Improved Cold Tolerance in *Brassica rapa*: Functional Diversification of BrICE1 and BrICE2 Paralogs via CBF and ROS Scavenging Pathways, Balancing Growth and Defense

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Article

Improved Cold Tolerance in *Brassica rapa*: Functional Diversification of BrICE1 and BrICE2 Paralogs via CBF and ROS Scavenging Pathways, Balancing Growth and Defense

Wangze Wu [1,*,†], Haobo Yang [2,†], Peng Xing [1], Guoting Zhu [1], Xueyan Han [1], Guotai Min [1], Haijun Ding [1], Guofan Wu [1] and Zigang Liu [3]

[1] College of Life Science, Northwest Normal University, Lanzhou 730070, China; xxfxpxp@163.com (X.P.); zhuguo2022212@163.com (G.Z.); 2021212777@nwnu.edu.cn (X.H.); 2174029198@qq.com (G.M.); 2975361560@qq.com (H.D.); wugf1971@163.com (G.W.)

[2] School of Life Sciences, Guangzhou University, Guangzhou, 510006, China; yhb998125@163.com

[3] State Key Laboratory of Aridland Crop Science, College of Agronomy, Gansu Agricultural University, Lanzhou 730070, China; lzgworking@163.com

* Correspondence: wangzew78@sina.cn

† These authors contributed equally to this work.

Abstract: Inducer of C-Repeat Binding Factor (CBF) expression (ICE), a well-known MYC-type bHLH transcription factor, plays a crucial role in the ICE-CBF-cold regulated (COR) cold signaling pathway. *Brassica rapa (B. rapa),* an important oilseed and vegetable crop, exhibits strong chilling and freezing tolerance. However, the exact molecular mechanisms by which ICE1 functions in *B. rapa* remain unclear. Here, 41 ICE1 homologous genes were identified from six widely cultivated Brassica species using a bioinformatics approach. These genes exhibit high conservation but reveal evolutionary complexity between diploid and allotetraploid species. Low-temperature stress induced ICE1 homolog expression, with patterns differing between strong and weak cold-tolerant varieties. Two novel ICE1 paralogs, BrICE1 and BrICE2, were cloned from Longyou 6. They positively regulate cold tolerance in *B. rapa* via a CBF-dependent pathway, contributing to reactive oxygen species (ROS) scavenfging and osmotic adjustment. Immunoblot analysis revealed that low temperatures induce BrICE1 and BrICE2 degradation via the 26S-proteasome pathway. In summary, ICE1 exhibits complex evolutionary relationships in Brassica species. BrICE1 and BrICE2 positively regulate cold tolerance via the CBF-dependent pathway and ROS scavenging mechanism and are also responsible for balancing the development and cold defense of *B. rapa*.

Keywords: BrICE1 and BrICE2; freezing tolerance; expression pattern; phylogenetic tree; *Brassica rapa*

1. Introduction

Low temperatures are a major ecological and environmental factor that strongly affects plant development and geographic distribution. Unlike animals, plants lack mobility and rely on sophisticated regulatory mechanisms for environmental adaptation [1]. Cold acclimation is crucial for plant responses to cold stress. During this process, the induction of cold-regulated (COR) genes is an important biological event [2]. COR genes encode key enzymes and cryoprotective proteins, such as soluble sugars, soluble proteins, and proline, which protect plant cells against cold-induced damage [1]. Under low-temperature stress, the C-Repeat-Binding Factor (CBF) is rapidly induced and regulates the expression of downstream COR genes by binding to their promoter regions [3–5]. CBFs are key upstream transcription regulators of COR genes, and their expression can be accurately

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controlled by upstream transcription factors. Among these factors, ICE1 (Inducer of CBF expression 1), a MYC-like bHLH transcription factor, is the best-characterized positive regulator of CBF genes identified to date [3–5].

Under cold stress, ICE1 directly binds to the MYC recognition motif of the CBF3 promoter, leading to the activation of *CBF3* expression [3]. In Arabidopsis, loss-of-function mutations in ICE1 lead to reduced resistance to cold stress, whereas ICE1 overexpression enhances the cold-induced upregulation of *CBFs* [3]. ICE2, a paralog of ICE1 with 61% identity, shares similar functions. Overexpression of either *ICE1* or *ICE2* enhances freezing resistance in transgenic Arabidopsis [3,6,7]. Notably, ICE1 primarily regulates *CBF3* expression in cold signaling [3], while ICE2 mainly targets CBF1 [7]. This suggests functional redundancy with distinct downstream targets. However, other studies using loss-of-function mutants suggest ICE1 may play a more dominant role compared with ICE2 [8].

Although ICE1 is the key regulator in the ICE1-CBF-COR cold signaling pathway, *ICE1* expression itself is not responsive to cold at the transcriptional level [3]. Its activity is controlled at the protein level by the 26S-proteasome pathway [9,10], highlighting the importance of post-translational regulation (PTM). Emerging evidence indicates that multiple PTMs control ICE1 cell turnover and duration at low temperatures. HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 (HOS1), a ubiquitin E3 ligase with a RING finger, directly interacts with ICE1, promoting its degradation and negatively regulating cold resistance [10]. Conversely, SAP AND MIZ1 DOMAIN-CONTAINING LIGASE1 (SIZ1), a SUMO E3 ligase, enhances cold tolerance by stabilizing ICE1 through SUMOylation, which reduces HOS1-mediated ubiquitination [11]. OPEN STOMATA 1 (OST1), a Ser/Thr protein kinase involved in Abscisic acid (ABA) signaling, can be activated by cold stress. Cold-activated OST1 phosphorylates ICE1 and enhances its stability by interfering with the interaction between HOS1 and ICE1, thereby enhancing freezing tolerance [9]. Beyond their role in cold signaling, ICE1/SCREAM (SCRM) and ICE2/SCRM2 are also involved in diverse processes, including stomatal development [12,13], flowering [14], primary seed dormancy [15], male fertility, and ABA signaling [16]. These findings suggest that ICE1 is not only a central component in the ICE1-CBF-COR cold signaling pathway but also serves as a convergence point, integrating multiple signals to regulate both cold tolerance and plant growth.

Given its crucial role, ICE1 homologs have been identified in numerous plants, including wheat (*Triticum aestivum*) [17], rice (*Oryza sativa*) [18], *Saussurea involucrata* [19], maize (*Zea mays*) [20], and tomato (*Solanum lycopersicum*) [21]. Although these ICE1 homologous genes are involved in cold stress, different species may display diverse ICE1-dependent cold regulation mechanisms [22–24]. For example, in Arabidopsis, cold-activated MPK3 and MPK6 kinases phosphorylate ICE1, targeting it for degradation and negatively regulating cold responses [23,24]. However, in rice, cold-activated OsMAPK3 phosphorylates and stabilizes OsbHLH002 (a rice ICE1 homolog), leading to OsTPP1 activation and increased OsTPP1-regulated trehalose content [22]. These contrasting findings indicate that ICE1 is evolutionarily conserved but contains functional divergence in cold signals in different species, particularly between Arabidopsis and rice. This functional differentiation of ICE1 was also supported by a recent study in which *PsnICE1*, a poplar ICE1 homolog (*Populus tomentosa Carr*), was not only involved in the CBF-dependent pathway but also involved in reactive oxygen species (ROS) scavenging in response to cold stress by binding to different cis-acting elements [25].

In addition to the functional diversity of different species, conflicting results exist regarding Arabidopsis ICE1's role in cold signaling. For example, *ice1* (a single substitution mutant of Arg-236 to His), a well-known dominant-negative mutant, exhibits reduced chilling and freezing tolerance, regardless of cold acclimation [3]. However, another study found that *ice1-2* and *ice2-2*, two T-DNA insertional mutants, did not exhibit any sensitive freezing tolerance phenotype in the absence of cold acclimation, suggesting that ICE1 and ICE2 may not be involved in basal cold resistance of plants [8]. Over the past 30 years, although the biological function of ICE1 has been well-understood in Arabidopsis and rice, neither are winter plants and, therefore, cannot survive safely in winter. However, some winter crops, such as winter rapeseed (*Brassica rapa* [*B. rapa*]), can even survive at

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extremely low temperatures (-20 °C to -30 °C) [26]. Theoretically, these winter crops may have evolved more effective cold acclimation mechanisms to respond to chilling and freezing stresses.

Winter rapeseed (*B. rapa*) is an important oilseed and economic crop worldwide. Some winter *B. rapa* varieties can even survive at extremely low temperatures (-32 °C) [27]. Considering ICE1's role in cold signaling, some researchers have investigated its physiological functions in *B. rapa*. However, unlike in Arabidopsis, the phylogenetic, evolutionary, and physiological functional divergence of ICE1 paralogs in cold signals remains unknown. This study aimed to investigate the functional divergence of ICE1 paralogs and their role in cold tolerance of *B. rapa* compared with Arabidopsis.

2. Results

2.1. Identification and Phylogenetic Analysis of ICE1 Homologous Genes in Brassica Species

Several studies have demonstrated that ICE1 in *B. rapa* is involved in cold pathway signaling [28,29]. However, no study has reported the functional redundancy and structural evolutionary relationship between ICE1 and ICE2 in Brassica species under cold signals. To identify the homologous genes of *ICE1* in six widely cultivated Brassica species (*B. rapa*, AA; *B. nigra*, BB; *B. oleracea*, CC; *B. juncea*, AABB; *B. napus*, AACC; and *B. carinata*, BBCC), the protein sequences of Arabidopsis ICE1 and ICE2 were used as queries to search the Brassicaceae Database (BRAD) (http://brassicadb.cn). Dicotyledon tomatoes (*Solanum lycopersicum*) and soybeans (*Glycine max*), and monocotyledon maize (*Zea mays*), foxtail millet (*Setaria italica*) and rice (*Oryza sativa*) served as controls for comparison with known ICE1 homologs. Phylogenetic analysis revealed distinct clusters for monocot and dicot ICE1 homologs (Figure 1). A total of 42 *ICE1* homologous genes were identified in six widely cultivated Brassica species, including a variant of *B. rapa* (Z1, yellow sarson, an oilseed crop). These genes were divided into two subgroups (ICE1 and ICE2) based on the phylogenetic tree (Figure 1).

