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RESEARCH

Mechanistic Basis of Silicon Mediated Cold Stress Tolerance in Alfalfa (*Medicago sativa* L.)

Md Atikur Rahman[1] · Yowook Song[1] · Md. Mahadi Hasan[2] · Mohammad Shah Jahan[3,4] · Manzer H. Siddiqui[5] · Hyung Soo Park[1] · Sang-Hoon Lee[1] · Deepti Singh[6] · Francisco J. Corpas[7] · Ahmad Humayan Kabir[8] · Ki-Won Lee[1]

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Abstract

Cold stress (CS) impact on crops is one of the critical constraints for sustainable and smart agricultural production. CS adversely affects plants leading to growth retardation, necrosis, chlorosis, and significant yield loss. The objective of this study was to explore the mechanistic basis of silicon (Si) in enhancing CS tolerance in alfalfa plants. The fluorescence staining indicated that Si-reduced the intensity of CS-induced superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) generation in plants that improved plant photosynthesis, cellular integrity, and alfalfa biomass production under CS. The exogenous supplementation of Si significantly restored the endogenous Si status accompanied by the upregulation of *NIP* (nodulin 26-like intrinsic protein) genes *NIP2*, *NIP5;1*, and *NIP6;1* in alfalfa. The elemental concentration analysis revealed that exogenous silicon (E-Si) triggers the increase of calcium (Ca), magnesium (Mg), and sulfur (S) in plants subjected to Si-supplementation compared to the plants cultivated without Si under CS. The application of Si significantly increased the activity of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). Furthermore, Si significantly enhanced the expression of CS-responsive candidate genes including *ICE1*, *CBF1/DREB1C*, *CBF2/DREB1B*, *CBF3/DREB1A*, *COR15A*, *COR47*, and *KIN1* in alfalfa. These findings together provide mechanistic insights into Si-involving CS tolerance in alfalfa. This eco-friendly SC management strategy using Si treatment can be useful to plant breeders and farmers for developing CS-resilient smart alfalfa production through breeding program.

Keywords Low temperature · Silicon transporter · Cold responsive marker gene · Alfalfa · Forage

✉ Ki-Won Lee
kiwon@korea.kr

[1] Grassland and Forage Division, Rural Development Administration, National Institute of Animal Science, Cheonan 31000, Republic of Korea

[2] State Key Laboratory of Grassland Agro-Ecosystems, College of Ecology, Lanzhou University, Lanzhou 730000, China

[3] Guangdong Key Laboratory for New Technology Research of Vegetables/Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China

[4] Department of Horticulture, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka 1207, Bangladesh

[5] Department of Botany and Microbiology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia

[6] Department of Botany, Meerut College, Meerut 250001, India

[7] Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Department of Stress, Development and Signaling in Plants, Estación Experimental del Zaidín, Spanish National Research Council (CSIC), Profesor Albareda 1, 18008 Granada, Spain

[8] School of Sciences, University of Louisiana Monroe, Monroe, LA 71203, USA

1 Introduction

Cold stress (CS) in winter through chilling (0–15 °C) and freezing (< 0 °C) is an environmental limiting factor that declines plant growth, survival rate, ecological distribution, and agricultural productivity [1]. In South Korea, crops are continuously exposed to freezing temperatures mostly from November to March of the year. The low temperature in the growing season severely limits plant germination, vegetative growth, reproductive development, and yield [2]. Severe freezing stress may cause considerable damage to aerial parts and even plant death of overwintering crops. Therefore, CS is one of the most challenging factors in forage crop production [1]. Considering these above limitations of crop production under CS, an eco-friendly, cost-effective, and sustainable CS-tolerance and mitigation strategies are highly demandable.

Mineral nutrition-mediated abiotic stress alleviation in plants is one of the promising approaches [3, 4]. Silicon (Si) is a beneficial element that efficiently mitigates multiple abiotic stresses and improves growth in plants [5–9]. Plant seed soaking, foliar spray, and root treatment with exogenous supplementation of Si mitigate the negative effects of frost-induced damage in plants [10]. Si enhances chilling tolerance in maize through the regulation of macronutrient homeostasis and hormonal balancing [11]. Si shows interaction with other essential and beneficial elements. For instance, exogenous Si enhances endogenous Si [6], declines the activity of Ca^{2+} [12], enhances the regulation of magnesium (Mg) and sulfur (S) [13]. These interactions lead to enhance plant growth, fitness, and stress adaptation in plants. Recently it has been reported that Si influx into the cells is mediated by nodulin 26-like intrinsic proteins (NIPs), which are family members of major intrinsic proteins (MIP) [14]. *NIP* genes are located in the plasma membrane (PM), endoplasmic reticulum, and vacuoles, and are reported to be expressed in the entire organelle or cell surface [15]. NIP transporters including *NIP2, NIP5;1*, and *NIP6;1* promote Si uptake in plants [6]. These transporters show higher permeability to low organic molecules (LOM), thereby transporting Si and other beneficial or toxic metals [6].

