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Polyploidization-driven differentiation of freezing tolerance in *Solidago canadensis*

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Abstract

Solidago canadensis, originating from the temperate region of North America, has expanded southward to subtropical regions through polyploidization. Here we investigated whether freezing tolerance of *S. canadensis* was weakened during expansion. Measurement of the temperature causing 50% ruptured cells (LT_{50}) in 35 *S. canadensis* populations revealed ploidy-related differentiation in freezing tolerance. Freezing tolerance was found to decrease with increasing ploidy. The polyploid populations of *S. canadensis* had lower *ScICE1* gene expression levels but more *ScICE1* gene copies than the diploids. Furthermore, more DNA methylation sites in the *ScICE1* gene promoter were detected in the polyploids than in the diploids. The results suggest that promoter methylation represses the expression of multi-copy *ScICE1* genes, leading to weaker freezing tolerance in polyploid *S. canadensis* compared to the diploids. The study provides empirical evidence that DNA methylation regulates expression of the gene copies and supports polyploidization-driven adaptation to new environments.

KEYWORDS

DNA methylation, freezing tolerance, gene expression, *ICE1* gene, polyploidization, *Solidago canadensis*

1 | INTRODUCTION

Cold stress, including chilling (0–15°C) and freezing (<0°C) temperatures, is one of the key factors limiting plant growth, development and distributions (Allen & Ort, 2001; Wang, Jiang, & Li, 2012). Plant response to cold stress is crucial for geographical expansion. At low but non-lethal temperature, most temperate plants can tolerate cold stress by increasing activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) and accumulating antioxidants including proline. These compounds protect plants from reactive oxygen species (ROS) such as H_2O_2 and peroxides such as malondialdehyde (MDA) (Deng et al., 2012; Mittler, Vanderauwera, Gollery, & Breusegem, 2004; Zhu, Dong, & Zhu, 2007). Many studies have revealed the underlying molecular mechanisms, which primarily depend on the C-repeat binding factor (*CBF*)/dehydration-responsive element binding protein (*DREB*) pathway (referred to as the *CBF* pathway) (Chinnusamy, Zhu, & Zhu, 2007; Cook, Fowler, Fiehn, & Thomashow, 2004; Zhu et al., 2007). The *CBF* transcription factors enhance plant cold tolerance by activating down-stream cold-regulated genes (*COR*) (Maruyama et al., 2004; Vogel, Zarka, Buskirk, Fowler, & Thomashow, 2005). The *CBF* genes are regulated by an up-stream gene, inducer of CBF expression 1 (*ICE1*). The *ICE1* gene encodes a myelocytomatosis (MYC)-like basic helix–loop–helix (bHLH) transcription factor. This transcription factor activates the *CBF* genes by binding to the MYC recognition elements in the promoter of *CBF* genes (Chinnusamy et al., 2007). The *ICE1* gene is constitutively expressed and its overexpression likely enhances the expression of *CBF* genes under cold conditions (Chinnusamy et al., 2007; Zhu et al., 2007). Further studies also found that the activation and degradation of ICE1 protein is mediated by sumoylation and ubiquitination, respectively (Dong, Agarwal, Zhang, Xie, & Zhu, 2006; Miura et al., 2007).

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Polyploidization (whole-genome duplication) is a process that an organism doubles its genome through unreduced gametes or somatic doubling. This event can originate from within or between populations of a single species or between different species (Lawton-Rauh, 2003; Ranney, 2006; Storme & Geelen, 2013). Many angiosperms have undergone rounds of genome duplication during long-term evolution (Jiao et al., 2011). Polyploid plants may occur physiological and morphological changes, leading to broader ecological niches than their diploid relatives (Hegarty & Hiscock, 2008; Paterson, Freeling, Tang, & Wang, 2010; Stebbins, 1971; Storme & Geelen, 2013). It has been found that polyploid plants usually have better adaptability and can endure extreme environmental conditions (Fawcett, Maere, & de Peer, 2009; Soltis & Soltis, 2000; Treier et al., 2009). Studies have revealed that polyploidization strengthens plant tolerance to cold, salt, water and drought stress (Allario et al., 2013; Deng et al., 2012; Maherali, Walden, & Husband, 2009; Wang, Wang, Liu, & Meng, 2013).

The genomes of polyploid plants contain a larger amount of duplicated genes, while the fates of these genes are concluded as: (a) neofunctionalization; (b) sub-functionalization; (c) non-functionalization and (d) selection for an increased gene product (Soltis & Soltis, 2012). Meanwhile, the expression of duplicated gene could be altered very rapidly in response to polyploidization (Osborn et al., 2003), leading to epigenetically induced gene silencing (Adams & Wendel, 2005a). Gene silencing, a common phenomenon in polyploid plants, may occur organ-specifically (Adams, Percifield, & Wendel, 2004). Some duplicated genes in cotton (*Gossypium hirsutum*) polyploids were reciprocally expressed with one copy being silenced in some organs while the other copies being silenced in other organs, indicating fast sub-functionalization (Adams, Cronn, Percifield, & Wendel, 2003). The occurrence of duplicated gene silencing can be stochastic or repeatable, but epigenetic modifications, such as DNA methylation and histone modification, are supposed to be one of the mechanisms (Adams & Wendel, 2005b). However, thus far few empirical evidences support the hypothesis that epigenetic modifications are responsible for silencing of duplicated genes.