Four ICE1-like homologous genes were each identified in the diploid species *B. rapa* (AA; Chinese cabbage) and *B. oleracea* (CC). Notably, the hybridized allotetraploid species *B. napus* (AACC) contained 10 *ICE1* homologous genes. The diploid *B. nigra* (BB) contained five *ICE1* homologous genes, while the hybridized allotetraploid *B. juncea* (AABB) contained 10 *ICE1* homologous genes. The hybridized allotetraploid *B. carinata* (BBCC) contained only five *ICE1* homologous genes. Among the three diploid Brassica species, *B. rapa* is a mesohexaploid diploid with a triplicated chromosomally segmented genome [30]. Amino acid sequence alignment of Z1 showed that two *ICE1* homologous genes belonged to the ICE1 subgroup, and the other two belonged to the ICE2 subgroup. Noteworthy, four *ICE1* homologous genes were identified in Chiifu-401-42 (*B. rapa*, Chinese cabbage, as a vegetable), among three belonged to the ICE1 subgroup and one belonged to the ICE2 subgroup. However, BraA06g038100.3C lost a partial domain in the MYC-like bHLH region, suggesting it may not be a true ICE1 homolog (Supplementary Figure S1). Phylogenetic tree analysis of *ICE1* homologous genes in Brassica species suggested that the evolution of *ICE1* homologous genes was complex during genome hybridization and polyploidization, particularly in allotetraploid Brassica species.

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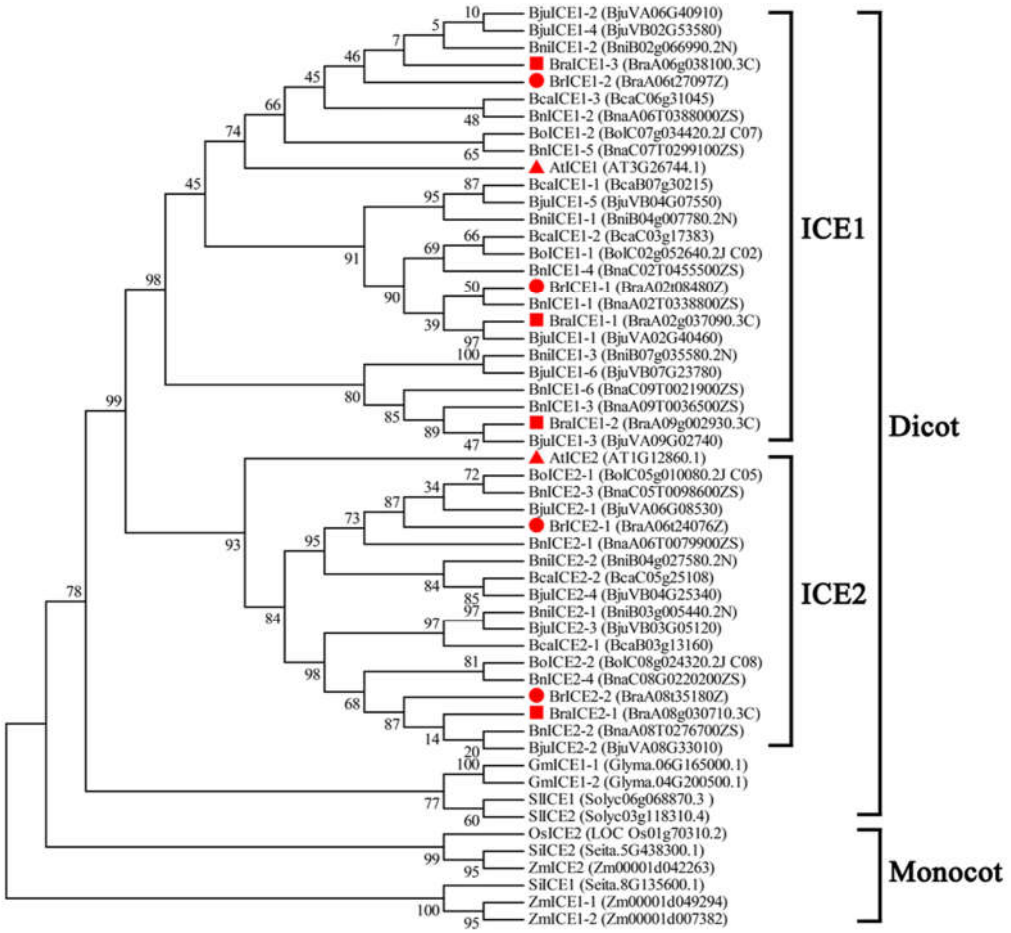

Figure 1. Phylogenetic analysis of *ICE1* homologous genes in Brassica species. The phylogenetic tree was constructed by neighbor-joining distance using MEGA 6.0. A total of 42 *ICE1* homologous genes was identified from Brassica species. Well-known *ICE1* and *ICE2* homologous genes of dicotyledon tomato (*Solanum lycopersicum*) and soybean (*Glycine max*), monocotyledon maize (*Zea mays*), foxtail millet (*Setaria italica*) and rice (*Oryza sativa*) were used as outgroup. *BrICE*, *BraICE*, *BoICE*, *BniICE*, *BnICE*, *BjuICE* and *BcaICE* mean the *ICE1* homologous genes of Z1 (*B. rapa*, yellow sarson, as oilseed crop), Chiifu-401-42 (*B. rapa*, Chinese cabbage, as vegetable), *B. oleracea*, *B. nigra*, *B. napus*, *B. juncea* and *B. carinata*, respectively.

2.2. Low Temperature Induces the Expression of BrICE1 Homologous Genes

To explore the expression patterns of *ICE1* homologous genes under cold stress, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using various freeze-resistant Brassica species varieties. Results showed that low temperatures induced the expression of *ICE1* homologous genes (Figure 2A–D). However, the expression patterns varied across different freeze-resistant varieties. As shown in Figure 2, chilling treatment for 6–12 h induced the expression of *ICE1* homologous genes in Tianyou 2 and Westar (weakly cold-resistant varieties) (Figure 2A,B). In contrast, Longyou 8 and Longyou 6 (strongly cold-resistant varieties) displayed significant induction only after 24 h (Figure 2C,D). Moreover, the expression levels of *ICE1* homologous genes peaked at 24 h of chilling stress in Longyou 8 and Longyou 6. However, in Tianyou 2 and Westar, the peak occurred at 6 h and then rapidly declined after 6 h. These results indicate that the *ICE1* homologous genes in Brassica species are involved in the cold response, with expression patterns that differ in cold-resistant varieties under cold stress.

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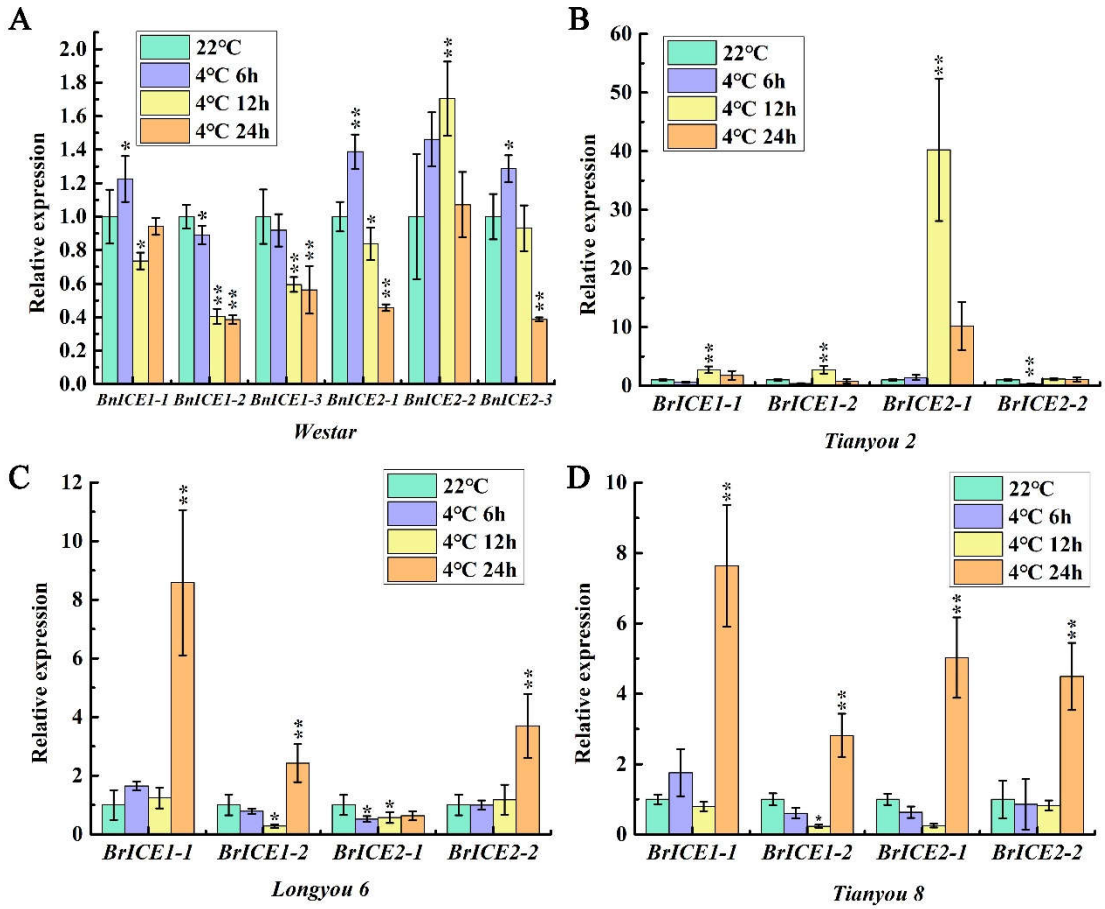

Figure 2. Low temperature induces the expression of *ICE1* homologous genes. 14-day-old seedlings were low temperature treated at 4 °C for 6 h, 12h and 24h, the expression levels of *ICE1* homologous genes were determined by qRT-PCR. BRACTIN2 were used as control. **(A)** The expression profiles of six *BnICE1* homologous genes in Westar. **(B-D)** The expression profiles of four *BrICE1* homologous genes in Tianyou 2, Longyou 6 and Longyou 8, respectively. Values are shown as means ± SD (n=3) of three independent experiments. Statistically significant differences are indicated by asterisks (Student's *t*-test, *, $p < 0.05$).

2.3. Cloning and Protein Structural Domain Analysis of BrRICE1 Homologous Genes

Based on the phylogenetic tree and protein structural feature analysis, two of the four *ICE1* homologous genes, *BrICE1-1* and *BrICE2-1*, were isolated from Longyou 6 using RT-PCR to investigate their role in cold signaling. The full-length cDNA of *BrICE1-1* contains 1,491 bps, encoding a protein of 497 amino acids, whereas the cDNA of *BrICE2-1* comprised 1,320 bps, encoding a protein of 440 amino acids (Figure 3). A BLASTp search against the BRAD database revealed 64.5% identity between BrICE1-1 and BrICE2-1 (data not shown). Protein structural domain analysis of BrICE1-1 and BrICE2-1 demonstrated that they share common structural domains, including conserved serine-rich (S-rich) region sites, ZIP region domain, conserved MYC-like bHLH domain, ICE-specific domain, potential SUMOylation site, and ACT-like domain, all of which are typical features of ICE1 [3,6,7]. BrICE2-1 contains a conserved glutamine-rich and leucine-rich region-specific domain in the variable N-terminal (Figure 3). Owing to the *ICE1* homologous genes of *B. rapa* being previously named *BrrICE1.1* (in *Brassica rapa* var. rapa) [28] or *BcICE1* (in *Brassica campestris*) [29], to maintain consistent nomenclature for *B. rapa* ICE1 homologs, as described previously, *BrICE1-1* and *BrICE2-1* were renamed *BrICE1* and *BrICE2*, respectively.

