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Overexpression of the ZmSAMDC Enhances Cold Tolerance in Transgenic Maize (Zea Mays L.)

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Abstract

Maize (Zea mays L.) is a food crop sensitive to low temperatures. Low temperature, as one of the abiotic stress hazards, seriously affects the yield of corn. However, the genetic basis of low-temperature adaptation in maize is still poorly understood. In this study, maize S-adenosylmethionine decarboxylase (SAMDC) was localized on the nucleus. We introduced the SAMDC gene into the excellent maize inbred line variety GSH9901 and used Agrobacterium-mediated transformation to produce cold-tolerant transgenic maize lines. After a 3-year single-location field trial, the contents of polyamine (PA), proline, malondialdehyde, an antioxidant enzyme, and APX in the leaves of transgenic maize plants overexpressing SAMDC were significantly increased, and the introduction of the SAMDC gene was significantly increased the expression of CBFs and cold-related genes.The agronomic traits of overexpression maize changed and the yield traits were significantly improved, but no significant changes were found in plant height, ear length, and shaft thickness.Thus, engineering the SAMDC enzyme is an effective strategy to improve the cold tolerance and value of maize.

Introduction

Maize (Zea mays L.) is an annual herbaceous plant and the second-largest food crop in the world (Jiao et al. 2021). Chilling damage has become a major adverse factor for the growth and development of corn, seriously affecting its growth and yield (He et al. 2020). Therefore, the differences in physiological and biochemical indicators and agronomic traits of plants were studied using biotechnological methods such as genetic modification under cold stress, providing an important theoretical basis for cultivating new cold-tolerant maize varieties.

S-adenosylmethionine decarboxylase (SAMDC) is one of the key enzymes (SAMDC, ODC, ADC) in the polyamine (PA) biosynthesis pathway, which can catalyze S-adenosylmethionine (SAM) after decarboxylation, thus providing aminopropyl group needed for synthesis reaction and promoting the conversion of putrescine into spermidine and spermine (Laha et al. 2019; Ji et al. 2019; Das et al. 2019). Meng et al. (2020) isolated the full-length cDNA of SAMDC (AhSAMDC) from peanut (Arachis hypogaea L.), which can effectively increase PA content and reduce membrane damage to enhance plant resistance to salt stress. Liu et al. (2018) found that the expression of the CmSAMDC gene in melon was induced by powdery mildew and may be involved in the response related to powdery mildew resistance. Luo et al. (2017) found that overexpression of the SAMDC gene could improve the cold tolerance of Fructus edulis by participating in H_2O_2 and NO signal transduction. Ifigeneia et al. (2016) found that overexpression of the SAMDC gene under salt stress could increase biomass and change developmental characteristics, such as increased height and leaf number. Osama et al. (2010) found that overexpression of the SAMDC gene could increase the level and ability of PA accumulation. Chen et al. (2018) found that cholesterol could induce the expression of SAMDC and promote the synthesis of Spd and Spm, leading to plant dwarf and drought tolerance. At present, the SAMDC gene has been cloned from many plants, such as Arabidopsis, rice, and wheat (Marco et al. 2014; Basu et al. 2014; Li et al. 2000), but the study of the SAMDC gene in maize has not been reported yet.

In this study, to cultivate cold-tolerant maize lines, Agrobacterium was used to transform callus, and the SAMDC gene was overexpressed into the excellent maize inbred line GSH9901. Under cold stress, the SAMDC gene was overexpressed to achieve the PA content and proteolytic content in the leaves. The significant increase in acid content, malondialdehyde content, antioxidant enzyme content, and yield proves that the SAMDC gene can improve the cold resistance of maize effectively.

Materials And Methods

Plant material and cold treatment

Wild-type (WT) and transgenic maize plants (C3) were grown in soil in a growth chamber at 25 °C, 75% humidity, under a 16 h light/8 h dark cycle. Three-leaf period plants were subjected to cold treatment by incubating them in the dark, at 4 °C, for 0,12, and 24 h. Control plants were incubated at 25 °C under the same conditions in the dark.

Sub-Cellular localization assay of ZmSAMDC

To study the subcellular localization properties of ZmSAMDC, we modified the pCAMBIA1302 vector to construct a fusion expression vector p1302-Ubi-ZmSAMDC-GFP for ZmSAMDC and the green fluorescent protein reporter gene GFP. The maize Ubiquitin promoter was used in this vector to regulate gene expression. A control vector p1302-Ubi-GFP was also constructed. Agrobacterium carrying both vectors was immersed in tobacco leaf cells. The infected tobacco leaf cells within 24 hours were observed with an LSM710 microscope.

Transformation and molecular characterization

In order to obtain SAMDC-overexpressed transgenic maize, primer pairs containing BstEII (5'-ACTCTTGACCATGGTAGATCTTCCCTCCATCTCCAGCATTG-3') and BglII (5'-GGGGAAATTCGAGCTGGTCACCAACCACGAAATTGCGACGAT-3') restriction enzyme sites were used, respectively and. The open reading frame of ZmSAMDC was amplified. The amplified product was inserted into the cauliflower mosaic virus (CaMV) 35S promoter downstream of the pCAMBIA3301 vector to replace the GUS encoding gene gusA. The recombinant plasmid pCAMBIA3301-ZmSAMDC was introduced into maize "GSH9901" using the Agrobacterium-mediated transformation method (Jiao et al. 2019) in the early stage of our research group.