DNA methylation in the promoter or the transcribed gene body plays an important role in regulation of gene expression (Jaenisch & Bird, 2003; Pecinka, Abdelsamad, & Vu, 2013; Phillips, 2008; Razin & Cedar, 1991). Previous studies have demonstrated that DNA methylation in the promoter and the coding region of *ICE1* gene down-regulated the expressions of genes in the *CBF* pathway in plants under freezing stress (Xie et al., 2015; Xie et al., 2019). Higher methylation level in the promoter, in the transcribed gene body, or in both promoter and transcribed gene body of *ICE1* gene has led to more sensitivity to cold stress in *Oryza sativa*, *Ageratina adenophora* and *Arabidopsis thaliana*, respectively (Xie et al., 2015; Xie et al., 2019; Xie et al., 2020). Moreover, genes in the downstream of the *CBF* pathway were determined that methylation levels of *CBF* and *COR* genes were not significantly correlated with cold tolerance in those plants (Xie et al., 2015; Xie et al., 2019; Xie et al., 2020).

Solidago canadensis is a perennial weed originating from the temperate regions of North America (Werner, Bradbury, & Gross, 1980). *S. canadensis* shows strong invasiveness, spreading globally to Europe, Asia and even Oceania (Lu et al., 2007; Weber, 2017). This weed was first introduced to China in the 1930s as an ornamental plant, then escaped to the wild and has become one of the most notorious invasive weeds in China (Guo, Jiang, Fang, & Chen, 2009). *S. canadensis* is a complex species with different ploidies, mainly including diploid (2n = 2x = 18), tetraploid (2n = 4x = 36) and hexaploid (2n = 6x = 54) (Werner et al., 1980). Normally, new *S. canadensis* seedlings are germinated from seeds or sprouted from rhizomes in both autumn and early spring. Therefore, the seedlings may face freezing stress during winter and early spring (Weber, 2000; Werner et al., 1980). Our previous survey found that diploid *S. canadensis* cytotypes distributed in high latitudes no matter in native or invasive regions, whereas the polyploids mainly spread in relatively low latitudes. Whether this distribution pattern is related to freezing tolerance differentiation and its underlying mechanisms are not clear. Therefore, the aims of this study were to: (a) characterize the freezing tolerance pattern of different *S. canadensis* populations; (b) confirm the relationship between the freezing tolerance and cytotypes; and (c) reveal the molecular mechanisms to this differentiation.

2 | MATERIALS AND METHODS

2.1 | Plant materials

In total, 35 *S. canadensis* populations were collected from native (Canada and USA) and invasive regions (China, Germany and Russia). The cytotypes of these populations were previously confirmed to be diploid, tetraploid and hexaploid in the native region (named as NA2X, NA4X and NA6X, respectively) and in the invasive region (named as IN2X, IN4X and IN6X, respectively) (Tables S5 & S6 in Data S1). All plants of different *S. canadensis* populations were grown in glasshouse located in the Pailou Experimental Base of Nanjing Agricultural University. The seeds were germinated in 5 cm diameter plastic cups containing potting mix. The seedlings were then transplanted into 13 cm diameter pots with four seedlings per pot. The seedlings were grown in the glasshouse with temperature of 20–25°C. Experiments were conducted when the plants were at the four-pair-leaf stage.

2.2 | Determination of freezing tolerance

Freezing tolerance was determined as described in Xie et al. (2019) with minor modifications. The fourth and fifth mature leaves of plants (three individual plants per population) were taken and washed by deionized water. All clean leaves were treated for 1 hr at varying temperatures of −2, −4, −6, −8, −10 and − 12°C, respectively, using a cooling bath (PolyScience, Division of Preston Industries, Inc., Niles, IL, USA). The treated leaves were then thawed at 4°C for 0.5 hr, followed by adding 8 ml deionized water. Freezing-injured electrical conductivity (FEC) was measured after vacuum pumping for 3 hr. All treated samples were boiled for 15 min and the boiling electrical conductivity (BEC) was measured after recovering to room temperature.

Relative electrical conductivity = (FEC / BEC) × 100%. The temperature causing 50% ruptured cells (LT_{50}) was estimated by the nonlinear sigmoidal-logistic model using Origin8.0 (OriginLab Corporation, Northampton, MA 01060, USA). Electrolyte leakage is a symptom of cold-induced membrane damage in the study, thus the LT_{50} value was used as an indicator of freezing tolerance.

2.3 | Changes of physiological parameters in different populations under freezing treatment

According to the results of freezing tolerance, several typical *S. canadensis* populations were selected to determine the physiological parameters. In total, 19 populations, including NA2X (CA09, CA11, CA14), NA4X (US34, US52, US60), NA6X (US06, US10, US30), IN2X (EU01, EU02, EU03, RUS01), IN4X (CN15, CN30, CN44) and IN6X (CN22, CN47, CN61), were selected to perform the assay. At the four-pair-leaf stage, seedlings from the mentioned populations were moved to the incubator (LRH250-G, LT-36VL, Percival Scientific Inc., Perry IA, USA) with temperature of −5°C, 12/12 hr photoperiod (300 μmol m^{-2} s^{-1}) and 60% relative humidity. The third and fourth mature leaves from each plant (three individual plants per population) were harvested at 2 and 4 hr after the treatment, respectively. The leaf samples were washed, blotted-dried, frozen in liquid nitrogen and stored at −80°C until use. The untreated plants from those populations were used as control.