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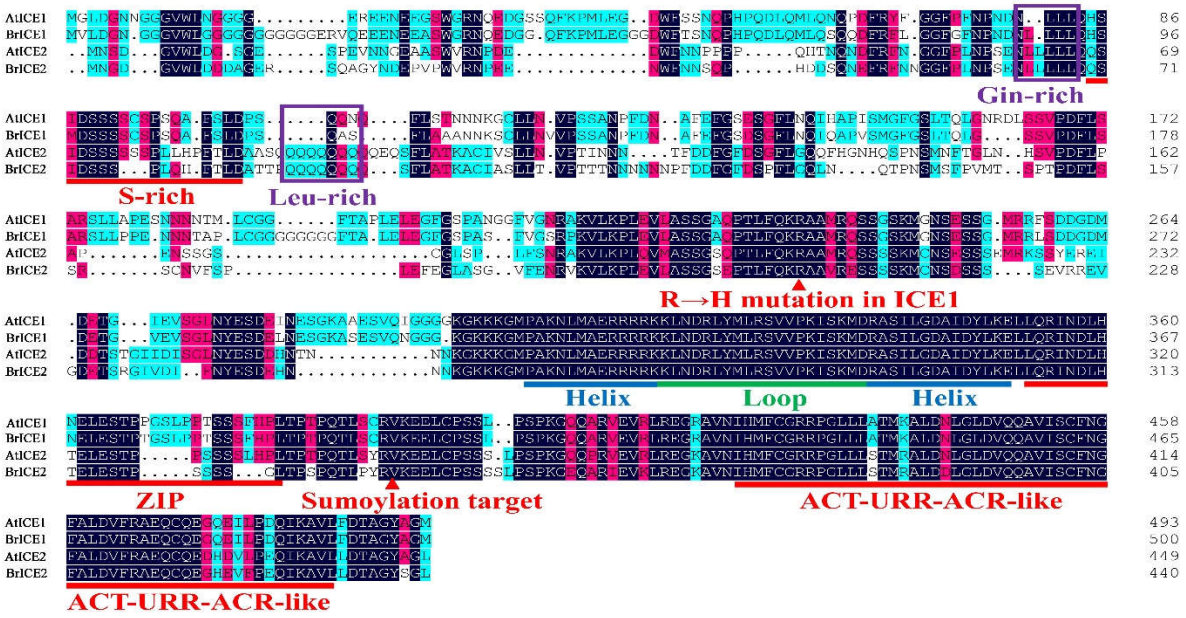

Figure 3. Multiple sequence alignment and domain structure analysis of BrICE1 and BrICE2 of *Brassica rapa*. DNAMAN v9.0 software was used to align the amino acid sequence of BrICE1 and BrICE2. Residues in red indicate conserved serine-rich (S-rich) region sites, ZIP region domain, ICE-specific domain, potential SUMOylation site, and ACT-like domain. Residues in blue and green indicate conserved MYC-like bHLH domain. Residues in purple indicate specific glutamine-rich and leucine-rich region domain of BrICE2.

2.4. BrICE1 and BrICE2 Localize to the Nucleus, and Low Temperature Does not Affect Localization

Previous studies have shown that AtICE1 in Arabidopsis functions within the nucleus [3]. To determine the subcellular localization of BrICE1 and BrICE2, 35S:BrICE1-GFP and 35S:BrICE2–GFP were constructed and transiently expressed in tobacco leaves (Figure 4). Meanwhile, Arabidopsis 35S:AtICE1-GFP and 35S:AtICE2-GFP were constructed as the positive control, and 35S:BAK1-GFP was constructed as a plasma membrane marker. Confocal imaging of GFP fluorescence showed that the BrICE1-GFP and BrICE2-GFP fusion proteins were present in the nucleus, consistent with the localization of AtICE1-GFP and AtICE2-GFP fusion proteins and previous research [3]. In contrast, the GFP fluorescence from BAK1-GFP was observed in the plasma membrane (Figure 4). These observations confirm that BrICE1 and BrICE2, similar to AtICE1 in Arabidopsis, localize to the nucleus.

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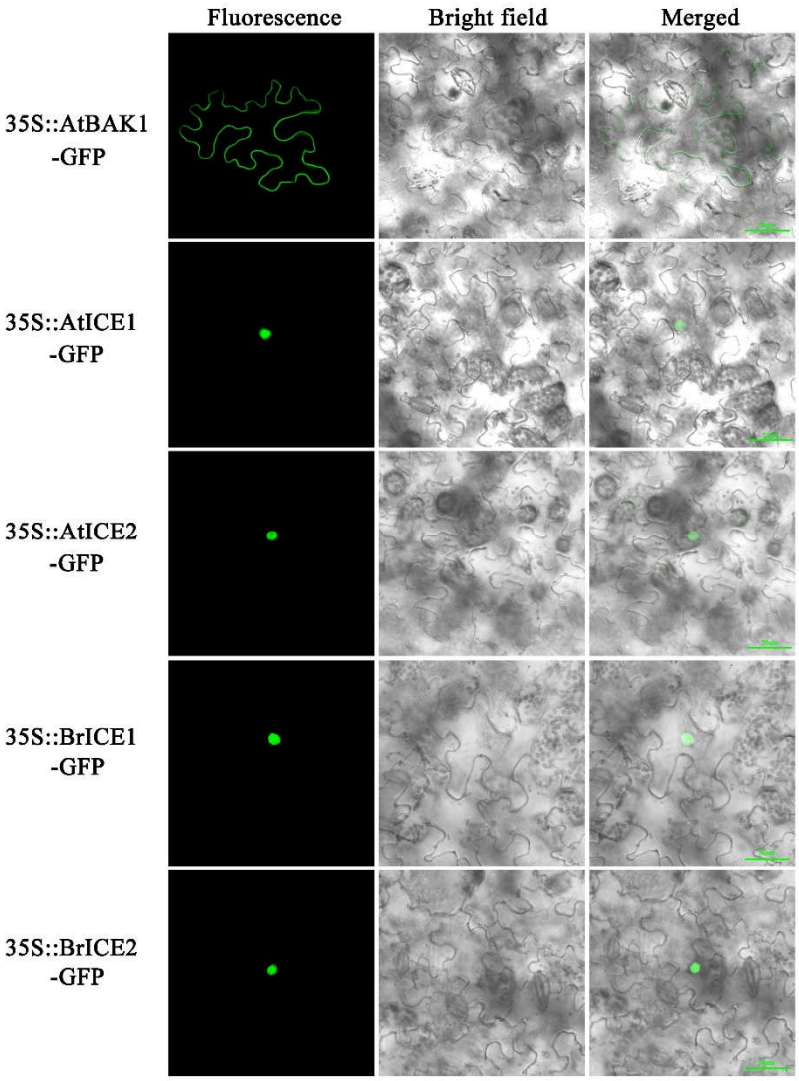

Figure 4. BrICE1 and BrICE2 are nuclear-localized protein. 35S:BrICE1-GFP, 35S: BrICE2-GFP, 35S:AtICE1-GFP, 35S:AtICE2-GFP and 35S:BAK1-GFP plasmids were constructed and were transiently expressed in tobacco leaves, the GFP signal was visualized under confocal microscope. From left to right, respectively: green fluorescence represents (GFP), bright field and merged image. Scale bar was 50 μm.

Several studies have demonstrated that AtICE1 is mainly localized to the nucleus, and cold stress does not significantly affect its localization [3,10]. To further investigate whether cold stress affects BrICE1 and BrICE2 localization, the roots of BrICE1-GFP and BrICE2-GFP transgenic plants were observed after cold treatment. Strong GFP fluorescence was observed in the nuclei at 22 ℃ (Figure 5A,B). While the fluorescence signal remained localized in the nuclei after cold treatment (at 4 ℃ for 12 h), it became weaker (Figure 5A,B). Similar changes were observed in the roots of AtICE1-GFP and AtICE2-GFP transgenic plants. However, we did not observe significant differences between BrICE1-GFP and BrICE2-GFP transgenic plants. Notably, the fluorescence signal was slightly stronger in BrICE1-GFP and BrICE2-GFP transgenic plants than in 35S:AtICE1-GFP and 35S:AtICE2-GFP. These results indicate that similar to AtICE1 and AtICE2 of Arabidopsis, BrICE1 and BrICE2 of *B. rapa* are nucleus-localized proteins, and their localization is not affected by cold stress.

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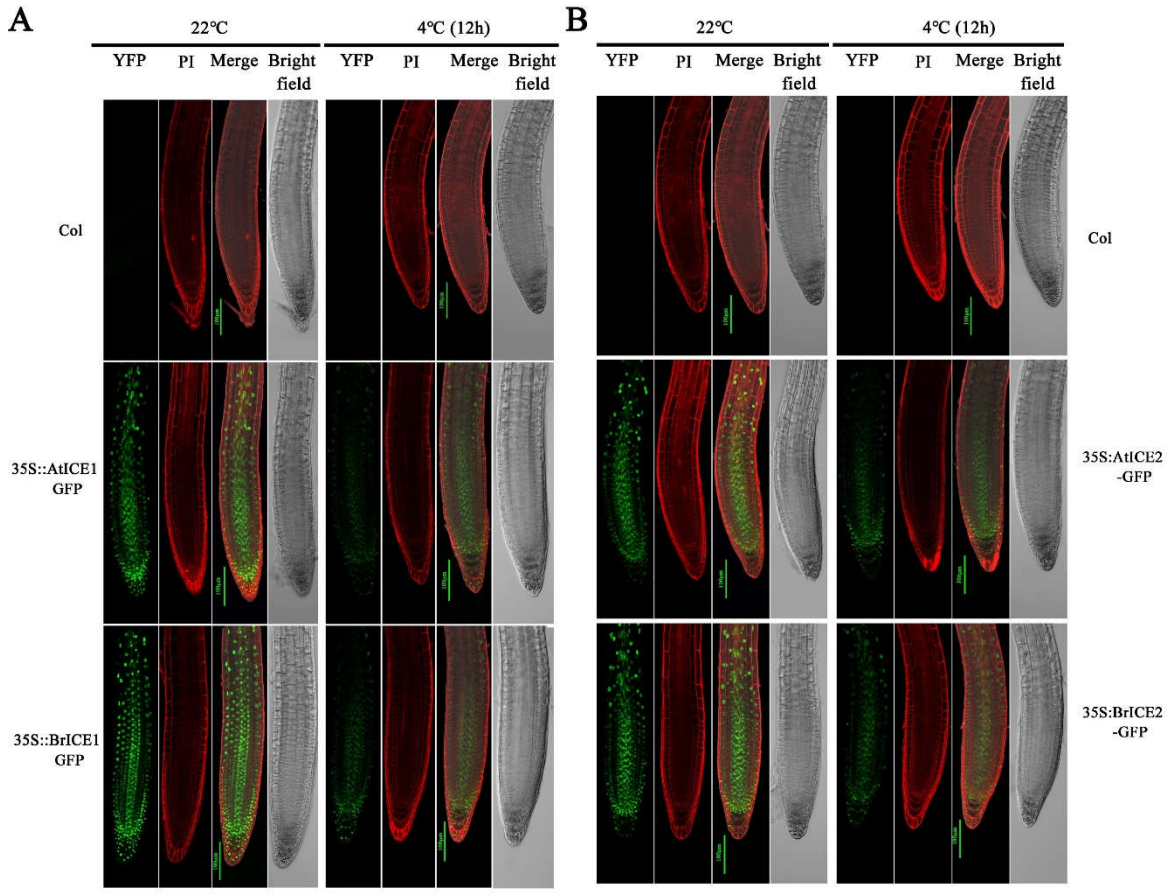