A series of physiological and molecular changes occur in plants under CS. For instance, growth delay, chlorosis, reduction of photosynthesis, retarded seedling growth, leaf curling, and tissue damage [1]. CS damages cell membrane (CM) by changing membrane fluidity, low water potential (LWP), lipid component, and accumulation of reactive oxygen species (ROS) [16]. Plants have evolved mechanisms to cope CS while exposed to non-freezing temperatures (> 0 °C) [17]. In cold adaptation processes, plant enhances the accumulation of osmolites, late-embryogenesis abundant (LEA) proteins, and ROS-scavenging antioxidant enzymes (e.g. SOD, CAT, APX, POD, GR, etc.). CS triggers the signaling pathways that regulate the expression of stress-responsive genes dehydration-responsive elements or C-repeat binding factor genes (*CBF/DREBs*), which regulates the expression of *COLD-REGULATED* (*COR*) candidate genes [1]. Another key candidate is *ICE1* (Inducer of CBF expression 1) which binds to MYC recognition cis-elements (CANNTG) in the promoter of *CBF3/DREB* and controls the transcription of CBF during CS adaptation in Arabidopsis [18]. Overexpression of *ICE1* enhances freezing tolerance in transgenic Arabidopsis [19], Zoysia grass [20], cold-drought-salt tolerance in transgenic tobacco and rice [21, 22]. However, it is still unclear how the combined roles of *ICE1* and *COR* in cold stress tolerance in *Medicago* species. Additionally, no report has yet been established on mechanistic insights of Si-mediated cold stress tolerance in alfalfa species.

Alfalfa (*Medicago sativa* L.) is an excellent forage legume that is extensively cultivated as a hay crop with excellent feeding value and high biomass production [23]. Alfalfa supplies N_2 benefits to soils that reduce the dependency on using commercial N_2-fertilizer to the soils [24]. In South Korea, the freezing temperature in the growing season shows negative impact on forages and other crop species. In this context, an eco-friendly and cost-effective strategy is highly demanded for sustainable plant production under cold stress. Si supplementation plays an excellent role in protecting cold stress tolerance in diverse plants [10]. However, the mechanistic basis of Si-mediated cold stress tolerance in alfalfa legumes has not yet been studied. Therefore, we analysed whether and how exogenous Si enhances cold stress tolerance in alfalfa to figure out the morphological, physiological, and molecular alterations underlying mechanisms of Si- mediated cold stress tolerance in alfalfa. Furthermore, it was investigated the interactome of cold-responsive candidates, which provides functional interaction of genes linked to cold stress tolerance alfalfa and other model plants.

2 Materials and methods

2.1 Plant Growth and Cold Treatment

Alfalfa (*Medicago sativa* L.) viable seeds were sterilized with 70% ethanol for 1 min, and rinsed thrice with distilled water. Then, the seeds were transferred to the germination tray for 2 days. Seedlings were placed to standard Hoagland nutrient described previously described [25]. In addition to the Hoagland solution, 1 mM potassium silicate (K_2SiO_3) was added to the four treatments as follows: control (C), cold stress (CS, 10 °C), cold stress + silicon (CS, 10 °C and 1 mM K_2SiO_3), + Si (1 mM K_2SiO_3 + control). The alfalfa

seedlings were cultivated individually for each treatment with three biological replications. Plants were grown in a control chamber with a 14 h light/10 h dark photoperiod (550–560 µmol s^{-1} per µA). The experiment was terminated after two weeks of treatment.

2.2 Determination of Photosynthetic and Morphological Parameters

Plant chlorophyll fluorescence as Fv/Fm (quantum efficiency of photosystem II) was determined using a portable fluorometer (LI-600 Porometer/ Fluorometer, Korea), leaf greenness (SPAD value) was recorded by SPAD meter (SPAD-502, Minolta, Japan). Root-shoot length (cm) and plant dry weight (g) were recorded by digital caliper and electronic weight machine, respectively.

2.3 Detection of ROS ($O_2^{\bullet-}$ and H_2O_2) in Alfalfa Seedling Root Tip by Fluorescence Microscope

Alfalfa seedling root tips (1–2 mm) were excised and washed gently with diethyl pyrocarbonate (DEPC)-treated water. The superoxide anion ($O_2^{\bullet-}$) was detected through incubating the root tips at 37ºC for 30 min in dark condition in 10 µM dihydroethidium (DHE, excitation and emission wavelength of 488 nm and 520 nm, respectively), as fluorescence probe prepared in 10 mM Tris–HCl (pH 7.4) [26]. In the case of H_2O_2 detection, it was used 25 µM 2′,7′-dichlorofluorescin diacetate (DCF-DA, Sigma-Aldrich, excitation and emission wavelength of 480 nm and 530 nm, respectively) [27].The root samples were then washed twice in the same buffer and mounted on a microscope slide for examination with a fluorescence microscope (CLS-01–00076, Logos Biosystem).

2.4 Determination of Elemental Concentration

The concentration of calcium (Ca^{2+}), silicon (Si), magnesium (Mg), and sulfur (S) in alfalfa samples were determined following the method used previously [28]. Root and shoot samples were digested using an acid solution containing $HClO_4/HNO_3$ (1:3 v/v). The elemental concentrations of digested solution were measured using ICP-spectrometry (Agilent 7700, Japan). Finally, these elemental concentrations were calculated based on standard known solutions.