The contents of H_20_2 and proline of plants from different *S. canadensis* populations were measured using Hydrogen Peroxide assay Kit and Proline Assay Kit, respectively (both kits were from Nanjing Jiangcheng Bio-Engineering Institute Co., Ltd., Nanjing, China). The contents of MDA and the activities of CAT, POD and SOD were examined using the separate assay kit from Comin Biotechnology Co., Ltd., Suzhou, China.

2.4 | Cloning of full-length *ICE1* gene, partial-length *CBF3* and *COR414* gene

Total RNA was extracted using the new leaf of *S. canadensis* plant from population CA11 (NA2X) using TRizol Total RNA Extraction Kit (TIANGEN Biotech, Beijing, China). Reverse transcription was conducted using MMLV reverse transcriptase (Takara Biomedical Technology Co., Ltd., Beijing, China). Degenerate primers (ICE1-CF/CR, CBF3-CF/CR, COR414-CF/CR and qACTIN-F/R, Table S1 in Data S1) for the conserved regions of *ICE1*, *CBF3*, *COR414* and *actin* genes in *S. canadensis* were designed according to the homologous gene sequences of *A. thaliana*, *Lactuca sativa* and *Chrysanthemum dichrum* (data from NCBI). The PCR reaction was conducted in a 25 μl volume consisting of 1 μl cDNA, 0.5 mM of each primer, 2.5 μl 10× Ex Taq Buffer, 2 μl $MgCl_2$ (25 nM), 2 μl dNTP Mix (2.5 nM) and 0.2 μl Ex Taq (5 U μl^{-1}) with the following steps: 94°C 5 min, 35 cycles of 94°C 30 s, Tm (Table S1 in Data S1) 30 s and 72°C 1 min, followed by an extension of 10 min at 72°C. The PCR product was purified from agarose gel using Gel/PCR Extraction Kit (BIOMIGA INC., San Diego, CA, USA). The purified fragment was cloned to the pMD 19-T vector (Takara Biomedical Technology Co., Ltd., Beijing, China) and transformed to *Escherichia coli*. White colonies were selected for sequencing. Sequencing was conducted by Shanghai Sangon Biotech and the results were analyzed using DNAMAN (version 7.0, Lynnon Corp, Quebec, Canada). The partial-length *CBF3*, *COR414* and *actin* gene were named as *ScCBF3*, *ScCOR414* and *Scactin*, respectively.

The 3′- and 5′-end of *ICE1* gene in *S. canadensis* were cloned using BD SMART RACE cDNA Amplification Kit (Takara Bio USA, Inc., Mountain View, CA, USA). The conserved region, 3′- and 5′-end of *ICE1* gene were assembled. The assembled sequence was BLASTed in the NCBI (Standard databases). The primers for full-length *ICE1* gene were designed (ICE1-FL-F/R, Table S1 in Data S1) and the PCR condition was the same as mentioned above except for the annealing temperature (Tm, Table S1 in Data S1). The full-length cDNA was amplified and sequenced. The final confirmed sequence of *ICE1* gene was named as *ScICE1*.

2.5 | DNA extraction and flank sequencing

DNA was extracted using the method described by Xie et al. (2015). The full-length primers (ICE1-DNAFL-F/R, Table S1 in Data S1) of the *ScICE1* gene were designed for the gene amplification. The PCR was performed in the same condition as described in Section 2.4.

The flank sequence of *ScICE1* (the promoter region) was obtained by the thermal asymmetric interlaced PCR (TAIL-PCR)(Liu & Whittier, 1995). Four specific primers (ICE1-1, 2, 3 and 4) were designed based on the sequence of *ScICE1*, and four anchor primers (ADP1, ADP2, ADP3 and ADP4) were selected from Liu and Whittier (1995) (Table S3 in Data S1). The TAIL-PCR was performed as described in Table S2 in Data S1. The PCR product was purified and sequenced as described in Section 2.4.

2.6 | Relative gene expression level of *ScICE1*, *ScCBF3 and ScCOR414*

Uniform plants from six *S. canadensis* populations, CA09 (NA2X), US52 (NA4X), US30 (NA6X), RUS01 (IN2X), CN44 (IN4X) and CN61 (IN6X), were moved to the incubator with temperature of 4°C at the four-pair-leaf stage (The six populations were used for the following experiments, hereafter named as na2x, na4x, na6x, in2x, in4x and in6x, respectively). The first new leaf of each plant was harvested for total RNA extraction at 0, 1, 2, 4, 8, 12 and 24 hr. Approximately, 2 μg total RNA (genomic DNA-free) was used for reverse transcription. Real-time quantitative PCR (qPCR) was conducted in a 20 μl volume consisting of 1 μl cDNA, 125 nM of each primer and 10 μl SYBR Premix Ex Taq (2X). The reaction was performed on the Mastercycler ep realplex Real-time PCR System (Eppendorf, Hamburg, Germany) with the following steps: 95°C 3 min, 40 cycles of 95°C 15 s and 55°C 30 s. A melting curve was performed at ranges of 60–95°C to test the

specificity of PCR amplification. The *actin* gene in *S. canadensis* was selected to be a reference gene to standardize the relative gene expression levels (the *actin* gene was determined to be stably expressed). Primers are listed in Table S1 in Data S1. Efficiencies of all primers used in the qPCR were 90–110%. Relative expression levels of *ScICE1*, *ScCBF3* and *ScCOR414* were determined using the $2^{-\Delta\Delta Ct}$ method according to Xie et al. (2015).