Figure 5. Cold induce the degradation of BrICE1 and BrICE2. Three-day-old seedlings grown on agar plates were treated (4°C, 12 h), the roots were incubated in 0.02 mg/mL PI for 12 min, the GFP signal in roots were visualized and photographed using confocal microscope. **(A)** Visualization of AtICE1-GFP and BrICE1-GFP transgenic plants. **(B)** Visualization of AtICE2-GFP and BrICE2-GFP transgenic plants. Scale bar was 100 μm.

2.5. BrICE1 and BrICE2 Positively Regulate Cold Tolerance Through the CBF-Dependent Pathway

To further elucidate the role of BrICE1 and BrICE2 in cold resistance, 35S:BrICE1-GFP and 35S:BrICE2-GFP were constructed and overexpressed in Arabidopsis. Additionally, 35S:AtICE1- GFP and 35S:AtICE2-GFP were overexpressed as positive controls. T_2 transgenic plants were used to evaluate cold resistance. The aerial phenotypes of *BrICE1* and *BrICE2* overexpression lines were not significantly different from the wild-type plants grown at a permissive temperature, except for slightly shorter petioles (Supplementary Figure S2A,B). 14-day-old seedlings grown on separate sections of the same agar plates were cold-treated at -6 °C for 1 h with or without cold acclimation (CA, 4 °C for 3 d) before undergoing a freezing tolerance assay. *BrICE1* and *BrICE2* transgenic plants showed significantly enhanced freezing tolerance compared with the wild-type plants under both non-acclimated (NA) or cold-acclimated (CA) conditions (Figure 6A). Without cold acclimation, only 25% of wild-type plants survived after freezing treatment (-6 °C for 1 h). While the survival rate of *BrICE1* transgenic plants was more than 58%, that of *BrICE2* transgenic plants was over 37%, and that of *AtICE1* and *AtICE2* transgenic plants was more than 35% (Figure 6B). As expected, cold acclimation not only significantly enhanced the freezing tolerance of transgenic plants but also enhanced the freezing tolerance of wild-type plants. After cold acclimation, approximately 80% of the *BrICE1* and *BrICE2* transgenic plants survived; however, the survival rate of the wild-type plants was only approximately 35% (Figure 6B). Furthermore, under non-acclimated conditions, the survival rate of *BrICE1* transgenic plants was significantly higher than that of *BrICE2* plants, suggesting that the

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overexpression of *BrICE1* conferred stronger basal resistance to transgenic plants compared with *BrICE2* transgenic plants (Figure 6B).

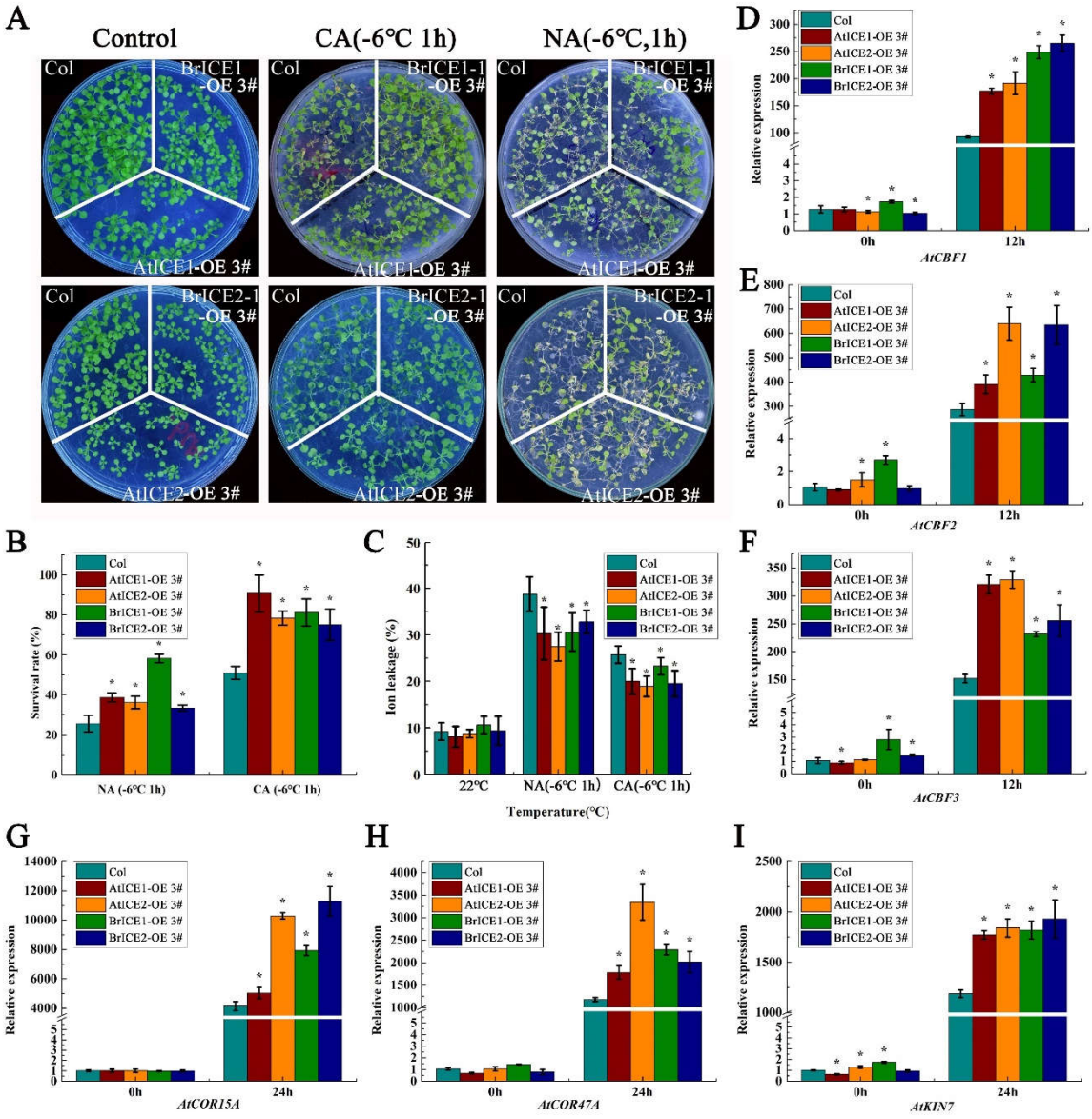

Figure 6. Overexpression *BrICE1* and *BrICE2* enhances the cold tolerance through the CBF-dependent pathway. Fourteen -day-old seedlings were subjected freezing at - 6 ℃ for 1 h with (CA, at 4 ℃ for 3 d) or without cold accumulation (NA), after 3 d recovering at 22 ℃, the survival rates, ion leakage were determined. For testing the expression levels of *CBFs* and their target genes, 14-day-old seedlings were low temperature 12 h or 24 h and subjected to qRT-PCR analysis. *ACTIN2* was used as the reference gene. **(A)** Freezing phenotypes. **(B)** The survival rates (n=120). **(C)** ion leakage rates (n=30). **(D-F)** The expression levels of *AtCBF1*, *AtCBF2* and *AtCBF3* (n=3). **(G-I)** The expression levels of *AtCOR15A*, *AtCOR47A* and *AtKIN7* (n=3). Values are shown as means ± SD of three independent experiments. Statistically significant differences are indicated by asterisks (Student's *t*-test, *, $p < 0.05$).

Subsequent electrolyte leakage assays supported these findings, where ion leakage in *BrICE1* and *BrICE2* transgenic plants was lower than that in wild-type plants (Figure 6C), indicating that cold stress-induced plasma membrane damage was mitigated in *BrICE1* and *BrICE2* transgenic plants. Similar phenotypes were observed in seedlings grown in the soil (Supplementary Figure S3). Consistent with previous reports, the overexpression of Arabidopsis *AtICE1* and *AtICE2* also enhances cold tolerance [3,7]. Regrettably, we were unable to obtain loss-of-function T-DNA homozygous lines for *ICE1* and *ICE2* from the Arabidopsis Biological Resource Center (ARBC);

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therefore, we did not conduct complementary experiments in *ice1* and *ice2* loss-of-function mutants. These observations indicated that BrICE1 and BrICE2 of *B. rapa* function as important positive regulators in response to cold stress, and BrICE1 and BrICE2 play overlapping roles with slightly unequal functional redundancy in acquiring freezing tolerance under the present experimental conditions.

AtICE1 of Arabidopsis is involved in the cold stress response by regulating CBF expression [3,32]. To investigate whether BrICE1 and BrICE2 regulate cold signaling through the CBF-dependent pathway, we examined the expression levels of *CBFs* and their target genes, *COR15A*, *COR47*, and *KIN1*, in transgenic and wild-type plants under cold conditions. As shown in Figure 6, cold stress significantly induced the expression of *CBFs* and their target genes in *BrICE1* and *BrICE2* transgenic and wild-type plants. However, their expression levels were lower in the wild-type plants than in the transgenic plants (Figure 6D–I). Notably, the basal levels of *CBFs*, *COR47*, and *KIN1* target genes were higher in *BrICE1* and *BrICE2* transgenic plants than in wild-type plants, particularly in BrICE1 transgenic plants.