2.5 Measurement of Antioxidant Enzyme Activity

The antioxidant enzyme activity in alfalfa seedlings was measured according to the protocol used previously [29]. A total of 0.1 g of plant tissue was mixed with potassium phosphate buffer (KPB-100 mM, pH 7.0) and vortexed for 3 min. The solution mixture was centrifuged at 16,000 × g for 20 min at 4ºC, the supernatant was used for enzymatic assays. The superoxide dismutase (SOD) activity was determined following the protocol [30]. To determine SOD, a solution was prepared that contained 1 ml of 0.1 mM EDTA, 50 mM $NaHCO_3$ (pH 9.8), 0.6 mM epinephrine, and plant extract (100 µL). The solution absorbance was read at 475 nm. The catalase (CAT) activity was determined according to the protocol used earlier [3]. To measure CAT activity a solution mixture containing 0.1 M KPB with 6% H_2O_2 and 100 µL plant samples. The diminishing of absorbance due to the decomposition of H_2O_2 was measured at 240 nm (0.036 mM^{-1} cm^{-1} extinction coefficient). The ascorbate peroxidase (APX) activity was measured using a mixed solution of 50 mL KP-buffer (pH 7.0) containing 0.1 mM EDTA (1 ml), 0.1 mM H_2O_2, 0.5 mM ascorbic acid, and 100 µL plant extract. The solution was exposed at 290 mm (extinction coefficient 2.8 mM^{-1} cm^{-1}). Glutathione reductase (GR) activity was measured according to the protocol used earlier [29]. Total 0.2 mol KPB (pH 7.0), 1 mM EDTA, 20 mM GSSG, 0.2 mM NADPH, and 100 µL plant extract were taken in a small EP tube. The reaction was triggered with GSSG and reduced in absorption at 340 nm in reaction to NADPH oxidation. The GR activity was measured using an extinction coefficient of 6.12 mM^{-1} cm^{-1}.

2.6 Analysis of Gene Expression

The response of candidate genes was investigated using the q-RT PCR approach. The gene-specific primers were used in this study (Supplementary Table S1) based on the *Medicago* gene database. To determine gene expression, total RNA was isolated from alfalfa plant tissue using an extraction kit (QIAGEN, USA), and the total RNA was quantified using Nanodrop Spectrophotometer. The RNA was converted to first-strand cDNA. The expression of cold-responsive candidate genes at the mRNA level was determined using a thermal-cycler system (CFX96 Dx, Biorad-USA). The q-RT-PCR was programmed as follows: 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s. The expression level of target genes was calculated following the $2^{-\Delta\Delta Ct}$ method [31], where *MsActin* is considered an internal control. A total of three individual replications were run for each condition.

2.7 Bioinformatics Analysis

A multiple sequence alignment (MSA) of the protein sequence of *Medicago ICE1* and *CBF1* genes with Arabidopsis homologs was implemented using Crustal omega

(https://www.ebi.ac.uk/Tools/msa/clustalo/). The tracking of sub-cellular localization of these homologs was predicted using CELLO v.2.5 tool. We have also predicted the sub-cellular localization of protein candidates using the CELLO (http://cello.life.nctu.edu.tw) network. Additionally, the interactions of cold-responsive genes were detected by the STRING network (http://string-db.org) and visualized in Cytoscape [32].

2.8 Statistical Analysis

All the physiological and molecular data were analyzed using analysis of variance (ANOVA). The significant level of data was tested by t-test at $P \leq 0.05$. Graphical data were prepared using GraphPad Prism software (V.8.0.2). At least three individual replications were considered for each treatment.

3 Results

3.1 Changes of Photosynthetic and Morphological Traits

Cold stress (SC) caused photosynthetic and morphological disturbance in alfalfa seedlings (Fig. 1). Photosynthetic chlorophyll fluorescence (Fv/Fm) efficiency and leaf greenness were significantly declined by 19% and 41% respectively in response to CS in alfalfa compared to control (Fig. 1A, B). The CS also showed the effect on plant growth and biomass production. The root-shoot length and their dry weights were significantly declined by 16%, 77%, 43% and 51% under CS compared to control (Fig. 1C-F). However, these parameters were reverted significantly after supplementation of Si with CS. These findings suggest that CS is critical for alfalfa seedlings that considerably inhibits plant growth and