2.7 | Confirmation of *ScICE1* gene copy number

Seedlings from six *S. canadensis* populations (na2x, na4x, na6x, in2x, in4x and in6x) were used for this assay. The *ScICE1* gene copy number was determined by Southern Blot, according to Mao, Xie, Chen, Valverde, and Qiang (2016) with minor modifications. A 10 μg DNA was digested overnight at 37°C with EcoR I, electrophoresed in agarose gel (0.7%), and then transferred to a positively charged nylon membrane (HyBond N+, Amersham Bioscience Corp., Piscataway, NJ, USA) using denaturing buffer. The *ScICE1* probe was amplified using the primers of F: 5'-GCTCGAAATACATCCAAAG-3′ and R: 5'-CATAACGAACTAGAGGCAAC-3′ and labelled with dUTP mix. The hybridization was conducted at 39°C overnight using 20 ml of the probe, followed by exposure to GelDocXR (Bio-Rad) at room temperature for 3 hr in the dark condition. Plasmid with target *ScICE1* gene fragment (426 bp) was used as positive control.

2.8 | Determination of DNA methylation sites

The methylation sites of *ScICE1* coding and promoter regions were determined using EZ DNA Methylation-Gold Kit (ZYMO Research, Irvine, CA, USA) based on the bisulphite sequencing method (Xie et al., 2015). Plants at the four-pair-leaf stage from the six typical *S. canadensis* populations (mentioned in section 2.6) were treated at 4°C for 1 hr, prior to DNA extraction and *ScICE1* gene amplification. The PCR was detailed in the Supplementary data (Table S4 in Data S1). The PCR product was sequenced for the determination of DNA methylation. The sequence obtained from each sample was compared with the original gene sequence, and a converted cytosine indicates an unmethylated site.

2.9 | Statistical analysis

The correlations between different parameters (LT_{50} & latitude, LT_{50} & mean temperature, LT_{50} & methylation number, *ScICE1* gene expression & methylation number and methylation number & ploidy) were analysed using SPSS17.0 software (IBM SPSS Statistics 20, Chicago, IL, USA). Significant differences among different *S. canadensis* cytotypes in each experiment were analysed by one-way analysis of variance (ANOVA) (Duncan post-hoc) using SPSS17.0 software. Figures were obtained using Origin8.0 software. Three biological replicates per population were used in each experiment. In addition, three individual plants per population with three technical replicates in each plant were used for gene expression experiments.

3 | RESULTS

3.1 | Freezing tolerance of 35 *S. canadensis* populations

The LT_{50} values of 35 *S. canadensis* populations were determined by measuring the relative electrical conductivity (Tables S5 & S6 in Data S1). The diploid cytotypes showed significantly lower LT_{50} values than the polyploids (Figure 1a), and NA2X had the lowest LT_{50} values among all cytotypes, indicating that *S. canadensis* has differentiated freezing tolerance among different cytotypes. Correlation analysis showed that the LT_{50} was negatively correlated with the latitudes of collection points in both native and invasive regions ($p < 0.05$, Figure 1b), and positively correlated with mean temperature of the coldest month in the native region ($p < 0.05$, Figure 1c), suggesting that the freezing tolerance was strengthened with the increasing latitudes.

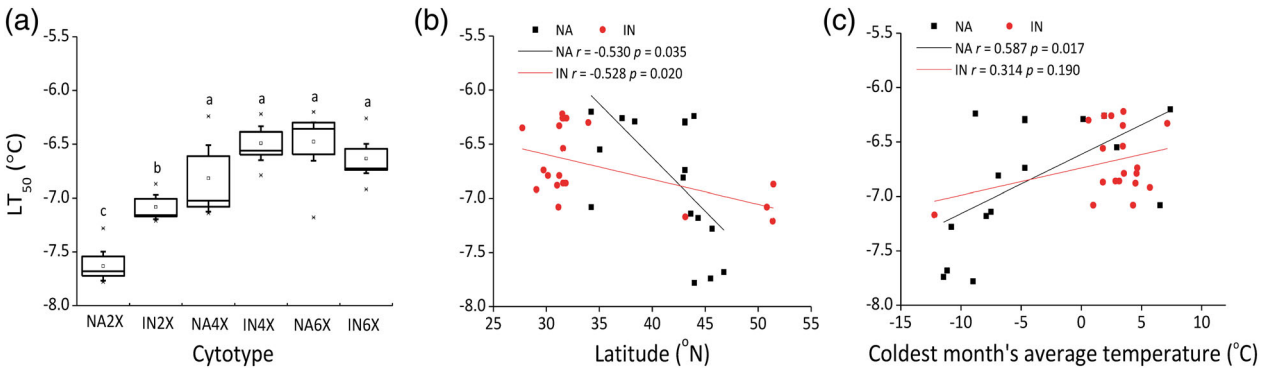

FIGURE 1 Relationship between the LT_{50} values and cytotype of *S. canadensis* populations (a), latitudes of collection points (b) and the mean temperatures of coldest month (c) (the letters in (a) represent significant differences of the LT_{50} values among different cytotypes, $p < 0.05$)