T3 generation plants were identified using PCR by selecting bar genes with the primer pair 5'-TCAAATCTCGGTGACGGGC- 3 ' and 5 '-ATGAGCCCAGAACGACGCC-3' (552 bp). ZmSAMDC protein expression was tested with western blot analysis in T3 generation plants. The protein from young leaves was fractionated by dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) using a Mighty Small II electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, USA) (Wang et al. 2019). The slab gel was composed of a 12% (w/v) separation gel and a 5% (w/v) concentration gel. The wet transfer technique used the wet transfer technique to separate the leaf protein and then blotted onto

the PVDF membrane. The primary antibody was added to the protein. Besides, the protein was incubated with the secondary antibody. Finally, the color with DAB horseradish peroxidase coloring solution was developed to stain the protein in the dark until obvious bands appeared. Then the sample was washed with PBST to stop the reaction, drain, and saved with pictures.

Physiological and biochemical index detection

The leaves of the three transgenic lines and WT were sampled at the three-leaf stage. High-performance liquid chromatography (HPLC) was used to determine the PA content of plant leaves (Xu et al. 2016). The acid ninhydrin method was used to analyze the proline content of maize leaves (E et al. 2015). The thiobarbituric acid method was used to analyze the malondialdehyde content of corn leaves (Yun et al. 2013). The dry weight and fresh weight analysis techniques were applied to determine the relative water content of plant leaves (Wopereis et al. 1996). The guaiacol method was used to analyze the peroxidase content of corn leaves (Li et al. 2020). The nitrogen blue tetrazolium method was used to analyze the superoxide dismutase content of maize leaves (Samuilov et al. 2021). The UV absorption method was used to analyze the catalase content of corn leaves (Svetlana et al. 2011). An absorbance photometer at 290nm (absorption coefficient 2.8mM-1cm-1) was used to determine APX activity (Hameed et al. 2012). The experiment was repeated three times, and the average refers to the calculation of various physiological indicators.

Expression analysis of cold-responsive genes regulated by ZmSAMDC

RT-qPCR was used to detect cold signal pathway genes CBF1/2/3, RD29A, COR15A, and COR47. WT plants were used as controls, and ACTIN2 was used as an internal reference gene. The 2-ΔΔCt method calculated the relative expression changes between samples (Livak and Schmittgen, 2001). Each experiment was repeated 3 times. The data represents the mean ± standard deviation. The single-factor Duncan, a multiple comparison test, was used to determine the significance of the experimental mean when $P<0.05$.

Field trial methods

The T3 transgenic lines "C3-1", "C3-3", "C3-7", and "C3-11" overexpressing ZmSAMDC and "GS9901" maize (WT) will be planted under natural conditions in Changchun, Jilin Province in 2020 City Nanguan District Jilin Agricultural University genetically modified crop test base (43°47'56"N, 125°24'2"E). The experiment adopted a completely randomized block design with 3 replicates. The plants were planted in rows of 5 meters long, 1 meter apart, and 25 cm apart. The leaves of 8 WT and 8 transgenic lines ("C3-1", "C3-3", and "C3-6") were randomly sampled at the seedling stage for physiological and biochemical index determination; 8 WT and 8 plants were randomly sampled at the maturity stage Agronomic traits were measured for transgenic lines.

Identification of Agronomic Characters of Transgenic Plants

Three transgenic plants and WT were planted in the transgenic base of Jilin Agricultural University. A total of 20 plants were randomly selected during the growth period to analyze their agronomic traits after they matured.

Statistical analysis

The statistical data analyses were performed using the IBM SPSS Statistics 19 (the general term for a series of software products and related services for statistical analysis operations, data mining, predictive analysis, and decision support tasks launched by IBM). The significant differences were determined using paired Student's t-test, and data were presented as means ± standard deviations. $P < 0.05$ were considered to be statistically significant.

Results

Identification of subcellular localization of ZmSAMDC in tobacco

To study the subcellular localization of **ZmSAMDC**, the target gene was cloned into the transient expression vector pCAMBIA1302-GFP using Gateway recombination technology. Due to the fusion of ZmSAMDC and GFP, the subcellular localization of ZmSAMDC can be observed under a confocal microscope. Besides, ZmSAMDC can be transformed into tobacco leaves using the green fluorescence characteristics of GFP. The results showed that the **ZmSAMDC** fusion protein was located only in the nucleus (Fig. 1).

The transgenic maize overexpressing ZmSAMDC

PCR analysis of T3 generation transformed plants using specific primers of the selection marker gene Bar showed that 6 independent transgenic lines were obtained (Fig. 2). WB results showed that compared with the control, the protein expression content of the transgenic lines was significantly increased, and all of them could successfully express 65.53 kDa protein (Fig. 3). Transgenic lines "C3-1", "C3-3", and "C3-6" were selected for analysis of physiological and biochemical indicators and yield traits.