Changes in the promoter region of some duplicate genes can lead to subfunctionalization [33]. Therefore, we analyzed *cis*-acting regulatory elements in the promoter region of the four *B. rapa* ICE1 homologous genes. As illustrated in Supplementary Figure S4, all four *B. rapa ICE1* homologous genes (*BrICE1-1*, *BrICE1-2*, *BrICE2-1*, and *BrICE2-1*) contained characteristic MYC recognition elements, suggesting that they are indeed *ICE1* homologous genes in *B. rapa*. Moreover, ABRE elements were highly conserved among the four *B. rapa ICE1* homologous genes. However, the AE-box was a specific element for BrICE2-1 and BrICE2-1, and the TGA and TCT motifs were specific elements for BrICE2-1 (Supplementary Figure S4). These *cis*-acting element analyses further support our conclusion that BrICE1 and BrICE2 of *B. rapa*, similar to AtICE1 and AtICE2 of Arabidopsis, regulate cold tolerance through the CBF-dependent pathway.

2.6. BrICE1 and BrICE2 Overexpression Inhibits Root Growth

ICE1 plays an important role not only in cold signaling but also in plant growth and development [5,12,15,16,34,35]. However, the mechanism by which ICE1 balances growth, development, and the cold stress response has not been extensively studied. Several studies have shown that constitutive overexpression of CBFs adversely affects plant growth under normal growth conditions [36,37], revealing that CBFs are master regulators of the trade-off between growth and development versus freezing tolerance.

To examine whether BrICE1 and BrICE2 can also influence this trade-off, root growth was analyzed under normal and cold stress conditions. As shown in Figure 7A, the aerial phenotype of *BrICE1* and *BrICE2* transgenic plants did not exhibit any detectable abnormalities compared with wild-type plants when grown on half-strength Murashige and Skoog (MS) medium at 22 °C. However, root elongation was significantly suppressed in *BrICE1* and *BrICE2* transgenic plants compared with wild-type plants (Figure 7A,B). The root length of wild-type plants was inhibited by approximately 60% when grown at 4 °C for 42 d compared with 22 °C for 7 days. Conversely, the suppression ratio in *BrICE1* and *BrICE2* transgenic plants was only 35% compared with the wild-type plants (Figure 7C). Notably, no significant differences in root growth were observed between *BrICE1* and *BrICE2* transgenic plants at either 22 °C or at 4 °C. Similar results were obtained for the *AtICE1* and *AtICE2* transgenic plants. These results suggest that BrICE1 and BrICE2, similar to CBFs, play critical roles in cold signaling by acting as regulators that balance growth and development with freezing tolerance, potentially through integration with unknown downstream target genes.

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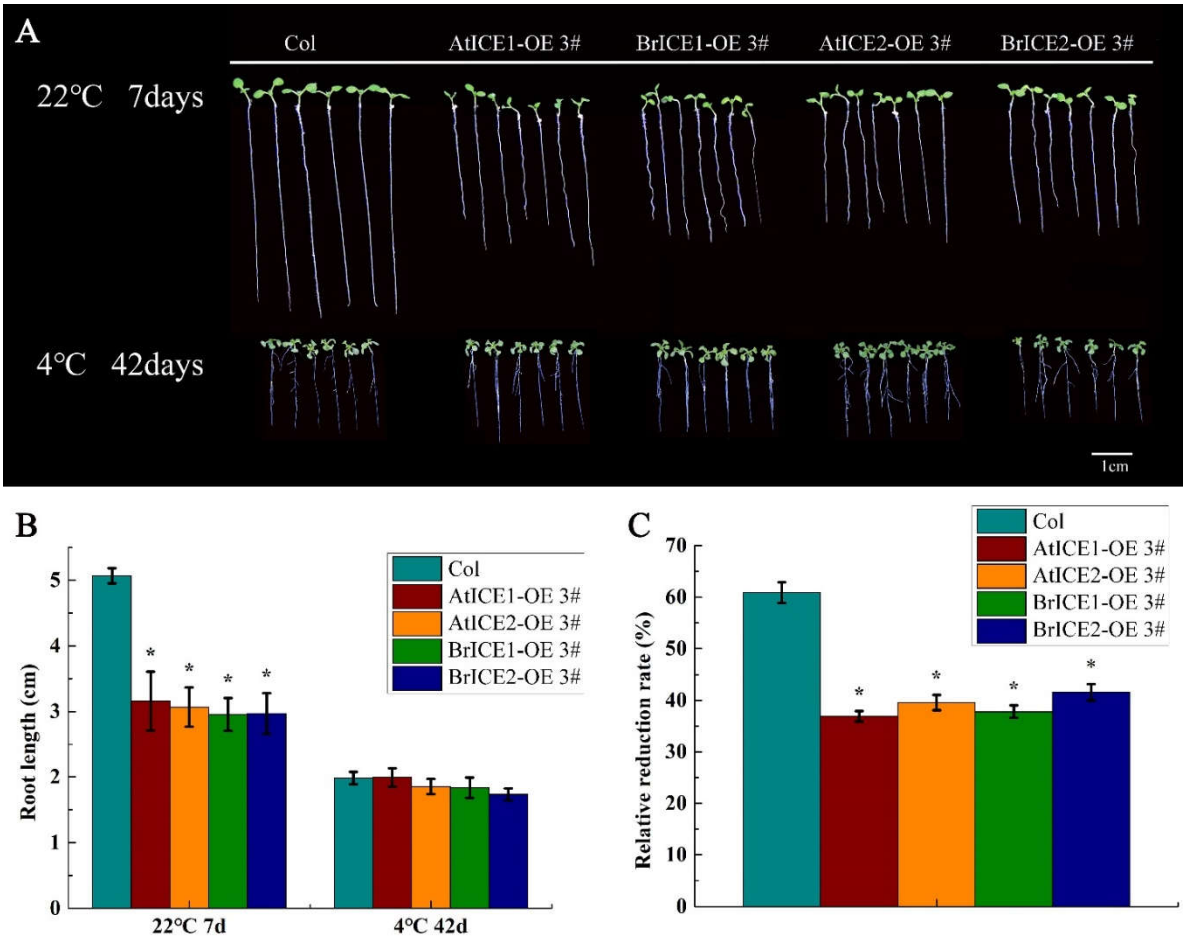

Figure 7. *BrICE1* and *BrICE2* overexpression inhibits root growth. Seedlings grown on half-strength MS at 22 ℃ for 7 d, roots length was measured using ImageJ software and designated as L1. After 3 d at 22 ℃, seedlings were cold treated for 42 d at 22 ℃, roots length was measured and designated as L2. The relative reduction rate of root length was calculated as (L1 − L2)/L1 × 100%. **(A)** Roots length phenotype. **(B)** Statistical analysis of root length (n=90). **(C)** Relative reduction rate of roots length under low treatment. Values are shown as means ± SD of three independent experiments, each with three technical replicates. Statistically significant differences are indicated by asterisks (Student's *t*-test, *, $p < 0.05$). Scale bar was 1 cm.

2.7. BrICE1 and BrICE2 Overexpression Enhances ROS Scavenging by Elevating Enzymatic Antioxidants

Our previous studies have revealed that ROS accumulation and response speed are critical for freezing tolerance in *B. rapa* [31,38]. To further explore the relationship between BrICE1 and BrICE2 involvement in low-temperature resistance and ROS, we assayed ROS accumulation in *BrICE1* and *BrICE2* transgenic plants using nitroblue tetrazolium (NBT) histochemical staining after chilling (at 4 ℃ for 3 or 6 h) or freezing treatment (at -4 ℃ for 3 or 6 h). Following chilling treatment at 4 ℃ for 3 or 6 h, NBT staining intensity was weaker in the leaves of *BrICE1* and *BrICE2* transgenic plants than in wild-type plants, suggesting higher levels of damage after cold stress (Figure 8A). Although freezing stress (-4 ℃ for 6 h) also caused significant injury to *BrICE1* and *BrICE2* transgenic plants, the degree of injury was milder compared with wild-type plants. The same phenomenon was observed in *AtICE1* and *AtICE2* transgenic plants. Quantitative measurements further confirmed these observations, revealing that the superoxide anion ($O_2^{\cdot-}$) content in *BrICE1* and *BrICE2* transgenic plants was lower than that in wild-type plants under both chilling and freezing stress (Figure 8B). Both histochemical staining and quantitative measurements demonstrated that overexpression of *BrICE1* and *BrICE2* resulted in lower ROS levels under chilling and freezing stress, suggesting that

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overexpression of BrICE1 and BrICE2 may confer a more efficient ROS scavenging system in transgenic plants.