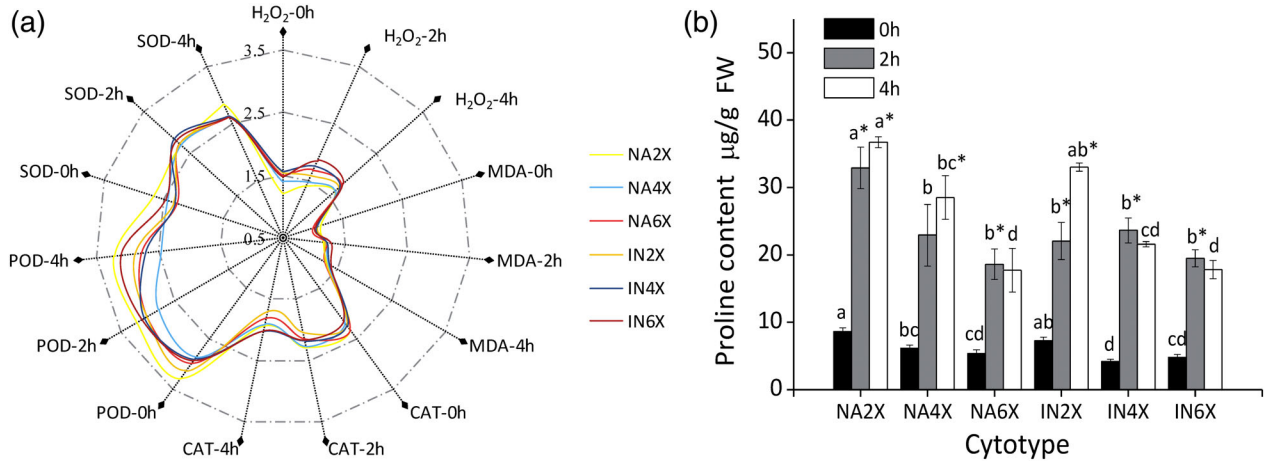

FIGURE 2 Comparison between cytotypes of *S. canadensis* populations and several physiological indicators, including the contents of H_2O_2, MDA and the activities of CAT, POD and SOD (a) and proline contents (b). The letters in (b) represent significant differences among different cytotypes with the same treatment, $p < 0.05$, and * represents significant differences to time point 0 in the same cytotype

3.2 | Changes of physiological parameters in selected populations

The contents of H_2O_2, MDA and Proline and activities of several enzymes in selected *S. canadensis* populations were measured after the freezing treatment. The results showed that the contents of H_2O_2 and MDA increased significantly in the tested cytotypes from 0 to 4 hr (Table S7 in Data S1). NA2X and NA4X had less H_2O_2 contents than other cytotypes at 4 hr, while all cytotypes showed similar MDA contents after the treatment (Figure 2a). In addition, the activities of CAT, POD and SOD decreased during the treatment (Figure 2a). NA2X had the highest POD activity, whereas the polyploids in both native and invasive regions had the relatively low activities at 4 hr, except for IN6X (Table S7 in Data S1). Moreover, the SOD activity was the highest in NA2X at 4 hr, similar to the result of POD activity experiment (Figure 2a). The proline contents were increased significantly in all cytotypes after freezing treatment, and the diploids (NA2X and IN2X) showed significantly higher proline content than the polyploids at 4 hr (Figure 2b).

3.3 | Relative expression of *CBF* pathway genes

The full-length of *ScICE1* gene was cloned and the sequence was shown in Supplementary data (Figure S1a, Genbank ID: MN172171). The sequence of *ScICE1* gene is 1,584 bp in length, encoding 527 amino acids and shows a highly conserved HLH domain (Supplementary data Figure S1b). The partial-lengths of *ScCBF3*, *ScCOR414* and *Scactin* gene were 207 bp, 134 bp, 138 bp in length, respectively (Supplementary data Figure S2).

Plants from six selected *S. canadensis* populations were treated under 4°C for 24 hr, and the relative expression levels of *CBF* pathway genes (Sc*ICE1*, Sc*CBF3* and Sc*COR414*) were examined. The *ScICE1* gene expressed differently in the absence of cold treatment, and the expression levels of *ScICE1* gene in all tested populations were increased to the peak at 1 hr after cold treatment, then declined until the end of treatment (Figure 3a). The diploids had significantly higher *ScICE1* gene expression levels than the polyploids during the whole process, while na2x had the highest expression level (Figure 3a). The *ScCBF3* and *ScCOR414* genes showed similar expression patterns to the *ScICE1* gene. The expression peaks of *ScCBF3* and *ScCOR414* gene were at 2 and 4 hr, respectively (Figure 3b,c). The diploids always showed higher expression levels of *ScCBF3* and *ScCOR414* gene, except for the *ScCBF3* gene expression in na4x and na6x at 2 hr (Figure 3b).

A correlation analysis revealed that the LT_{50} value was strongly correlated with the expression levels of three *CBF* pathway genes (Table 1), suggesting that gene expressions in the *CBF* pathway are associated with response to cold stress in *S. canadensis*.