Analysis of PA content in plants overexpressing ZmSAMDC

As a low molecular weight aliphatic nitrogenous base with strong biological activity, PA can bind to the phospholipids of cell membranes under cold stress to prevent intracellular solutes from exuding and improve the cold resistance of plants. The average content of the three PAs of the transgenic line C3 was higher than that of the control (Table 1). The absolute content of Put, Spd, and Spm was 0.04, 0.048, and 0.02 mmol/g higher than that of the control. The relative proportions of Put, Spd, and Spm in the leaves of transgenic plants have also changed. The proportion of Spd in plants overexpressing the ZmSAMDC gene was significantly increased, while the proportion of Put and Spm decreased, and the relative proportion of Put decreased.

Overexpression of ZmSAMDC enhances the cold tolerance of maize

In order to study the role of ZmSAMDC in cold tolerance, we further analyzed the three transgenic maize plants (C3-1, C3-3, and C3-6) overexpressing ZmSAMDC. RT-qPCR analysis of 3 transgenic lines showed that ZmSAMDC was highly expressed in maize (Figure 4A). After 0, 2, 4, and 6 days of cold stress at 4℃, the germination ability of transgenic seeds was higher than that of the control group (Figure 4B). Under normal circumstances, the morphological difference between the transgenic line and the wild-type plant is not statistically significant. After 0, 8 and 12 hours of cold stress at -4 ℃, the damage of the transgenic line at the seedling stage was significantly lower than that of the control group (Figure 4C). The survival rate and relative water content of transgenic lines were significantly higher than those of WT (Figure 4D and 4E). The results indicate that overexpression of ZmSAMDC improves the cold tolerance of transgenic maize.

Overexpression of ZmSAMDC under cold stress significantly increased leaf proline content and malondialdehyde content

Under cold stress, the proline content in plants increases, and varieties with strong cold resistance tend to accumulate more proline. With the increase of the time of low temperature (4 ℃), the Pro content of transgenic plants (C3-1, C3-3, and C3-6) showed an upward trend (Figure 5A). Among them, we found that the most significant change was the 24 h treatment at 4℃. The average proline content of the transgenic lines was 6.1 μg/ml higher than the control group. The results indicate that overexpression of ZmSAMDC changes the protein composition of transgenic maize leaves, resulting in a large accumulation of Pro in plant cells.

Under cold stress, the content of malondialdehyde which could reflect the stress resistance of plants, increased with the increase of the active oxygen content of plant leaves. With the increase of the time of low temperature (4 ℃), the MDA content of transgenic plants showed an upward trend (Figure 5B). These results indicate that the transgenic lines accumulate relatively low ROS under cold stress.

Overexpression of ZmSAMDC under cold stress enhance plant cold resistance by increasing leaf antioxidant enzymes

The metabolic system of the plant will undergo significant changes in a cold environment. The amount of oxygen absorbed by the plant will be reduced, and a large amount of harmful active oxygen will be accumulated, thus causing certain damage to the plant. The level of antioxidant enzyme activity can measure the strength of plant resistance. With the increase of the time of low temperature (4 ℃), the contents of POD, SOD, CAT, and APX of the transgenic plants showed an upward trend (Figure 6A-D). When the transgenic plants were treated at 4 ℃ for 48 h, the average content of POD, SOD, and CAT was higher than the control group. When the transgenic plants were treated at 4 ℃ for 12 h, the average ascorbate peroxidase content of the transgenic line was 5.58 μmol/mg higher than that of the control group. Therefore, the overexpression of ZmSAMDC changes the oxidative stress response of plants, improves the ability of plants to resist oxidation and scavenging cationic free radicals, and promotes the decomposition of H2O2 and the ability to catalyze AsA in plants.

ZmSAMDC positively regulates CBFs and cold-responsive gene expression under cold stress

In order to further clarify the molecular mechanism of ZmSAMDC overexpression lines responding to cold stress, we used qRT-PCR to study the expression patterns of cold-induced CBF family genes and downstream cold-responsive genes. In the transgenic lines and WT plants, the CBF family genes CBF1, CBF2, and CBF3 were induced rapidly and peaked 12h after cold stress. However, the expression levels of these three CBF genes in all transgenic lines were higher than those of WT. RD29A, COR15A, and COR47 are the downstream target genes of CBF, and these COR genes are gradually induced to express under cold stress (Figure 7). These results indicate that overexpression of ZmSAMDC positively regulates the expression of the CBF gene and downstream COR gene, thereby improving the cold tolerance of maize.

Overexpression of ZmSAMDC significantly increased the yield of maize

Field experiments were conducted to observe the agronomic traits of transgenic plants. The results are shown in Table 2. The transgenic materials for plant height, ear length, shaft thickness, and other traits have no significant difference with the control group, indicating that the ZmSAMDC gene may not affect these traits. However, the number of rows and 100-seed weight of ZmSAMDC plants were significantly higher than those of the control group, and the bald tip length was significantly lower than that of the control group. Therefore, the SAMDC gene can effectively increase the yield of maize.