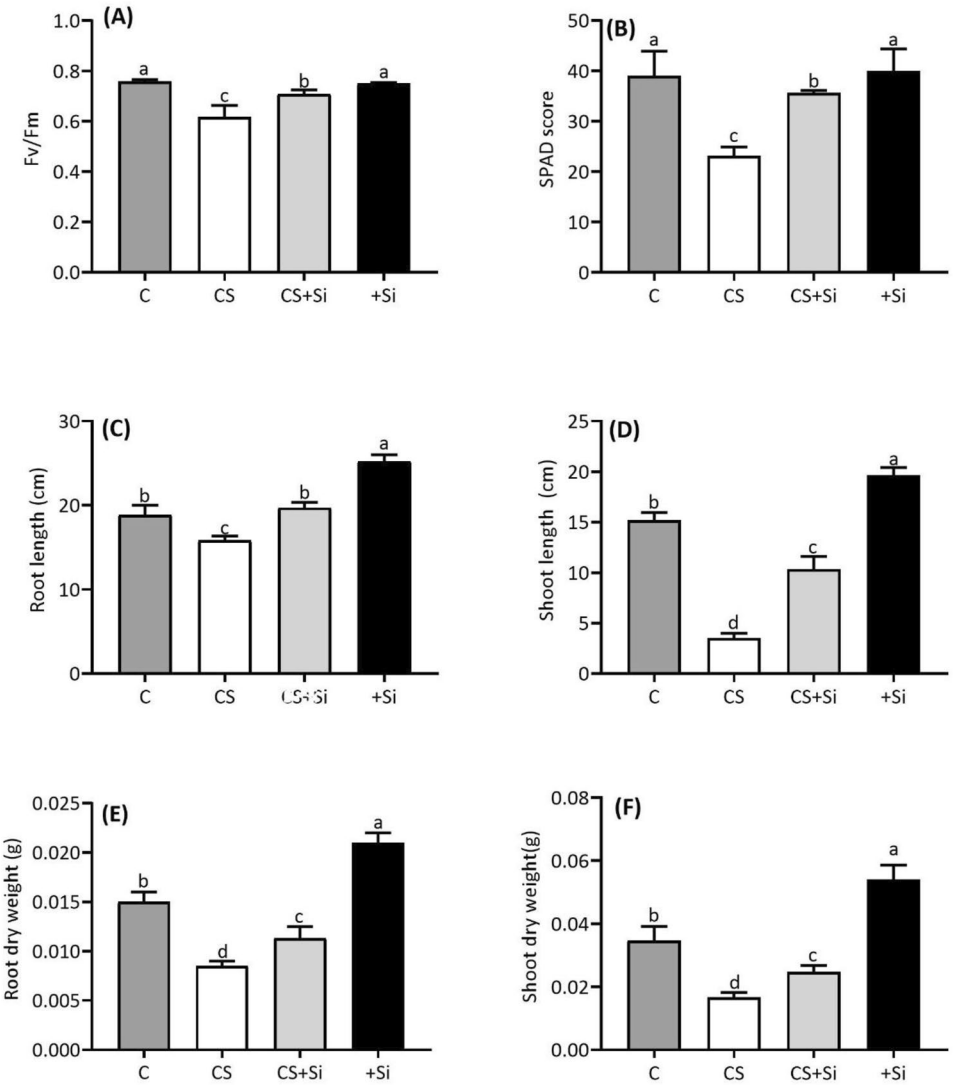

Fig. 1 Cold stress (CS)-induced photosynthetic and morphological changes in alfalfa seedlings. The 2-week-old alfalfa seedlings show phenotypic differences after exposure to cold stress. Fv/Fm (**A**); SPAD score (**B**); root length (**C**); shoot length (**D**); root dry weight (**E**); shoot dry weight (**F**) of alfalfa seedlings. Different letters above the bar column show significant differences ($P \leq 0.05$) among the group means with standard error (SE). At least three individual replications were considered for each treatment. Abbreviation, C, control; CS, cold stress; + Si, silicon supplementation with control