3.4 | Gene copy number of plants with different ploidy levels

The results of *ScICE1* gene copy number determined by Southern Blot showed that the diploids had the least copies (two copies) (Figure 4). The gene copy number was the same within each cytotype with an exception of in6x, which had four gene copies. The gene copy number was linear-related with ploidy ($r = 0.913$, $p < 0.05$).

3.5 | DNA methylation sites of plants with different ploidies

The full-length *ScICE1* gene at the DNA level was 2,135 bp in length and contained three introns (Supplementary data Figure S3). The promoter region of *ScICE1* gene is an inducible promoter and 1,320 bp in length. Bioinformatic analysis predicted that the promoter contains a

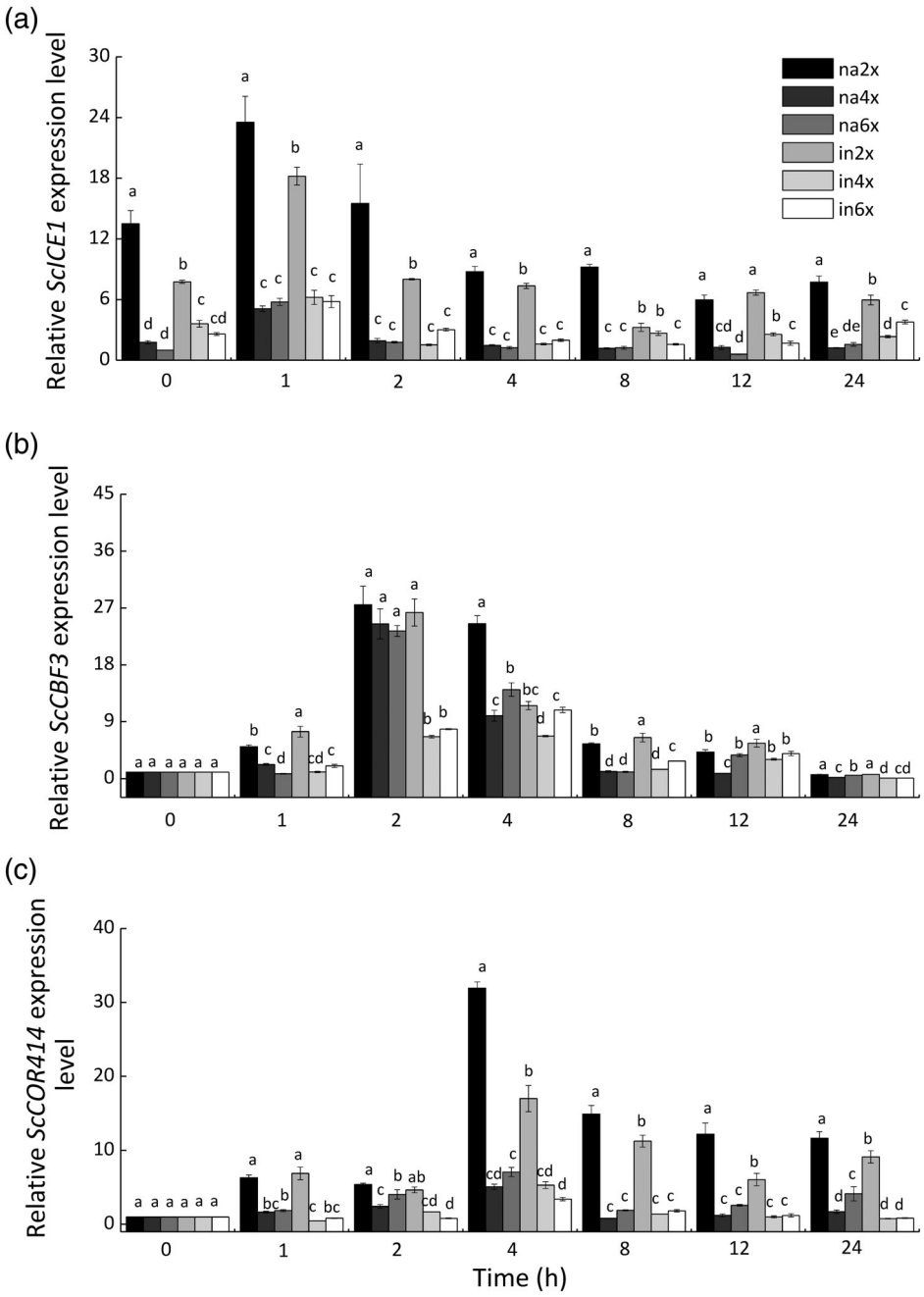

FIGURE 3 Relative expression levels of *ScICE1* (a), *ScCBF3* (b) and *ScCOR414* (c) gene in six selected *S. canadensis* populations with the cold treatment (4°C for 0–24 hr). Different letters in different populations at a time point indicate significant difference using Duncan post-doc analysis, $p < 0.05$

TATA Box and several CAAT Boxes. In addition, the promoter has a CpG-island at −800 bp (the adenine in the initiation codon, ATG, was counted as 1) and contains binding sites for MYB, light-responsive elements and stress-related *cis*-elements, such as TC-rich repeats, MBS, TGACG-motif, and so on (Supplementary data, Figure S4).

