study, the CG methylation level of the OsICE1 promoter was not correlated with cold tolerance. CHG and CHH methylation of the OsICE1 promoter was negatively correlated with CBF pathway gene expression, which may contribute to the cold tolerance differentiation in weedy rice and rice. These findings indicate that DNA methylation of the ICE1 coding regions and promoters may play different roles in different plant species.
Besides broad geographical patterns of climatic adaptation, there was also cold tolerance differentiation among weedy rice and rice populations at similar latitudes within the high- and mid-latitude regions. The cold tolerance differentiation among populations at high latitudes was correlated with the CHG methylation of the OsICE1 promoter, while for mid-latitude populations, cold tolerance was correlated with both CHG and CHH methylation levels.

It has been reported that the methylation of CG sites is affected by the sample collecting site temperatures in Arabidopsis (Dubin et al., 2015). However, our findings indicate that CHG and CHH methylation of the OsICE1 promoter could produce different responses to low temperatures. In moderately low-temperature regions (June average temperatures of 19 – 25°C, annual average temperatures of 10.7 – 25.7°C, minimum temperatures of −22 – 2°C), plants tend to have low CHG and CHH methylation levels. In colder regions (June average temperatures of 16.8 – 18.9°C, annual average temperatures of 1.6 – 11.8°C, minimum temperatures of −37 – 19°C), plants have even lower CHG methylation levels. These findings point to an epigenetic mechanism of the spread of weedy rice concomitant with rice cultivation into northern climates and provide a clue for understanding the morphological and physiological similarity between rice and weedy rice (Delouche et al., 2007; Qiu et al., 2017; Ziska et al., 2015).

The CG methylation sites on the OsICE1 promoter were relatively stable among the different weedy rice and rice populations, while the CHH methylation sites exhibited the most diversity. The relationship between the cold tolerance differentiation and methylation of the OsICE1 promoter suggests that the CG methylation of weedy rice and rice cultivars may not play a role in responding to cold temperature and are stable under various climates. The CG methylation did not differ significantly under different temperature conditions and was not correlated with temperature. The CHH methylation differed significantly under various temperatures, leading to the primary adaptation to low temperatures and widespread cultivation areas of weedy rice and rice. The CHG methylation differed in only lower environmental temperatures at high latitudes and had lower methylation diversity than CHH, as it did not differ under primary low-temperature stress. Previous studies have shown that CHH methylation in the gene body is significantly associated with Arabidopsis habitat temperatures (Dubin et al., 2015; Kawakatsu et al., 2016), and our study suggested that CHH and CHG methylation of the OsICE1 promoter is also associated with response to low temperatures. However, additional studies will be required to establish a definitive causal relationship between the observed OsICE1 promoter methylation patterns and cold tolerance.

The above results reveal for the first time that the convergence of cold tolerance in rice varieties and co-occurring weedy rice populations is correlated with similar methylation levels of their OsICE1 promoter regions. While the data from this study do not provide evidence on the mechanism by which this adaptive convergence has occurred, we suggest that there are three possibilities. Numerous genetic studies, including recent genome resequencing and population genomic analyses.
(Li et al., 2017; Qiu et al., 2017), indicate weedy rice strains worldwide have evolved repeatedly through a process of de-domestication (feral-ization) from cultivated rice (Wedger & Olsen, 2018). Given that cultivated rice underwent strong selection for local climatic adaptation as rice farming spread into temperate regions, the ability of weedy rice to rapidly adapt across this same range of climates could be a legacy of earlier selection that occurred in its domesticated ancestors. Thus, standing variation in weedy rice populations could provide a reservoir of adaptive variation upon which selection has acted. A second possibility is that, provided sufficiently high mutation rates, adaptive mutations could arise de novo within the weed populations that confer the cold tolerance (and associated changes in CBF pathway gene expression and OsICE1 promoter methylation). The third possibility is local weed populations have acquired cold tolerance through hybridization with local crop varieties (e.g., Zhang et al., 2015, 2018) and adaptive introgression of cold tolerance. Although both cultivated rice and weedy rice are predominantly self-fertilizing, outcrossing and hybridization does occur (Chen, Dong, Song, Suh, & Lu, 2004; Cui, Dai, Qi...


### Supporting Information
Additional supporting information may be found online in the Supporting Information section.