Discussion

As a key enzyme in synthesizing spermine and spermidine, SAMDC participates in the resistance reaction of most plants. In many cases, H_2O_2 produced by the PA catabolism pathway is a protective measure (Cona et al. 2005). In 2017, Diao et al. also demonstrated in tomato that Spd and Spm induced the generation of H_2O_2 by increasing the activities of diamine oxidase and PA oxidase and prompting the ROS system to respond. In 2015, Saha et al. (2015) also confirmed that PA may trigger ROS synthesis or scavenging ROS, depending on the concentration of PA in the cell. In the stress response process, PA can stabilize the composition of molecules and maintain the integrity of cell membranes by multiple binding proteins (H et al. 2004; Liu et al. 2011), thereby eliminating ROS in plants (Kolupaev et al. 2021) and reducing lipids. Peroxidation maintains membrane stability and reduces oxidative stress damage. In this study, the contents of PRO, CAT, POD, SOD, and MDA in the overexpressed plants were all higher than those of the negative control group, indicating that the overexpression of the ZmSAMDC gene had an impact on the reactive oxygen species and ROS system during cold stress. The results further prove the hypothesis that PA affects the ROS system indirectly. The above results are basically consistent with previous studies on overexpression of the SAMDC gene to improve cold tolerance, drought tolerance, and salt tolerance (Basu et al. 2014; Li et al. 2006; Meng et al. 2020).

Agronomic traits are an important index for breeding excellent varieties of maize. In this study, the baldness, number of rows, and 100-seed weight of the overexpression plants were improved, indicating that the ZmSAMDC gene could effectively hinder the decreased yield caused by low temperature. Bais et

al. (2002) found that PA was widely involved in life activities and growth and development processes in plants, including fruit development and maturation, senescence, and stress response. Zhu et al. used the antisense RNA of the SAMDC gene to decrease the transcription level of SAMDC rapidly. With decreased Spd and Spm content, plants showed growth inhibition, internode shortening, stem branching, and leaf reduction (Zhu et al. 2020).

The most important finding of this study is that overexpression of the SAMDC gene increases the PA content, proline content, malondialdehyde content, and antioxidant enzyme content in the leaves, thereby increasing the yield of maize under cold stress. Therefore, engineering the SAMDC enzyme is an effective strategy to improve the chilling tolerance and value of maize.

Declarations

Acknowledgments We thank SYG and YYM conceived research plans and designed experiments.

Author's contributions SYG and YYM conceived research plans and designed experiments. PJ, NNC and CLW, conducted experiments. PJ and SYJ wrote the draft. SYL and JQ analyzed the data. PJ SYG and YYM reviewed and edited this article and provided helpful comments and discussions. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Tables

Table 1 Determination of the average content of three polyamines in transgenic strain C3

Genotype	Put (mmol/g)	Spd (mmol/g)	Spm (mmol/g)	PAs (mmol/g)	Put/PA	Spd/PA	Spm/PA
C3-1	0.06a	0.064a	0.03a	0.16a	0.375b	0.4a	0.1875b
C3-3							
C3-6							
WT	0.02b	0.016b	0.01b	0.05b	0.4a	0.32b	0.2a

Table 2 Agronomic performance of overexpressing ZmSAMDC lines and wild-type plants in field

Genotype	Plant height (cm)	Ear henght (cm)	Ear diameter (cm)	The average bald tip (cm)	Kernel numbers	100-grain weight (g)
C3-1	121.72±0.42	14.3±0.16	5.58±0.07	1.38±0.02*	34*	29.32±0.12**
C3-3	121.64±0.4	14.3±0.07	5.65±0.06	1.43±0.15*	35*	29.35±0.25**
C3-6	121.57±0.57	14.2±0.13	5.45±0.2	1.28±0.75**	34*	29.79±0.24**
WT	121.82±0.41	13.9±0.67	5.66±0.15	2.28±0.13	30	26.32±0.01