Three individual plants from each selected *S. canadensis* population were tested for the methylation sites in coding and promoter regions of *ScICE1* gene. Results showed no methylation sites in the coding region (Supplementary data Figure S5), and hence the promoter region of *ScICE1* gene was focused to detect the methylation site. The six populations showed varying numbers of methylation sites from 59 to 76 in the *ScICE1* gene promoter (Table 2, and Supplementary data Figure S6). Correlation analysis showed that the number of methylation sites in the *ScICE1* gene promoter was positively correlated with the LT_{50} values ($p < 0.01$, Figure 5a), and negatively correlated with expression level of the *ScICE1* gene ($p < 0.05$, Figure 5b), indicating that *ScICE1* methylation is likely involved in freezing tolerance in *S. canadensis*. Furthermore, the methylation sites significantly increased with the ploidy level ($p < 0.01$, Figure 5c), suggesting that polyplidization may have an effect on the *ScICE1* gene methylation.

4 | DISCUSSION

The study reported for the first time the negative relationship between freezing tolerance and ploidy in *S. canadensis* and suggested

TABLE 1 Correlation between the LT_{50} value and expression levels of different genes in *CBF* pathway

	LT_{50}	*ScICE1*	*ScCBF3*	*ScCOR414*
LT_{50}	1.00			
ScICE1	−0.791[b]	1.00		
ScCBF3	−0.826[b]	0.576[a]	1.00	
ScCOR414	−0.856[b]	0.951[b]	0.611[b]	1.00

[a]represents significant differences between two indexes as shown in the table at the level of $p < 0.05$.
[b]represents the significant differences at the level of $p < 0.01$.

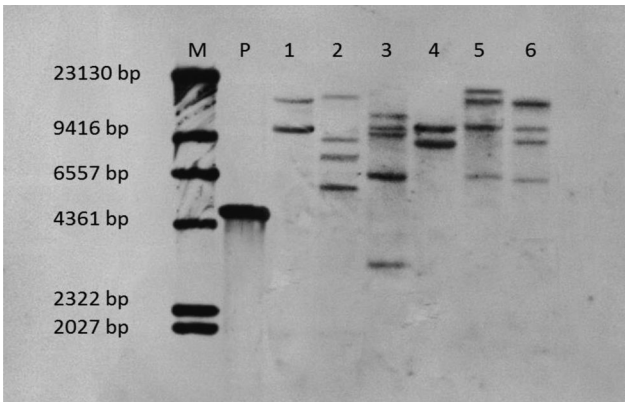

FIGURE 4 Southern blot of *ScICE1* gene with the EcoR I digestion in six selected *S. canadensis* cytotypes (M represents marker, P represents plasmid with the target *ScICE1* gene fragment used as positive control, 1–6 represent six selected *S. canadensis* plants with different populations of na2x, na4x, na6x, in2x, in4x and in6x, respectively)

that methylation in the *ScICE1* gene promoter repressed the *ScICE1* gene expression, resulting in weaker freezing tolerance in the polyploids than in the diploids.

4.1 | Freezing tolerance differentiation in *S. canadensis* cytotypes

The results of LT_{50} experiment indicated freezing tolerance of *S. canadensis* has already differentiated in the native region, with the stronger tolerance in the diploids (distributed at higher latitudes). Plant cold tolerance has a close relationship with distribution (Daly, Widrlechner, Halbleib, Smith, & Gibson, 2012). An investigation on freezing tolerance of 71 *A. thaliana* populations confirmed that populations in higher latitudes had enhanced freezing tolerance than in lower latitudes (Zhen & Ungerer, 2008). Moreover, polyploidization usually enhances cold tolerance in plants. Meta-analysis on the global biogeography of polyploid plants showed that polyploidy frequency increased away from the equator (Rice et al., 2019) and the Arctic Circle has the highest proportion of polyploid plants in the world (Brochmann et al., 2004). Polyploid plants usually show greater tolerance when distributing at high latitudes and elevations with cold condition (Soltis, Marchant, de Peer, & Soltis, 2015). However, polyploidization has weakened the freezing tolerance in *S. canadensis* in the present study (Figure 1a). It is rarely reported that polyploids have weaker cold tolerance than diploids. So far, only a few cases had a similar conclusion with our study (Sugiyama, 1998; Tyler, Borrill, & Chorlton, 1978), which gave rise to a question: why do diploid *S. canadensis* populations show higher freezing tolerance than the polyploids?

TABLE 2 Number of methylation sites and relative information of six selected *S. canadensis* populations

Accession	Latitude (°N)	Coldest month's average temperature (°C)	LT_{50} (°C)	Number of methylation sites
na2x	43.9800	−9.0	−7.78	59
na4x	43.6433	−7.5	−7.14	68
na6x	38.3472	0.2	−6.29	73
in2x	43.1202	−12.2	−7.17	64
in4x	31.2208	2.5	−6.33	71
in6x	27.7300	0.6	−6.30	76