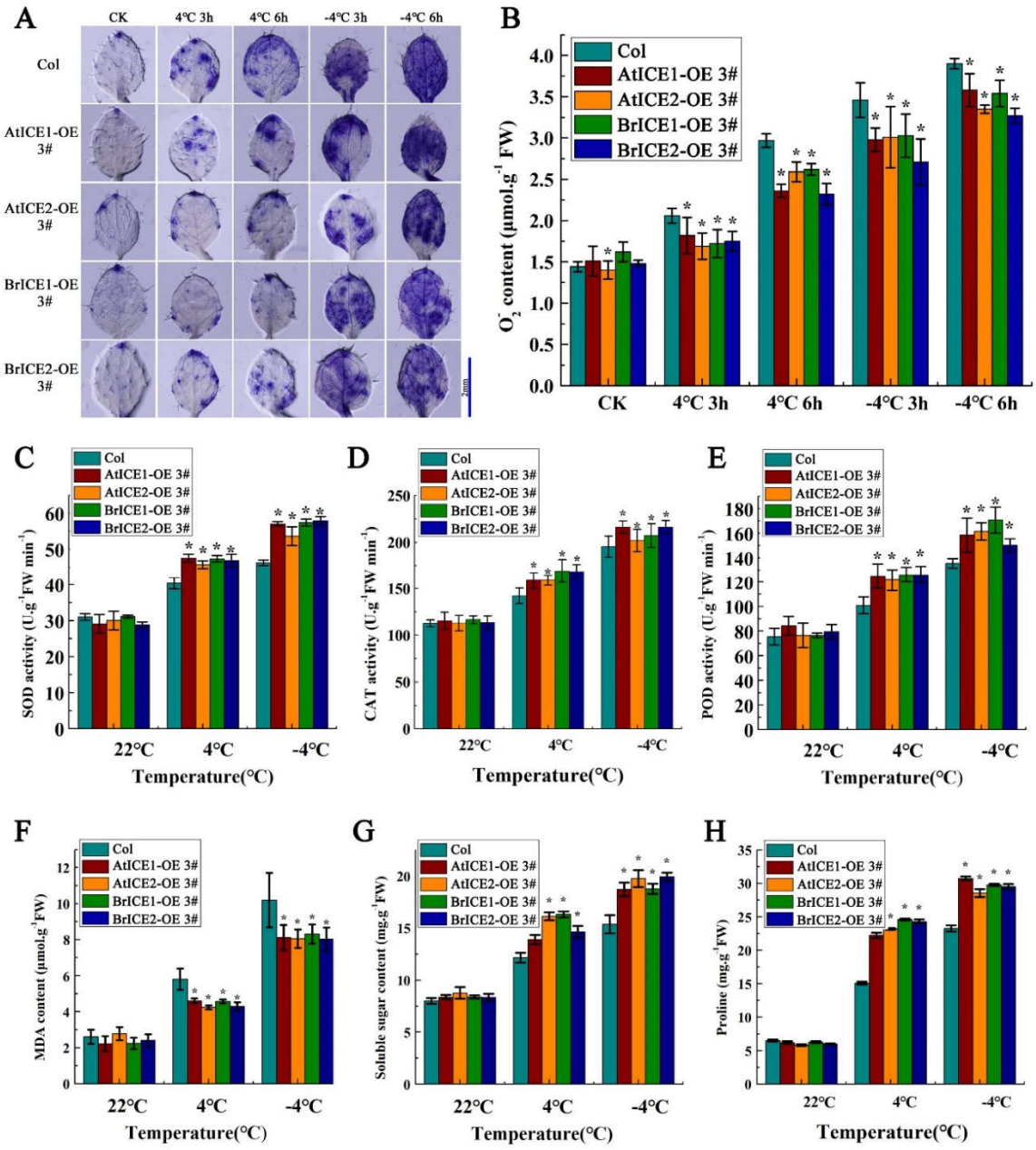

Figure 8. *BrICE1* and *BrICE2* overexpression enhances ROS scavenging by elevating enzymatic antioxidants. Ten-day old seedlings were chilling and freezing treated 3 or 6 h, Leaves were stained using NBT solution. The phenotype was photographed, and the activity of SOD, CAT, POD, O_2^-, MDA, soluble sugars, and proline content was detected. **(A)** The phenotype of ROS accumulation. **(B)** The changes of $O_2^{\cdot-}$ content. **(C-E)** The activity of SOD, CAT, POD. **(F-H)** The content of MDA, soluble sugars, and proline content was detected. Values are shown as means ± SD (n=30) of three independent experiments, each with three technical replicates. Statistically significant differences are indicated by asterisks (Student's *t*-test, *, $p < 0.05$). Scale bar was 2 mm.

To determine whether rapid ROS scavenging observed in *BrICE1* and *BrICE2* overexpressing plants is attributable to enzymatic antioxidative processes, the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were investigated. Under normal conditions, the enzyme activities of SOD, CAT, and POD were not significantly different between *BrICE1* and *BrICE2* transgenic and wild-type plants (Figure 8C–E). However, after chilling (at 4 °C for 3 h) or freezing

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treatment (at -4 ℃ for 3 h), the activities of all three enzymes in transgenic plants were significantly higher than in wild-type plants, with the difference between more significant after freezing treatment (at -4 ℃ for 3 h). These results suggest that overexpression of BrICE1 and BrICE2 enhances ROS-scavenging ability by increasing the activities of SOD, CAT, and POD, potentially contributing to cold tolerance by rapidly balancing ROS accumulation. Furthermore, malondialdehyde (MDA) content was measured, as shown in Figure 8F. Chilling or freezing stress caused membrane damage, leading to elevated MDA levels. However, the elevation of MDA was lower in *BrICE1* and *BrICE2* transgenic plants than in wild-type plants, suggesting that overexpression of BrICE1 and BrICE2 relieved low-temperature-induced membrane injury.

Plants can increase their tolerance to cold stress by rapidly synthesizing numerous soluble sugars and proline protective substances [39]. Our physiological results showed that *BrICE1* and *BrICE2* overexpression increased the content of soluble sugars and proline compared with the wild-type plants after both chilling and freezing treatments (Figure 8G, H). Therefore, these physiological results suggest that *BrICE1* and *BrICE2* overexpression increases cold resistance and is closely correlated with ROS scavenging and osmotic adjustment.

2.8. BrICE1 and BrICE2 are Degraded via the 26S-Proteasome Pathway in Response to Cold Stress Pathway

Previous studies have identified OST1, a well-known Ser/Thr protein kinase, as an upstream of CBFs and positively regulates freezing tolerance by interacting with ICE1 [9]. To determine whether BrICE1 has transcriptional activity, we used BrOST1 as a target protein to test BrICE1's transactivation potential. BrICE1 and BrOST1 of *B. rapa* were cloned into pGBKT7 and pGADT7 vectors, respectively. Yeast cells harboring pGBKT7-BrICE1 and pGADT7-BrOST1 grew well on the screening medium (SD/Trp-Leu-His-Ade), whereas transformants carrying the empty vector pGADT7 and pGBKT7-BrICE1 could not grow on the same medium (Figure 9A). These results demonstrate that BrICE1 has transcriptional activity and can interact with BrOST1.

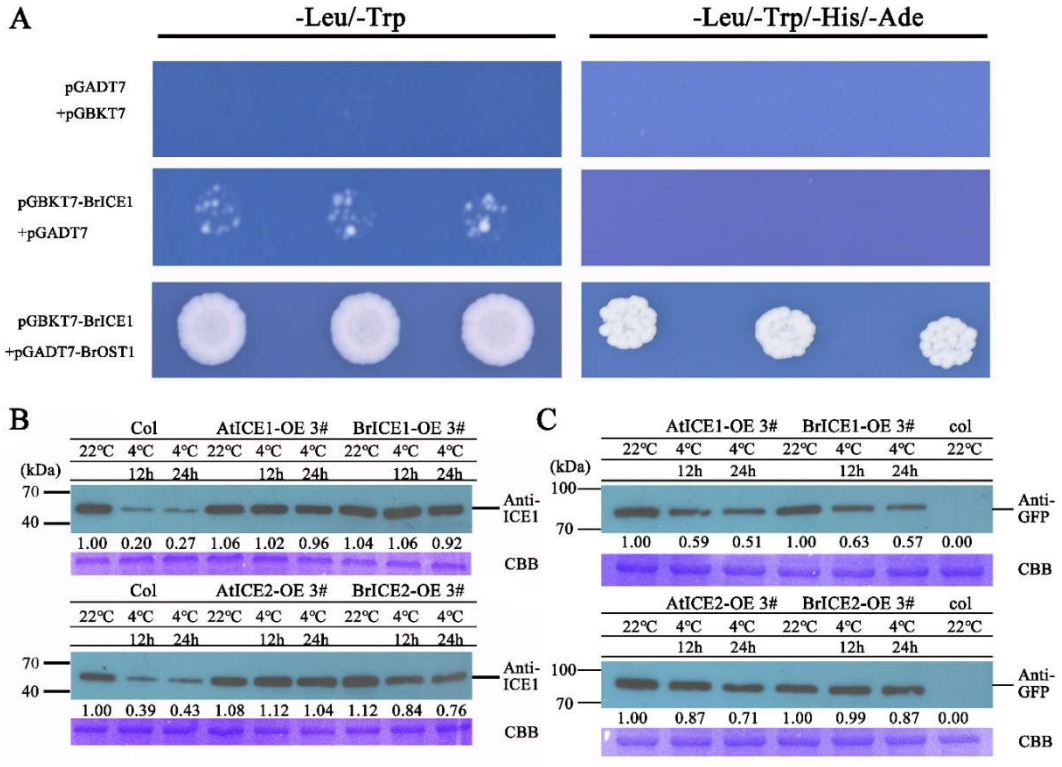

Figure 9. BrICE1 has transcriptional activation activity and low temperature induces their degradation. The full-length of BrICE1and BrOST1 were fused into pGBKT7 and pGADT7, respectively, and were expressed in the yeast strain AH109. 14-day-old wild-type and transgenic seedlings were treated at ℃ for 12 h or 24 h, total protein was extracted and immunoprecipitated with

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specific anti-ICE1 antibody and anti-GFP antibody. **(A)** The interaction of BrOST1 and BrICE1 in yeast. **(B, C)** Immunoblotting assays the protein level in wild-type and transgenic seedlings using specific anti-ICE1 (B) and anti-GFP (C) antibody.

Accumulating evidence suggests that ICE1 functions in cold signaling through PTMs [40]. To investigate whether BrICE1 and BrICE2 also function in cold tolerance through PTMs, BrICE1 and BrICE2 protein levels were determined in transgenic and wild-type plants before and after cold treatment using a specific anti-ICE1 antibody. As shown in Figure 9B, in wild-type plants, a 12 h cold treatment at 4 °C induced a substantial reduction in ICE1 protein abundance. However, the protein levels of ICE1 and ICE2 in BrICE1 and BrICE2 transgenic plants did not decrease significantly until 24 h of low-temperature treatment. This phenomenon was also observed in *AtICE1* and *AtICE2* transgenic plants. The anti-GFP antibody was used to assess the fusion protein levels of BrICE1-GFP and BrICE2-GFP in transgenic plants after cold treatment. The results showed that cold treatment induced a substantial reduction in both BrICE1-GFP and BrICE2-GFP fusion proteins, with the reduction of the BrICE2-GFP fusion protein being lower than that of BrICE1-GFP. Similarly, the AtICE2-GFP fusion protein reduction was lower than that of AtICE1-GFP in transgenic plants after cold treatment (Figure 9C). These western blot results are consistent with the observed root phenotypes in the nuclear localization experiment, suggesting that, similar to AtICE1 in Arabidopsis, low temperatures can also induce the degradation of BrICE1 and BrICE2 in vivo.

Furthermore, the stability of BrICE1-GFP and BrICE2-GFP fusion proteins was investigated in the presence of MG132 (a 26S-proteasome inhibitor) and cycloheximide (CHX) (a protein synthesis inhibitor) using anti-GFP and specific anti-ICE1 antibody. As shown in Figure 10, low temperature obviously induced the degradation of BrICE1 and BrICE2 proteins, but this degradation could be dramatically blocked by MG132. It is noteworthy that the degradation of ICE1 was less pronounced in *BrICE1* and *BrICE2* transgenic plants than in *AtICE1* and *AtICE2* transgenic plants. These western blot results are consistent with above root phenotype observed in the nuclear localization experiment. Interestingly, cold-induced degradation of BrICE1 and BrICE2 proteins was weak when using specific anti-ICE1 antibody than using anti-GFP antibody. Anyhow, these data suggest that in *B. rapa*, low-temperature-induced degradation of BrICE1 and BrICE2 occurs via the 26S-proteasome pathway.