Figures

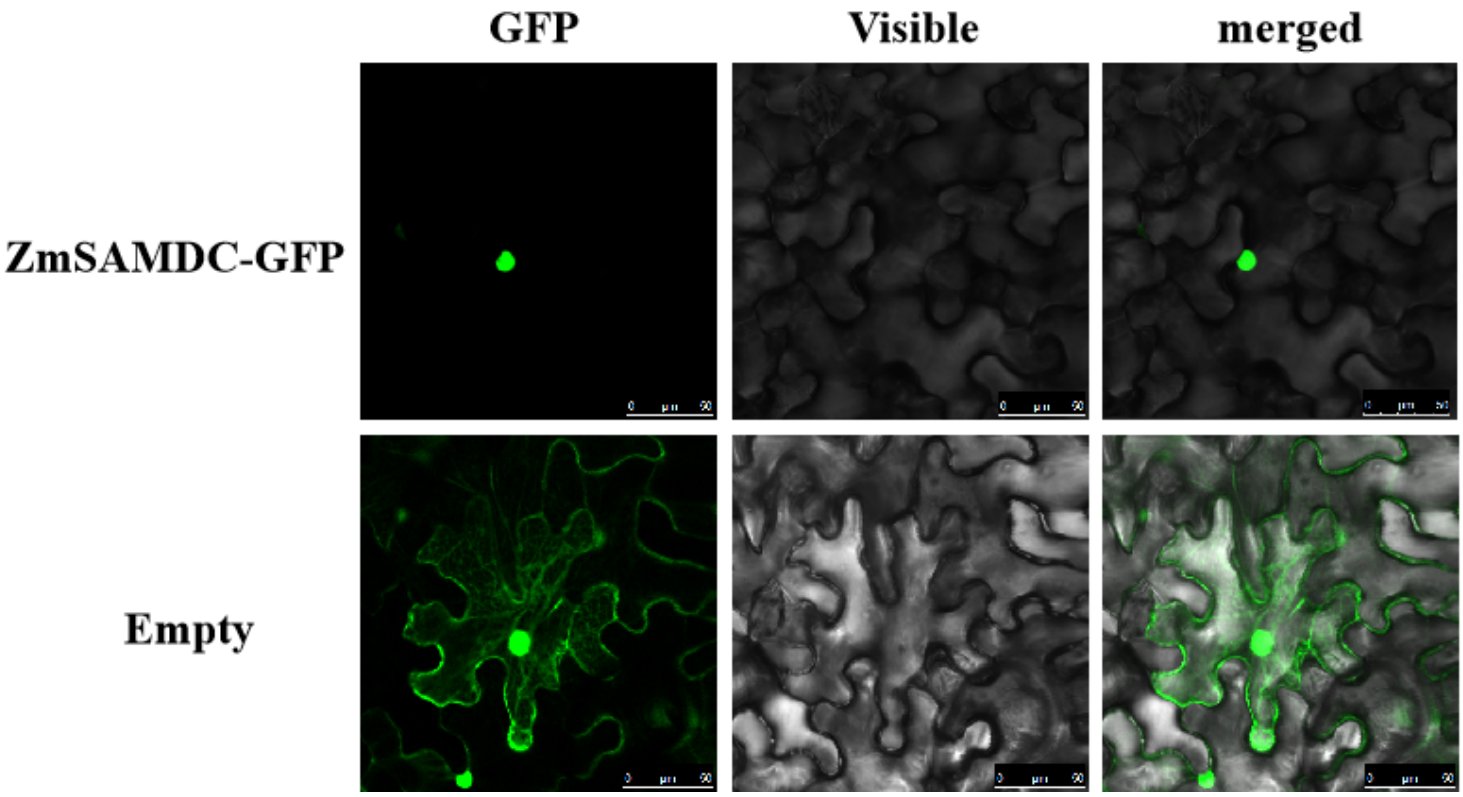


Figure 1

Sub-cellular localization analysis of the ZmSAMDC gene bar=50 μm

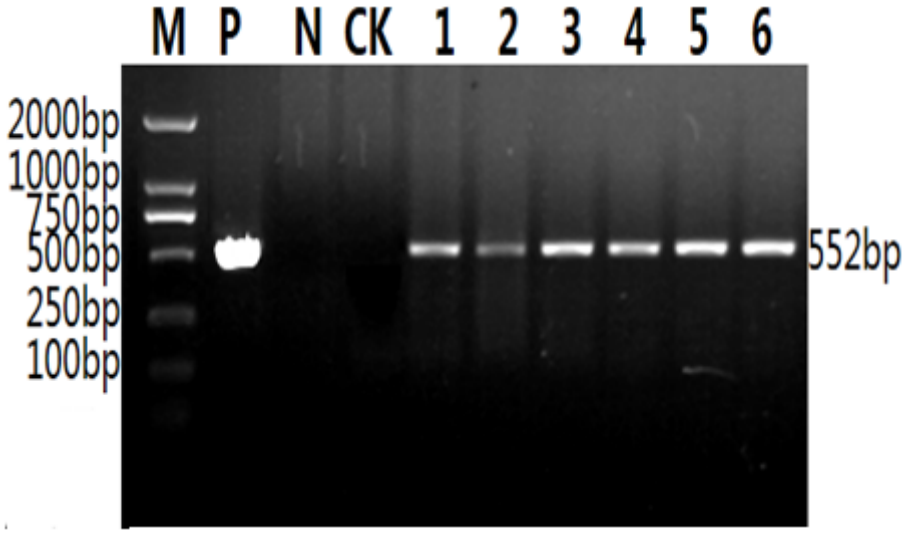


Figure 2

Identification of transgenic maizes overexpressing ZmSAMDC by PCR analysis(bar gene).M: DL2000 ladder, P: positive control (plasmid as template), N: negative control (H2O as template), CK: negative control (“GSH9901” DNA as template), 1–6: transgenic plants.

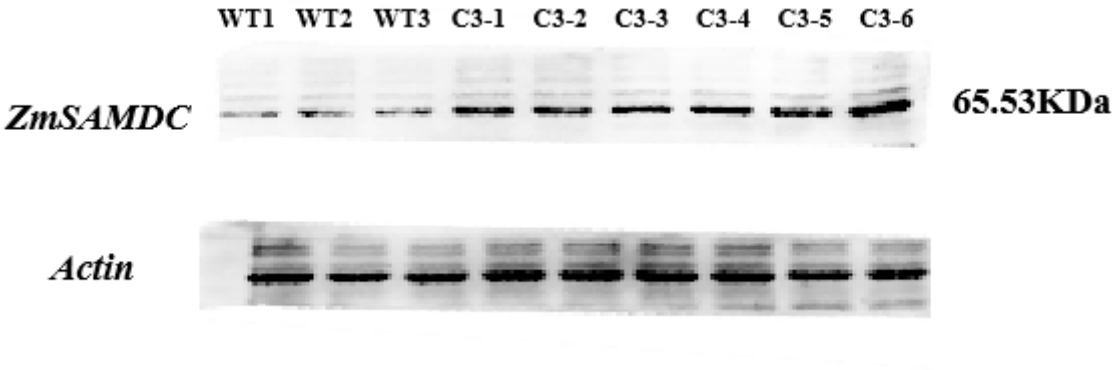


Figure 3

western blot analyses of transgenic maizes overexpressing ZmSAMDC. WT1-WT3: negative control. C3-1-C3-6: transgenic plants.