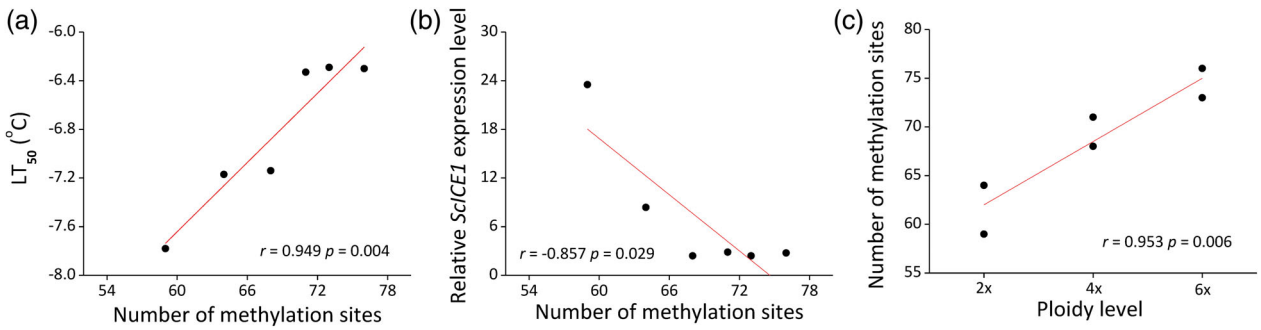

FIGURE 5 Correlations between number of methylation sites in the promoter region of *ScICE1* gene and the LT_{50} values (a), the relative *ScICE1* gene expression level (b), and the ploidy level of *S. canadensis* (c)

4.2 | Molecular basis of the freezing tolerance differentiation

The differentiation in freezing tolerance between the diploids and polyploids is significantly related to *ScICE1* gene expression level (Table 1). The higher *ScICE1* gene expression level likely up-regulated the down-stream *CBF* and *COR* gene expressions (Figure 3), thus endowing stronger freezing tolerance in the diploids than in the polyploids. In addition, increased *ScICE1* gene copies were detected in the polyploids (Figure 4). Gene copy number has a close correlation with gene expression level (Coate & Doyle, 2015). In *Glycine* genus, putatively gene dosage-sensitive networks showed reduced expression variance across seven species, and coordinated expression response to recent whole-genome duplication (Coate, Song, Bombarely, & Doyle, 2016). In our study, the increased *ScICE1* gene copies are likely correlated with the gene expression in the polyploids, resulting in decreased *ScICE1* expression and weak freezing tolerance.

Previous studies have reviewed reasons for gene expression divergence in polyploid plants, which mainly includes that (a) most homoeologous genes are co-expressed, (b) a few genes are lost or mutated and (c) epigenetic modifications may reprogram gene expression (Chen, 2007). Polyploidization rearranges the genome and induces a large quantity of epigenetic modifications, leading to different DNA methylation levels compared to their diploid relatives (Ding & Chen, 2018; Liu, Vega, & Feldman, 1998; Song & Chen, 2015; Weiss-Schneeweiss, Emadzade, Jang, & Schneeweiss, 2013). DNA methylation usually regulates gene expressions negatively in plants, such as *A. thaliana*, *A. adenophora*, *Nicotiana tabaccum* and *Zea mays* (Choi & Sano, 2007; Steward, Ito, Yamaguchi, Koizumi, & Sano, 2002; Xie et al., 2015; Xie et al., 2019). However, in polyploid plants, it has been hypothesized that DNA methylation plays a role in silencing one or several copies of duplicated genes (Chen & Tian, 2007; Xu, Zhong, Wu, Fang, & Wang, 2009). In our study, higher levels of DNA methylation in the *ScICE1* gene promoter were observed in the polyploids than in the diploids, indicating that *ScICE1* gene methylation is possibly involved in the gene expression regulation. Both DNA methylation and ploidy are vital for shaping gene expression diversity in polyploid plants. Moreover, transgenerational effects, defining as the influence of the maternal environment on the phenotype and performance of progenies, could be also associated with the DNA methylation (Angers, Castonguay, & Massicotte, 2010; Walter, Harter, Beierkuhnlein, & Jentsch, 2016), as different *S. canadensis* cytotypes distribute under different environmental conditions.

4.3 | Trade-off strategy for resource balancing

Polyploid plants can potentially invade new niches due to enhanced adaptability (Lowry & Lester, 2006; Treier et al., 2009). Therefore, polyploid plants are usually weedier and more invasive compared to the diploid relatives (Levin, 1983; Pandit, 2006; Thompson & Lumaret, 1992). Although *S. canadensis* polyploids have weak freezing tolerance, they show faster growth and have trended to expand southward (Cheng et al., 2020), where the temperature is higher than the original distributions. The phenomenon that the ability to tolerate environmental stress likely induces a growth cost (Growth-stress survival trade-off) has been observed in several plants (Bristiel et al., 2018). However, the opposite results (faster growth vs. weak tolerance) were observed in *S. canadensis* polyploids. In order to balance growth and freezing tolerance, the polyploids probably convert energy provided for freezing tolerance to energy for growth. This trade-off strategy between freezing tolerance and growth may endow *S. canadensis* becoming a successful invasive weed worldwide.

In summary, we demonstrate for the first time that methylation of the promoter region may repress overall expression levels of the multicopy *ScICE1* genes, contributing to weaker freezing tolerance in polyploid *S. canadensis* compared with the diploids. However, a question is still unclear yet. Is the weakened freezing tolerance attributed to homolog-specific expression and methylation of the *ScICE1* gene or the combined action of all copies in the polyploids? With the development of omics, combination of comparative genomics and methylomics is worthwhile to be performed to examine homolog-specific expression and methylation of *ScICE1* gene in the near future.

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CONFLICT OF INTEREST

There is no conflict of interest.

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SUPPORTING INFORMATION

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