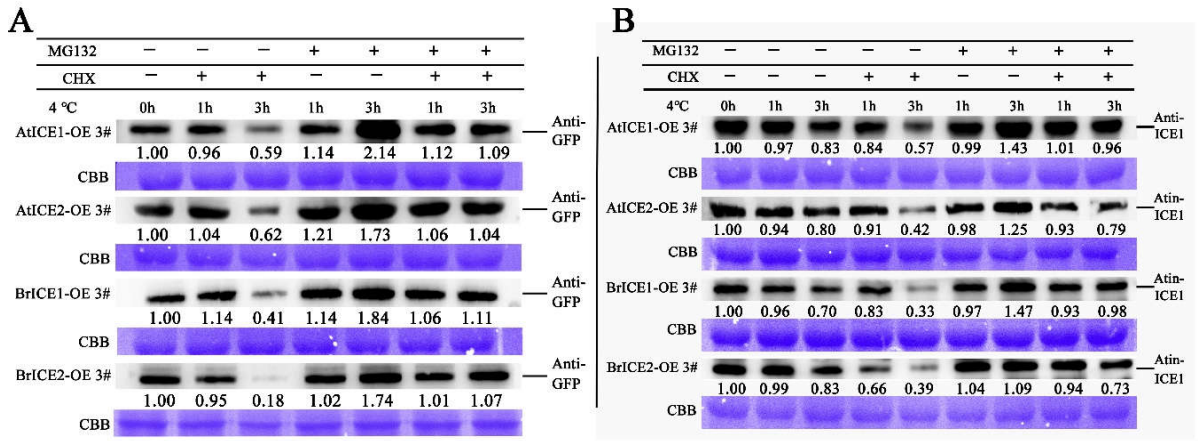

Figure 10. Cold induce the degradation of BrICE1 and BrICE2 through 26S-proteasome pathway. Fourteen-day-old wild-type and transgenic seedlings were treated at 4°C for 1 h or 3 h with or without 100 mM CHX and 50 mM MG132, total protein was extracted and immunoblotting were performed using specific anti-ICE1 antibody and anti-GFP antibody. Coomassie brilliant blue (CBB) was as the control for protein loading. The integrated optical density (IOD) values of ICE1bands were quantified. **(A)** Immunoblot assay of ICE1 protein using anti-GFP antibody. **(B)** Immunoblot assay of ICE1 protein using specific anti-ICE1 antibody.

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3. Discussion

Several studies reported that some *B. rapa* varieties can survive extremely low temperatures (-32 °C) during overwintering [27,41]. However, the underlying molecular mechanisms remain unclear. The ICE1-CBF-COR regulatory cascade is regarded as the most essential cold signaling pathway in Arabidopsis, with ICE1 acting as a crucial regulator. Consequently, several ICE1 paralogs from Brassica species have been cloned and characterized [28,29,42]. However, the gene structure and roles of ICE1 and ICE2 in *B. rapa* cold signaling have not been extensively investigated.

3.1. ICE1 Homologs Exhibit High Conservation Across Brassica Species

In this study, four ICE1 paralogs were identified in Z1 (*B. rapa*, yellow sarson) and three in Chiifu-401-42 (*B. rapa*, Chinese cabbage). Although both Chiifu-401-42 and Z1 are diploid *B. rapa* varieties, the number of ICE1 and ICE2 paralogs differs between them. This discrepancy suggests a complex evolutionary process within *B. rapa* species. One potential explanation is that ICE2 in Arabidopsis arose from a recent duplication event within the Brassicaceae family, estimated at around 17.9 million years ago [6]. Additionally, Z1 may represent a variant within *B. rapa* crops. This hypothesis is supported by the phylogenetic analysis of ICE proteins in *B rapa*. In Chiifu-401-42, two BraICE1 paralogs reside on chromosomes 2 and 9, while a single BraICE2 paralog is located on chromosome 8 (data not shown). Similarly, Z1 possesses two BrICE1 paralogs on chromosomes 2 and 6, with two additional BrICE2 paralogs on chromosomes 6 and 8. These findings suggest a convoluted process of paralogous gene selection on different chromosomes between Chiifu-401-42 and Z1, potentially reflecting a complex evolutionary mechanism in other cultivated Brassica species.

BrICE1 and BrICE2 amino acid sequences exhibit high conservation with their homologous genes in Brassica species. However, the identity between BrICE1 and BrICE2 is only 64.5% (data not shown), implying an unequal evolutionary event. Gene duplication events can lead to the retention of some transcripts [43]. Paralogous genes, such as *BrICE1* and *BrICE2*, may persist after undergoing subfunctionalization, neofunctionalization, or experiencing gene dosage effects [33,44]. In Arabidopsis, ICE2 presumably originated from a duplication event in early Brassicaceae species approximately 17.9 million years ago. This was followed by the sequence and functional diversification of ICE1 [6]. The duplication and subsequent subfunctionalization of BrICE2 might explain the low sequence identity observed between BrICE1 and BrICE2.

Brassica species belong to the Brassicaceae family with 3,700 known species across 340 genera [45], which includes three diploid species (*B. rapa*, *B. nigra*, and *B. oleracea*) and three amphidiploid species (*B. juncea*, *B. napus*, and *B. carinata)*. The complex history of genome hybridization and polyploidization within this family has resulted in intricate genomic information between Brassica species, often referred to as the "U's triangle" [46]. For instance, the diploid species *B. rapa* (Chiifu-401-42) and *B. oleracea* contain three and four ICE1 paralogs, respectively, while their allotetraploid offspring, *B. napus,* contains ten ICE1 homologous genes (Figure 1). Due to the limited scope of our current data, definitively elucidating the evolutionary relationship between ICE1 and ICE2 in Brassica Species remains challenging.

ClustalW protein sequence alignment revealed the presence of glutamine-rich and leucine-rich region domains in BrICE2 (Figure 1), similar to those found in Arabidopsis ICE2. This suggests a conserved evolutionary trajectory for ICE2 in Brassica species. Future studies will investigate whether these specific domains govern unknown physiological functions in BrICE2. Therefore, our study identified 41 ICE1-like homologous genes in six widely cultivated Brassica species, distinguishing between ICE1 and ICE2. The gene structure of ICE is highly conserved in Brassica species; however, the gene duplication events are complicated.

3.2. BrICE1 and BrICE2 Enhance Cold Tolerance Through CBFs and ROS Scavenging

Over the past two decades, research has established that ICE directly binds to CBF promoters, regulating the cold signaling cascade, a key regulating mechanism of ICE1 in many species [17–21,25]. Our expression pattern analysis revealed that low temperatures induced the expression of *ICE1*

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homologous genes in all tested varieties (Figure 2). However, the expression patterns differed between freezer-resistant varieties. Strong cold-tolerant varieties required longer low-temperature stress to activate *ICE1* expression compared with weak cold-resistant varieties. We speculated that strong cold-resistant varieties might have stronger basal cold resistance than weak cold-resistant varieties. Under low-temperature stress, strong cold-resistant varieties may not require promoting *ICE1* expression until basal cold resistance is exhausted. Conversely, varieties with weaker cold resistance need to promote *ICE1* expression earlier due to their weaker basal cold resistance. This regulated molecular mechanism requires further investigation.

BrICE1 and BrICE2, isolated from the strong cold-resistant variety Longyou 6, were found to localize to the nucleus (Figure 4), similar to AtICE1 and AtICE2 of Arabidopsis. This localization was not affected by cold stress, consistent with a previous study in Arabidopsis [3]. Cold-activated upregulation of the expression of *CBFs* and their target genes (*COR15A*, *COR47*, and *KIN1*) was higher in transgenic plants than in wild-type plants (Figure 6), suggesting that overexpression of *BrICE1* and *BrICE2* enhanced cold resistance was dependent on the CBF signal pathway. Additionally, the basal expression of these genes was also higher in transgenic plants than in wild-type plants. Moreover, similar to Arabidopsis AtICE1 and AtICE2, BrICE1 and BrICE2 contain MYC recognition elements in their promoter region. These results collectively indicate that *BrICE1* and *BrICE2* are novel putative *ICE1* homologs in *B. rapa* and that cold tolerance is also dependent on the CBF pathway. While BrrICE1.1 in *B. rapa var. rapa* [28] can directly bind to the promoter of BrrADC2.2, positively regulating its expression and response to cold stress, this suggests that some *ICE1* homolog genes might also be involved in non-CBF-dependent pathways under cold stress. Our results further demonstrate that *BrICE1* or *BrICE2* overexpression could elevate ROS scavenging ability via enzymatic antioxidative processes and increase the accumulation of proline and soluble sugars in response to cold stress (Figure 8). These findings suggest that BrICE1 and BrICE2 may have evolved multiple regulatory mechanisms to adapt to environmental stress.

Previous studies demonstrated that ICE1-mediated cold tolerance requires a period of cold acclimation. For example, overexpression of *Hevea brasiliensis HbICE1* and wheat *TaICE87/41* in Arabidopsis enhanced freezing tolerance after cold acclimation [17,48]. However, our data indicated that BrICE1 plays a role in both cold acclimation-dependent and basal freezing tolerance. *BrICE1* transgenic plants exhibited significantly higher survival rates compared with wide-type plants, even without cold acclimation. This discrepancy may be due to the functional differentiation of ICE1 in different species. In Arabidopsis, AtICE1 and AtICE2 play overlapping roles in cold signaling, but ICE1 plays a predominant role [8].

3.3. Balancing Development and Cold Defense: The Role of BrICE1 and BrICE2

ICE1 is not only a central component of the ICE1-CBF-COR cold signaling pathway but also serves as a convergence point, integrating multiple signals to regulate cold tolerance and plant growth development. Our root growth assay revealed that overexpression of BrICE1 and BrICE2 suppressed root growth under normal conditions but not under cold stress (Figure 7). This suggests that BrICEs functions as a positive regulatory factor that balances plant defense and development. Under a constant energy supply, overexpression of *ICE1* enhances cold tolerance, which requires more energy. Consequently, less energy is distributed for development. This observation is supported by a study where overexpression of *CBF1* or *CBF2* transgenic plants resulted in smaller stature, slower growth rates, and a more prostrate growth habit compared with wild-type plants [49]. These findings revealed that BrICE1 and BrICE2 function as network nodes, integrating different signals to regulate cold tolerance and root growth in *B. rapa.* However, the detailed molecular mechanisms underlying this regulation require further investigation.

3.4. Post-Translational Regulation: Crucial for BrICE1 and BrICE2 Response to Cold Stress

ICE1-regulated cold tolerance involves the activation of downstream CBFs and their target COR genes [3,49]. However, *ICE1* expression remains unresponsive to cold treatment at the transcriptional level, suggesting that PTM mechanisms play a crucial role in ICE1 function during cold signaling.

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Further, studies have shown that ubiquitination and SUMOylation regulate ICE1 stability, allowing plants to balance growth and development under cold stress [11]. Our immunohistochemical analysis and bimolecular fluorescence results revealed that cold induces the degradation of BrICE1 and BrICE2 (Figure 5, 9, 10). This suggests that similar to AtICE1, BrICE1 and BrICE2 rely on PTM mechanisms for their involvement in cold tolerance. This conclusion is further supported by our yeast two-hybrid assay of BrICE1 and BrOST1 (Figure 9A). OSTI interacts with ICE1, stabilizing it by preventing its degradation. This reduced degradation observed in *BrICE2* transgenic plants was lower than that in *BrICE1* transgenic plants because of the unequal functional redundancy between BrICE1 and BrICE2 in cold signaling, albeit with varying degrees of effectiveness. Similar results were observed in *AtICE1* and *AtICE2* transgenic plants. In summary, our findings suggest that BrICE1 and BrICE2 function as ICE1 paralogs in *B. rapa*, similar to AtICE1 and AtICE2, and that their roles in cold signaling involve PTM.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 and transgenic seedlings used in this study were grown on half-strength MS medium supplemented with 1% sucrose and 0.8% agar at 22 ℃ under a 16-h light/8-h dark photoperiod. For soil growth, Arabidopsis and *B. rapa* seeds were vernalized at 4 ℃ for 3 d and then grown at 22 ℃ in a greenhouse under a 16-h light/8-h dark cycle condition [31,38].