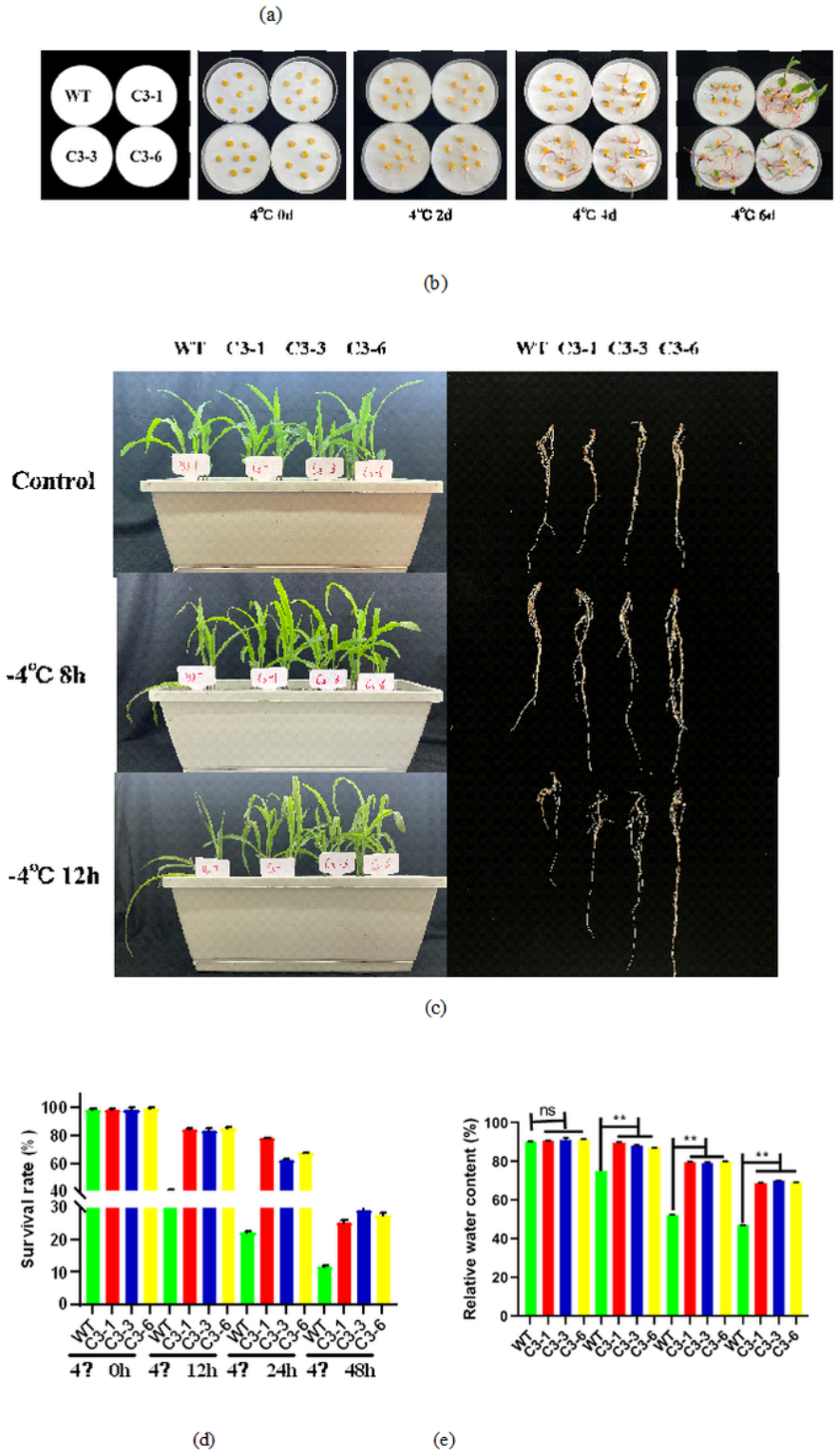
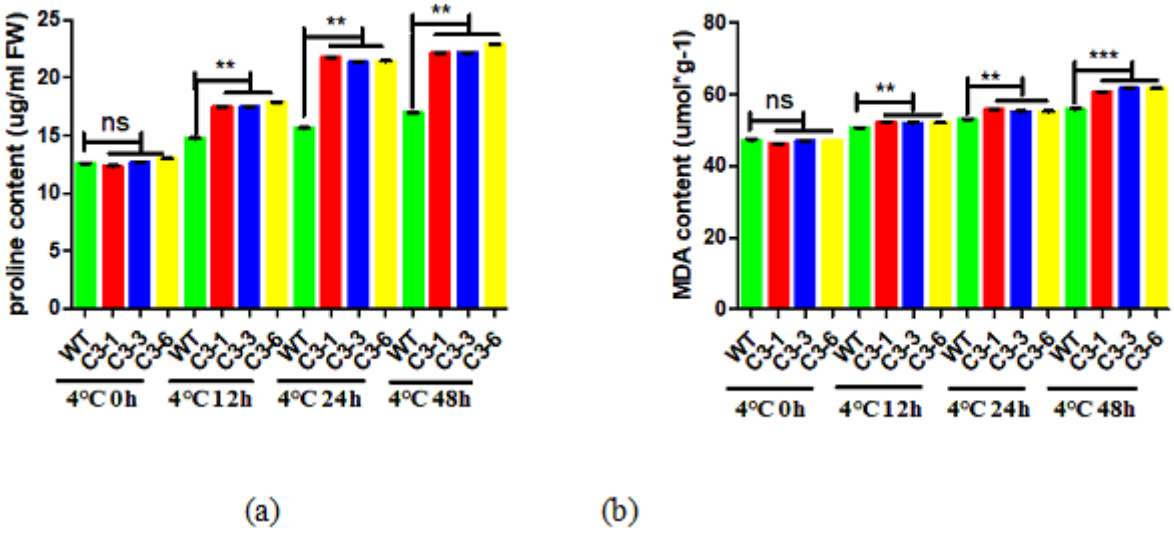