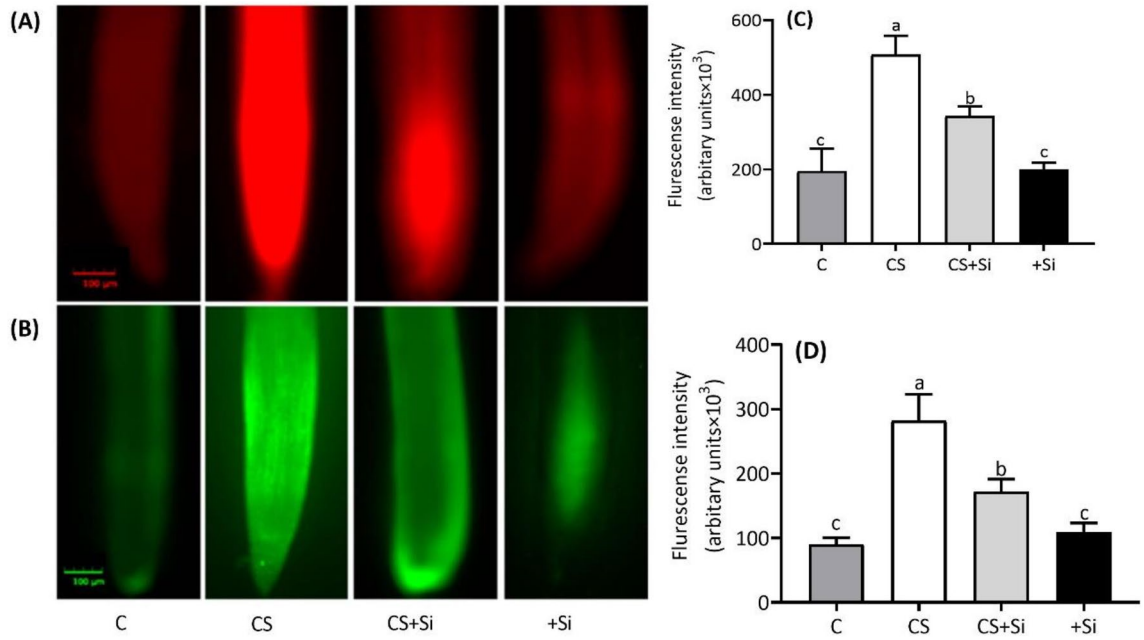

Fig. 2 Representative pictures illustrating the fluorescence microscopy detection of ROS ($O_2^{\bullet-}$ and H_2O_2) in root tips of alfalfa seeding under different treatments. **A** Superoxide radical ($O_2^{\bullet-}$) detection (red fluorescence). **B** H_2O_2 detection (green fluorescence). **C** Fluorescence intensity of $O_2^{\bullet-}$. **D** Fluorescence intensity of H_2O_2. Fluorescence is expressed as arbitrary units (A.U.). Different letters above the bar column show significant differences ($P \leq 0.05$) among the group means with standard error (SE). Abbreviation, C, control; CS, cold stress; + Si, silicon supplementation with control

photosynthesis, while exogenous Si application protects plants from CS by improving plant physiological and morphological attributes.

3.2 Cold-Induced Regulation of ROS

CS triggered the intensity of superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in alfalfa seedling root tips (Fig. 2A-B). The $O_2^{\bullet-}$ fluorescence intensity level significantly increased in response to CS compared to control plants (Fig. 2C), while it declined following Si supplementation with CS (CS + Si). However, the $O_2^{\bullet-}$ fluorescence intensity did not show any significant difference between positive control (+ Si) and control plants (Fig. 2C). Almost the same fashion was observed in the case of H_2O_2, though the $O_2^{\bullet-}$ generation intensity was higher than H_2O_2 (Fig. 2D). These data indicate that CS leads to the generation of ROS while exogenous Si can mitigate their toxicity level in alfalfa.

3.3 Accumulation of Mineral Nutrition

In this study, we found that mineral accumulation was significantly influenced by CS, and subsequent Si application in alfalfa seedlings under CS (Fig. 3). CS-signal significantly triggered the Ca^{2+} level in root and shoot, while it declined after adding Si (Fig. 3A). Interestingly, the Ca^{2+} level was higher in shoot than root tissue. Possibly, the CS signal was properly sensed by aerial parts that induced a Ca^{2+} signal. In opposition, no significant difference was observed in single Si treatment and control. Therefore, it is clear that Si has not any significant effect in inducing Ca^{2+} level (Fig. 3A). Interestingly, endogenous Si concentration significantly enhanced in combined treatment (CS + Si) and single Si treatment (Fig. 3B). Exogenous Si application showed another promising finding that significantly increased magnesium (Mg) level in alfalfa roots and shoots under CS (Fig. 3C). However, CS and even Si supplementation were not showed any significant impact in elevating sulfur (S) level in alfalfa root and shoot (Fig. 3D). The regulation of mineral nutrition in response to CS and Si indicates that especially SC signal is capable for triggering cellular Ca^{2+} level, while Si is effective for boosting endogenous Si (E-Si) and Mg, which might help to alleviate cold stress in alfalfa seedlings.

3.4 Regulation of Antioxidant Defense

CS treatment significantly increased the SOD activity compared to control plants (Fig. 4A). Interestingly, this activity was higher in combined treatment which means that Si supplementation boosted the activity of SOD under CS. The CAT activity was also significantly higher in combined treatment (CS + Si) in comparison to control plants (Fig. 4B). However, no significant variation was observed between