4.2. Identification and Phylogenetic Analysis of ICE1 Homologous Genes

To identify ICE1 homologs in six widely cultivated Brassica species (*B. rapa*, AA; *B. nigra*, BB; *B. oleracea*, CC; *B. juncea*, AABB; *B. napus*, AACC; and *B. carinata*, BBCC), the amino acid sequences of Arabidopsis ICE1 (At3g26744) and ICE2 (At1g12860) were used as queries to search against the BRAD (http://brassicadb.cn) with an e-value threshold of 1e−05 and a maximum identity of 50%. Pfam (http://pfam-legacy.xfam.org/) and the National Center for Biotechnology Information (NCBI) Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) were used to evaluate the conserved domains of ICE1 homologs, and redundant sequences were removed. Dicotyledon tomato and soybean (*Glycine max*), monocotyledon maize (*Zea mays*), foxtail millet (*Setaria italica*) and rice (*Oryza sativa*), known to possess ICE1-like homologous genes, were searched in the Phytozome (https://phytozome-next.jgi.doe.gov/) public databases as controls. DNAMAN v9.0 software (Lynnon Corporation, San Ramon, CA, USA) was used to align the amino acid sequences. MEGA 6.0 software (Molecular Evolutionary Genetics Analysis, The Pennsylvania State University, University Park, PA, USA) [50] was employed to construct a phylogenetic tree based on the full-length protein sequences of ICE1 homologous genes.

4.3. Plant Freezing Tolerance and Physiological Assays

Arabidopsis freezing tolerance and physiological assays were performed as previously described [38]. For the non-acclimated (NA) treatment, 14-day-old seedlings grown on a half-strength MS medium were directly subjected to a freezing chamber for the freezing assay, as described in the figure legends. For the cold-acclimated (CA) treatment, 14-day-old seedlings were pre-treated at 4 ℃ for 3 d before the freezing assay, as described in the figure legends. After the freezing treatment (specific time and temperature details provided in the figure legends), the seedlings were kept at 4 ℃ for 12 h and then recovered for 72 h at 4 ℃. Subsequently, the survival rates [51], ion leakage [52], POD [53], SOD [54], and CAT activities and MDA content [55] were determined. For soil-grown seedlings, 28-day-old seedlings were subjected to the freezing assay treatment as described in the figure legends (specific time and temperature details provided). Phenotypes were photographed, and survival rates were counted.

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4.4. RNA Preparation and qRT-PCR Assays

Total RNA was extracted from Arabidopsis and *B. rapa* seedlings using an RNAprep Pure Plant Kit (TIANGEN, No. PD423) with or without freezing treatment as described in the figure legends. cDNA was synthesized using the Hifair® II 1st Strand cDNA Synthesis Kit (YEASEN, No. 11120ES60). qRT-PCR was performed using SYBR Green Master Mix (YEASEN, No. 11202ES08) on a QuantStudioTM 5 System. Arabidopsis and *B. rapa ACTIN2* was used as the reference gene. Primers used for qRT-PCR are listed in Supplementary Table S1.

4.5. Gene Cloning and Plasmid Construction

Full-length cDNA fragments of *BrICE1*, *BrICE2*, *AtICE1*, and *AtICE2* were cloned by RT-PCR and transferred into the plant expression vector pBIB-BASTA-35S-GWR-GFP [56] using gateway technology. The expression vector containing the target gene fragments was transformed into the *Agrobacterium* GV3101 recombination strain. Arabidopsis plants were transformed using the floral dip method [57]. Furthermore, T_1 seedlings were screened on 0.1% (v/v) Basta in the soil, and T_2 transgenic plants were verified by RT-PCR and western blotting with anti-GFP antibodies (Roche, No. 1181446001). All primers used for cloning and qRT-PCR analyses are listed in Supplementary Table S1.

4.6. GFP Fluorescence Assay

Subcellular localization of BrICE1 and BrICE2 was determined as previously described [38]. Plasmids encoding 35S:BrICE1-GFP and 35S:BrICE2-GFP fusions were constructed and transformed into *Agrobacterium* GV3101 recombinant strain. Following incubation at 28 ℃ for 18–20 h, the *Agrobacterium* cultures were injected into tobacco leaves. These tobacco plants were then kept at 22 ℃ in darkness for 12 h, followed at 22 ℃ under light conditions for 48 h. GFP fluorescence was visualized under a confocal microscope (Leica, TCS SP8). 35S:AtICE1-GFP and 35S:AtICE2-GFP constructs were also generated as positive controls for nuclear localization, and 35S:BAK1-GFP was used as a negative control for plasma membrane localization.

Protein degradation assays for BrICE1 and BrICE2 under cold stress were performed as previously described [38]. The roots of 3-day-old seedlings were incubated in 0.02 mg/mL propidium iodide (PI) solution for 12 min. The GFP signal in the roots was then visualized and photographed using a confocal microscope (Leica, TCS SP8). To verify cold-induced degradation of BrICE1 and BrICE2, 3-day-old wild-type and transgenic seedlings were incubated at 4 ℃ for 12 h. Subsequently, the GFP signal in the roots was visualized and photographed.

4.7. cis-Element and Conserved Domain Analysis

cis-Elements within the promoters of the four BrICE1 homologous genes (*BrICE1-1*, *BrICE1-2*, *BrICE2-1*, and *BrICE2-2*) were analyzed using the PlantCare database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) as previously described [38]. TBTools v1.045 (https://github.com/CJ-Chen/TBtools-II) and the SMART database (https://smart.embl.de/) were used to evaluate gene structures and conserved domains, respectively.

4.8. Root Growth Inhibition Assays

Arabidopsis thaliana ecotype Col-0 and transgenic seedlings were grown on half-strength MS at 22 ℃ for 7 d under a 16-h light/8-h dark photoperiod. Root length was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and designated as L1. For testing the growth development of roots under cold stress, Arabidopsis Col-0 and transgenic seedlings were grown on half-strength MS at 22 ℃ for 3 d with a 16-h light/8-h dark photoperiod. These seedlings were then grown at 4 ℃ for an additional 42 days under the same photoperiod. Root length was subsequently measured as L2. The relative reduction rate in root length was calculated as (L1－L2)/L1 × 100%.

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4.9. Histochemical Staining and $O_2^{\cdot-}$ Detection of ROS

Histochemical staining and $O_2^{\cdot-}$ detection were performed as previously described [38]. Notably, 4-day-old seedlings were subjected to chilling (at 4 ℃, 3 or 6 h) and freezing (at -4 ℃, 3 or 6 h) treatments. Leaves were then incubated in an NBT solution (Med Chem Express, 0.1% NBT in 10 mM sodium azide and 10 mM phosphate buffer, pH 7.8) overnight. The next day, these leaves were decolorized with 95% ethanol 3–4 times and photographed. $O_2^{\cdot-}$ content was detected as previously described [38].

4.10. Yeast Two-Hybrid Assays

BrOST1, a protein kinase known to interact with ICE1, was cloned into the pGBKT7 vector as a positive control. BrICE1 was cloned into the pGADT7 vector, and both constructs were co-transformed into the yeast strain AH109. Yeast cells were grown on synthetic complete (SC) medium lacking leucine and tryptophan (SC-Leu-Trp) or SC-Leu-Trp-His-Ade medium supplemented with 2 mM 3-amino-1,2,4-triazole (3-AT) for 5 d at 30 ℃. Growth on the medium containing 3-AT indicates an interaction between BrICE1 and BrOST1.

4.11. Protein Extraction and Immunoblotting Assays

Total protein extraction and immunoblotting were performed as previously described [31]. For transgenic plant authenticity testing, immunoblot analysis was performed using an anti-GFP antibody (Roche, No. 1181446001) to detect GFP-fusion proteins, and coomassie brilliant blue (CBB) was as the control for protein loading.

For the protein degradation assay, 14-day-old wild-type and transgenic seedlings were treated according to the specific time and temperature conditions described in the figure legends. Total protein was then extracted and subjected to immunoblot analysis. ICE1 protein was detected using a specific anti-ICE1 antibody (Agrisera, AS16 3971). The ICE1-GFP fusion protein was detected with an anti-GFP antibody (Roche, No. 1181446001), and an and coomassie brilliant blue (CBB) was as the control for protein loading.

To verify whether the low-temperature-induced degradation of BrICE1 and BrICE2 was dependent on the 26S-proteasome pathway, 14-day-old wild-type and transgenic seedlings were treated with or without 100 mM CHX and 50 mM MG132, as described in the figure legends. Total protein was subsequently extracted and subjected to immunoblot analysis as described above. The Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA)) was used to quantify the integrated optical density (IOD) values of ICE1 and actin bands.

4.12. Statistical Analysis

All statistical analyses and qRT-PCR experiments were repeated in at least three independent experiments, each with three technical replicates. Data was analyzed using IBM SPSS Statistics 26.0 (IBM Corporation, Armonk, NY, USA) and are presented as means ± SD. Significance tests were performed using Student's t-test (*, $p < 0.05$).

5. Conclusions

In this study，41 ICE1-like homologous genes were identified in six widely cultivated Brassica species. These ICE1-like homologs exhibit high conservation in Brassica species, but the gene duplication events are complicated. Low temperatures induced expression patterns of *ICE1* homologous are different between freezer-resistant varieties. Two cloned novel ICE1 paralogs *BrICE1* and *BrICE2* are nuclear-localized protein, their localization are not affected by cold stress. BrICE1 and BrICE2 of *B. rapa* positively regulate cold tolerance via the CBF-dependent pathway and ROS scavenging mechanism, and this regulating mechanism are also responsible for balancing the development and cold defense of *B. rapa*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, W.W.; Data curation, W.W., H.Y., P.X., G.Z., X.H., G.M., H.D., G.W. and Z.L.; Formal analysis, H.Y., G.Z. and G.W.; Funding acquisition, W.W.; Methodology, H.Y., P.X., G.Z., X.H. and G.M.; Project administration, W.W.; Software, H.Y., H.D. and Z.L.; Writing – original draft, W.W. and H.Y.; Writing – review & editing, W.W.

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Conflicts of Interest: The authors declare no conflict of interest.

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