Figure 4

Overexpression of ZmSAMDC enhances the cold tolerance of maize.a Analysis of ZmSAMDC expression in positive transgenic maize. Using ACTIN2 as an internal reference gene, the mRNA levels in transgenic lines C3 and WT were detected by RT-qPCR.The frozen phenotype (b and c), survival rate (d) and relative water content (e) of transgenic lines C3-1, C3-3 and C3-6 and WT. Corn seeds germinate after cold acclimation (2-6 days at 4°C), and are treated at -4°C for 8 hours and 12 hours after the three-leaf period.

The data represents the mean ± standard deviation (SD) of three biological replicates. Non-significant (ns) ,P<0.01 (***) and P<0.05 (**).



(a) (b)

Figure 5

Leaf proline content (a) and MDA content (b) of three ZmSAMDC overexpression transgenic and wild-type plants.The data represent the means ± standard deviations of three replicate experiments. Non-significant (ns) ,P<0.01 (***) and P<0.05 (**).

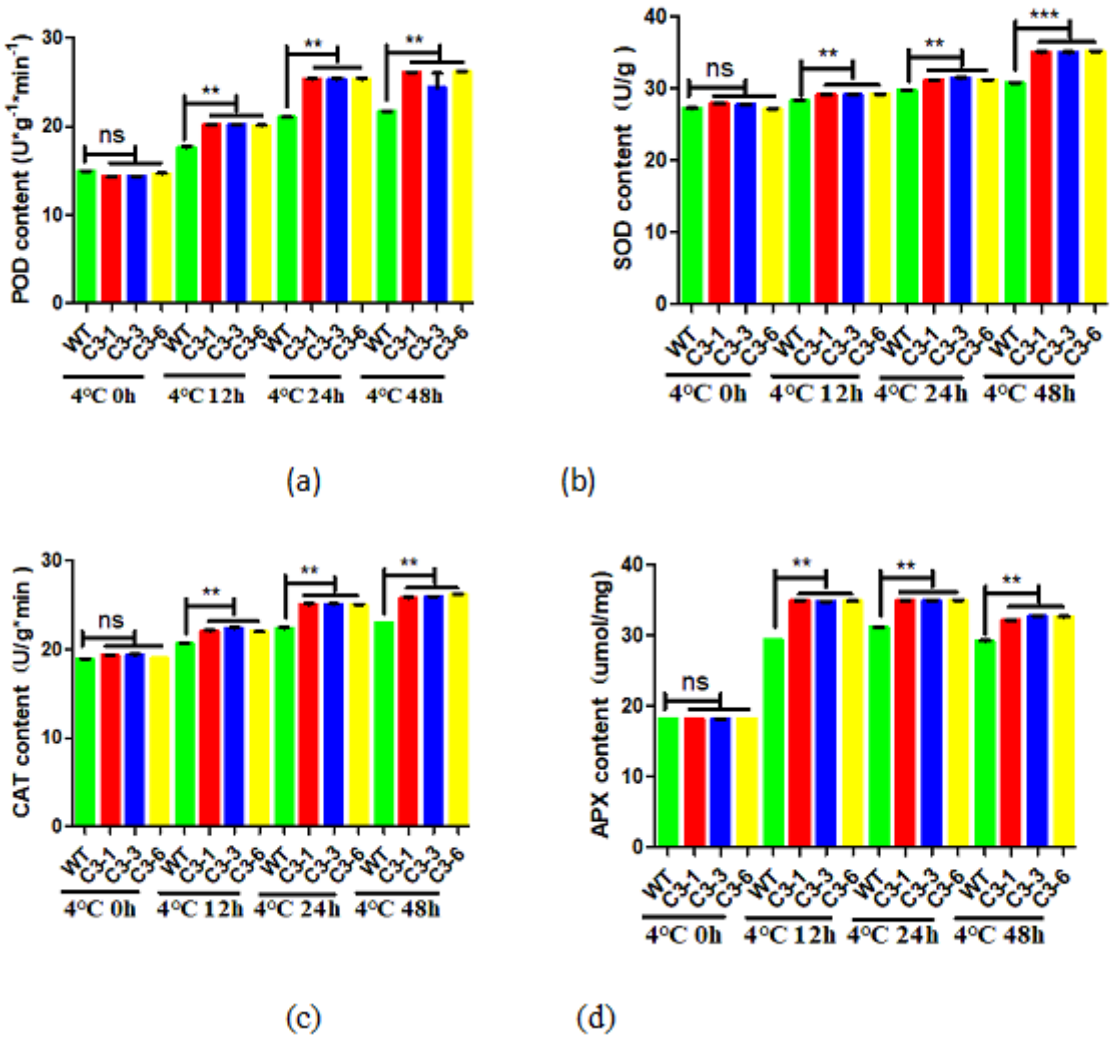


Figure 6

Three ZmSAMDCs overexpress the leaf antioxidase (a:Peroxidase; b: superoxide dismutase; c:catalase; d:ascorbate peroxidase) content of transgenic and wild-type plants. The data represent the means ± standard deviations of three replicate experiments.Non-significant (ns) ,P<0.01 (***) and P<0.05 (**).

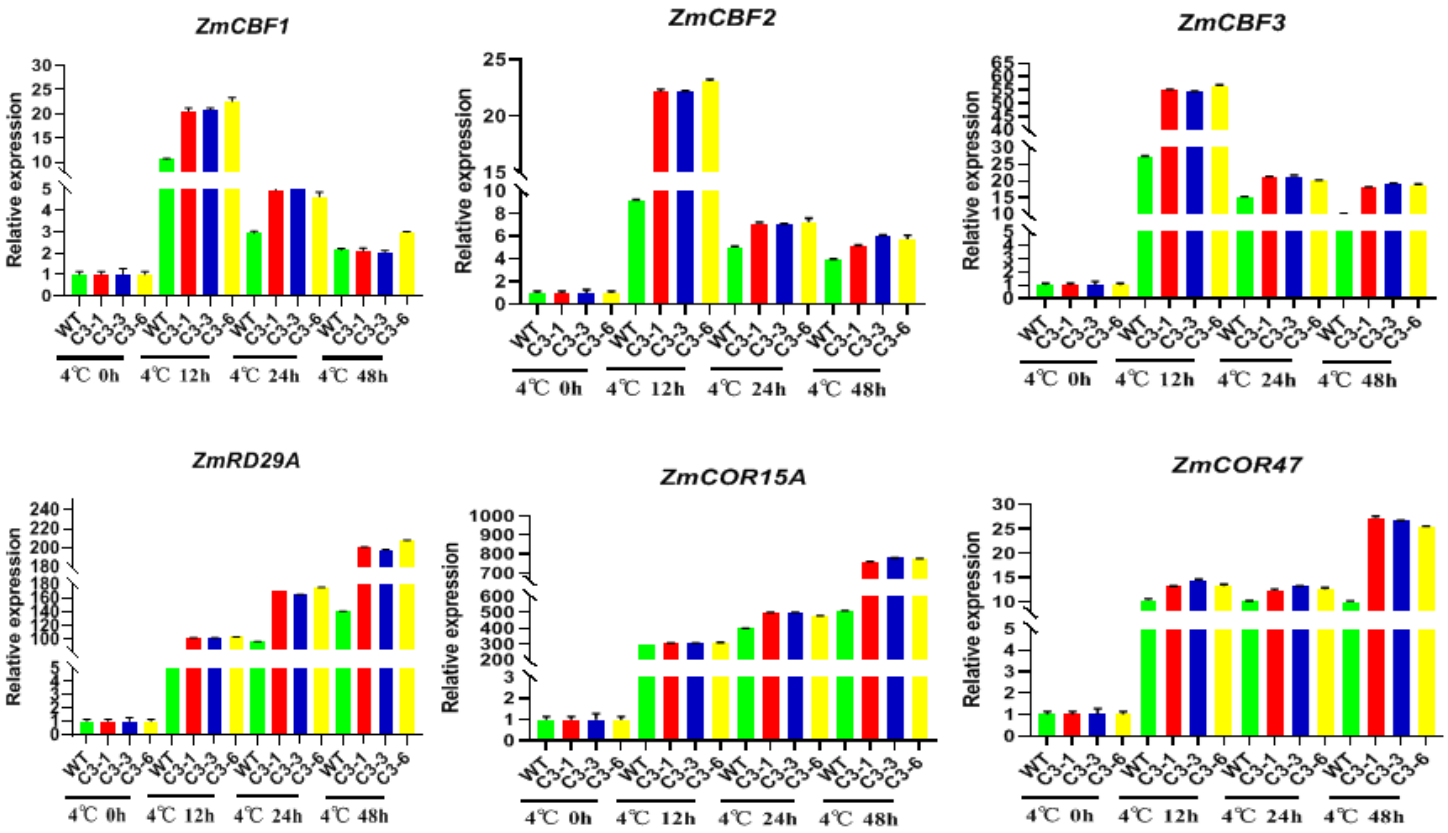


Figure 7

Expression of cold-responsive genes in WT and the transgenic lines under cold treatment.