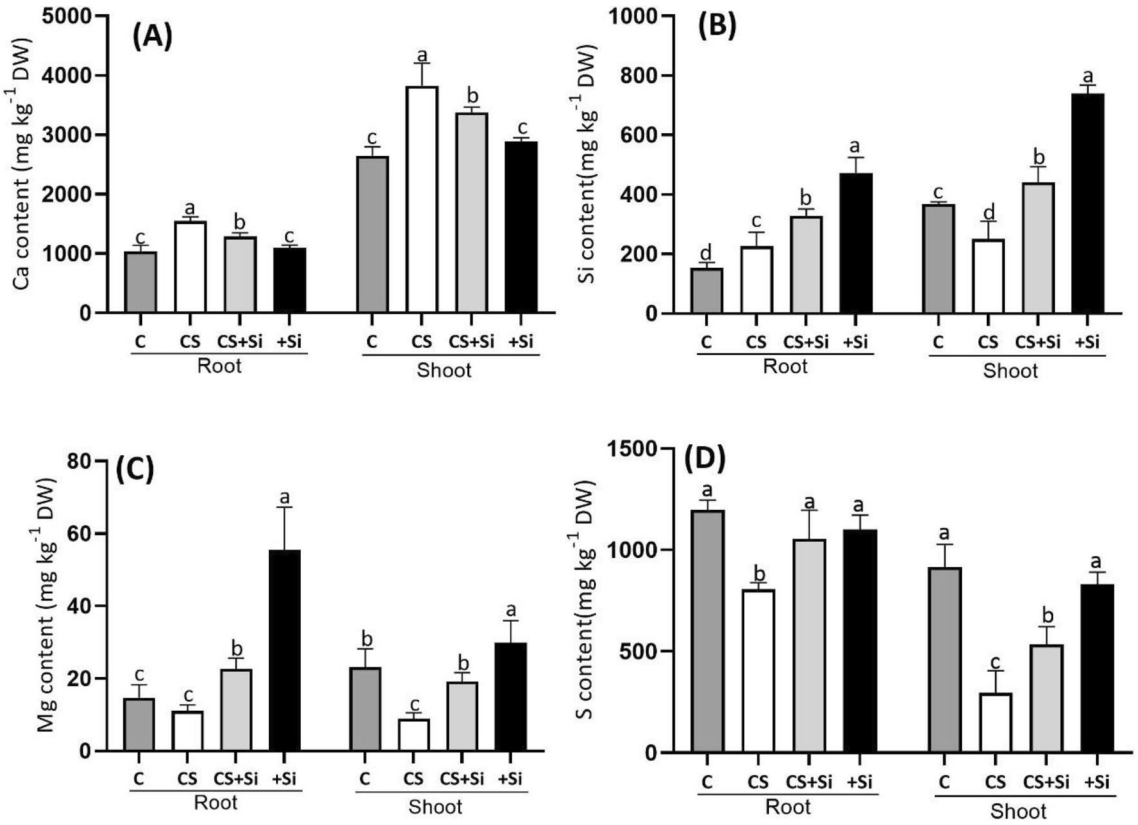

Fig. 3 Mineral content in roots and shoots of alfalfa seeding under different treatments. **A** Ca^{2+} content. **B** Endogenous Si content. **C** Mg content. **D** S content. Different letters above the bar column show significant differences ($P \leq 0.05$) among the group means with standard error (SE). Abbreviation, C, control; CS, cold stress; + Si, silicon supplementation, Ca^{2+}, calcium; Si, silicon; Mg. magnesium; and S, sulfur

combined and single Si treatments. Interestingly, the highest APX activity was observed in combined treatment compared to CS (Fig. 4C). CS also triggered an increase in APX activity. In the case of GR activity, no significant change was observed among the different treatment groups (Fig. 4D). These data indicate that the antioxidant defense is triggered by CS but is also boosted in response to Si supplementation, indicating that it was effective for alleviating CS in alfalfa.

3.5 Regulation of Cold Responsive and Silicon Transporter Genes

Expression of cold-responsive and Si transporter genes were differentially regulated in alfalfa seedlings (Figs. 5–6). *Cold-regulated gene 15A* (*COR15A*) expression significantly increased by 95% under CS compared to control plants (Fig. 5A). However, the expression was highly increased after exogenous application of Si with CS, and the gene expression was not significantly different between a single dose of Si and control plants. The expression of *COR47* was unaffected in response to CS and combined treatment (CS + Si) (Fig. 5B). Another cold-responsive gene *KIN1* showed the highest upregulation by 295% under combined treatment (Fig. 5C). Si-transportation related several genes including *NIP2*, *NIP5;1* and *NIP6;1* showed differential expression patterns. The *NIP2*, *NIP5;1*, and *NIP6;*1 showed the highest expression patterns, especially under Si supplementation. The *NIP5;1* exhibited upregulation in Si treatment and combined treatment compared to control plants. However, *NIP2* and *NIP6;1* showed no significant difference between combined (CS + Si) and single dose of + Si treatment (Fig. 5D-F). The overall expression analysis suggests that cold-responsive genes, specially *COR15A* and *COR47*, were fully activated in response to CS and/or CS + Si treatment, while Si transporters were active in response to + Si supplementation in alfalfa seedlings.

Cold treatment combined with Si supplementation, showed higher expression of *ICE1* and candidate members of the *CBF/DREB* family (Fig. 6). The expression of *ICE1* was initially induced by cold stress and it showed the highest expression in response to combined treatment (Fig. 6A). The *CBF1/DREB1C* and *CBF2/DREB1B* were significantly upregulated by 375% and 108% respectively under combined treatment compared to controls (Fig. 6B-C). However, *CBF3/DREB1A* did not show any significant variation

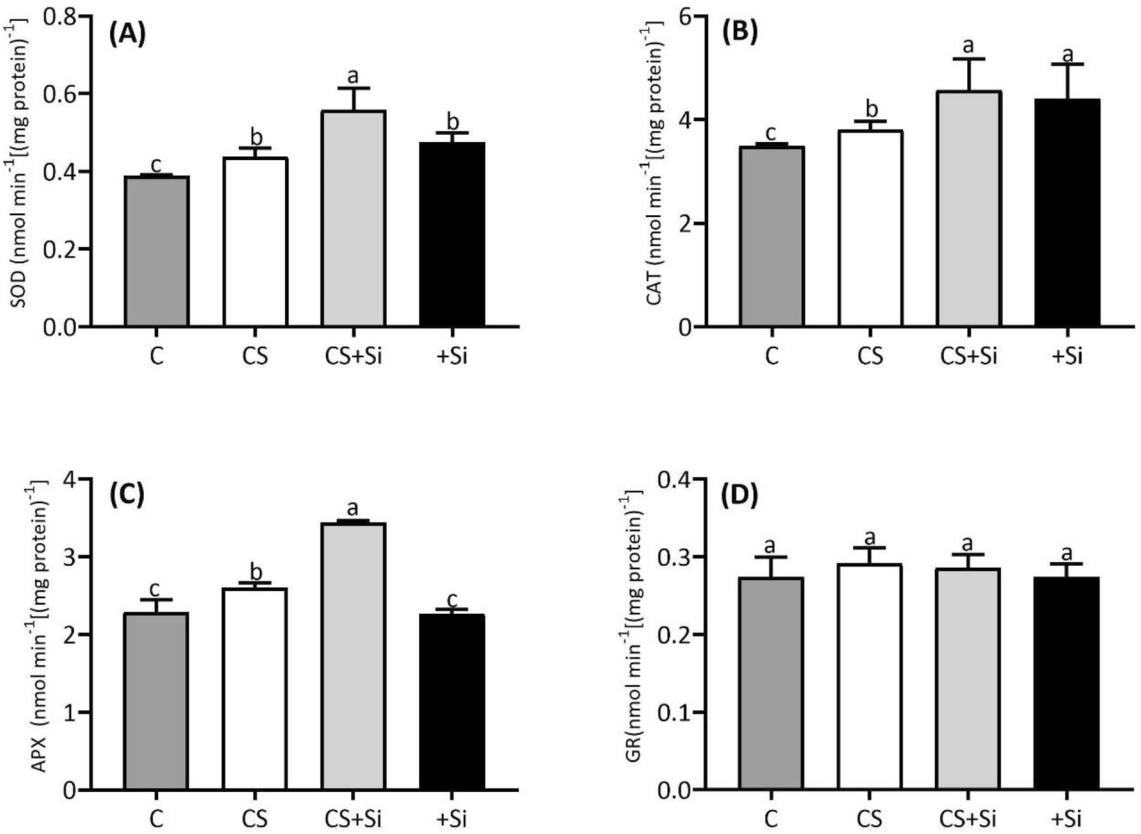

Fig. 4 Antioxidant enzyme activities in alfalfa seedlings under different treatments. **A** Superoxide dismutase (SOD) activity. **B** Catalase (CAT) activity. **C** Ascorbate peroxidase (APX) activity. **D** Glutathione reductase (GR) activity. Different letters above the bar column show significant differences ($P \leq 0.05$) among the group means with standard error (SE). Abbreviation, C, control; CS, cold stress; + Si, silicon supplementation with control

between CS and CS + Si treatments (Fig. 6D). The *ICE1*, and *CBF1/DREB1C* exhibited almost the same expression patterns. This suggests that *ICE1* could be a regulator of *CBFs* expression in response to CS signals in alfalfa seedlings.

Bioinformatics predictor CELLO indicated that the proteins encoded by the genes *ICE1* and *CBF1* are located in the nucleus of plant cells (Supplementary Table S2). The *ICE1* and *CBF1* exhibited a shared gene network in which *CBF1* (C-repeat binding factor 1), *CBF2* (C-repeat binding factor 2), and *COR15A* (cold regulated gene 15A), *COR47* (cold regulated gene 47), along with *ICE1* (inducer of CBF expression 1) were most common in the interactome analysis (Fig. 7A-D). Moreover, multiple sequence alignment indicated that Medicago *ICE1* and *CBF1* are closely associated with Arabidopsis *ICE1* and *CBF1*, respectively.

4 Discussion

This study implies the combined physiological and molecular mechanisms associated with Si-mediated cold stress (CS) tolerance in alfalfa seedlings. This research with the beneficial implication of Si improved features of major alfalfa traits along with antioxidant and transcriptional responses linked to CS tolerance. The Si-mediating improved traits can be utilized by forage breeders and farmers to improve alfalfa production at low temperatures.

4.1 Si-Mediated Improvement of Photosynthesis, Plant Biomass, and Cold Acclimation

The data showed that CS negatively affected on alfalfa photosynthesis process and photosynthetic pigment (chlorophyll), while Si maintained the photosynthetic pigment and photosystem II efficiency. This indicates that Si is capable of reducing the detrimental effects of CS on membrane stability and photosynthetic process in alfalfa. CS has exhibited negative effects on photosynthesis and chlorophyll (Chl) content in plants [33]. In this study, Fv/Fm ratio was found to be correlated, this the Chl score after supplementation of cold-stressed plants. CS reduced root-shoot biomass, along with retarded plant growth. We noticed that morphological attributes and biomass yield traits significantly improved after Si supplementation, indicating its potential role in alleviating

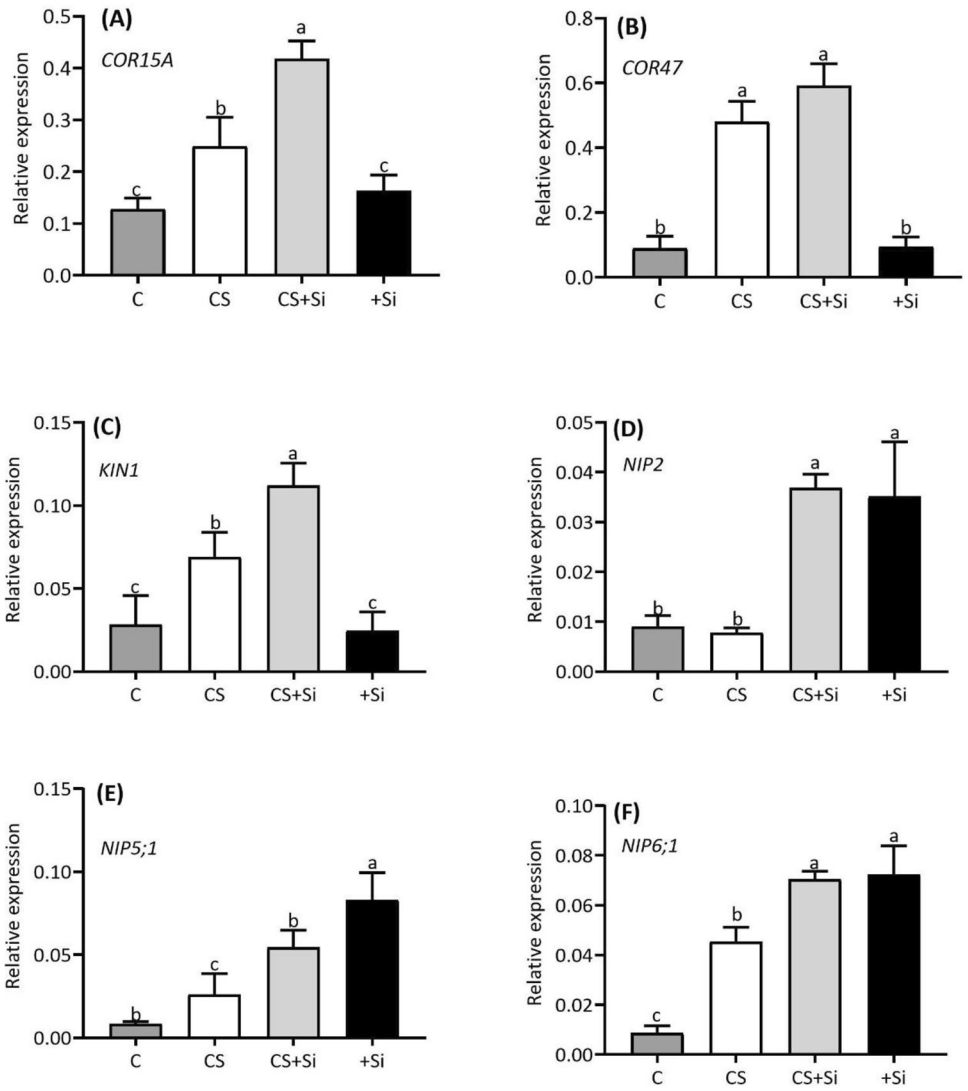

Fig. 5 Relative **e**xpression analysis of cold regulated and silicon transporter genes in alfalfa seedlings under different treatments. **A** *COR15A* (cold regulated gene 15A). **B** *COR47* (cold regulated gene 47). **C** *KIN1* (Kinesin-like protein gene 1). **D** *NIP2* (nodulin 26-like intrinsic protein 2). **E** *NIP5;1* (nodulin 26-like intrinsic protein 5;1). **F** *NIP6;1* (nodulin 26-like intrinsic protein 6;1). The *MsActin* was used as internal control. The expression level of target genes was calculated following the $2^{-\Delta\Delta Ct}$ method that was mentioned detail in material and method section. Different letters above the bar column show significant differences ($P \leq 0.05$) among the group means with standard error (SE). Abbreviation, C, control; CS, cold stress; + Si, silicon supplementation with control

CS in alfalfa seedlings. This efficiency of Si under CS is consistent with other findings in plants [11, 34, 35].

4.2 Si-Enhanced ROS Scavenging Activity

The CS signals induce the generation of superoxide radicals in diverse plant species [36–38]. In a similar study, it has been reported that non-enzymatic ROS scavenging efficiency was increased after Si supplementation under CS [34]. Our current study further implies that the enhanced activity of SOD, CAT, and APX play crucial roles in alleviating CS-induced cellular damage in alfalfa seedlings. This is consistence with the increased ROS content induced by CS, and further, it declined due to the supplementation of Si under CS. The SOD activity is involved in $O_2^{\bullet-}$ dismutation and is considered a first-line defense against multiple abiotic stimuli in plants [3, 5, 39]. In our investigation, we noticed that increased activity of SOD, CAT, and APX in response to Si-supplementation helps to enhance cold stress tolerance in alfalfa. The GR and APX as component of the ascorbate–glutathione cycle are key enzymes that plays a pivotal role against H_2O_2 [40]. We previously found that alfalfa seedlings were not capable of inducing GR [23], though GR activity slightly was induced under CS and after supplementation of Si. Therefore, our data indicate that the CS-induced ROS accumulation was efficiently diminished by the simultaneous increase of the SOD, CAT, and APX activities in response to Si, which greatly enhanced plant CS tolerance in alfalfa.

4.3 Interaction of Si with other Elements during Cold Acclimatization

Silicon (Si) showed potential interaction with other essential and beneficial elements in plants [13]. Our data show that exogenous supplementation of Si has a potential impact in

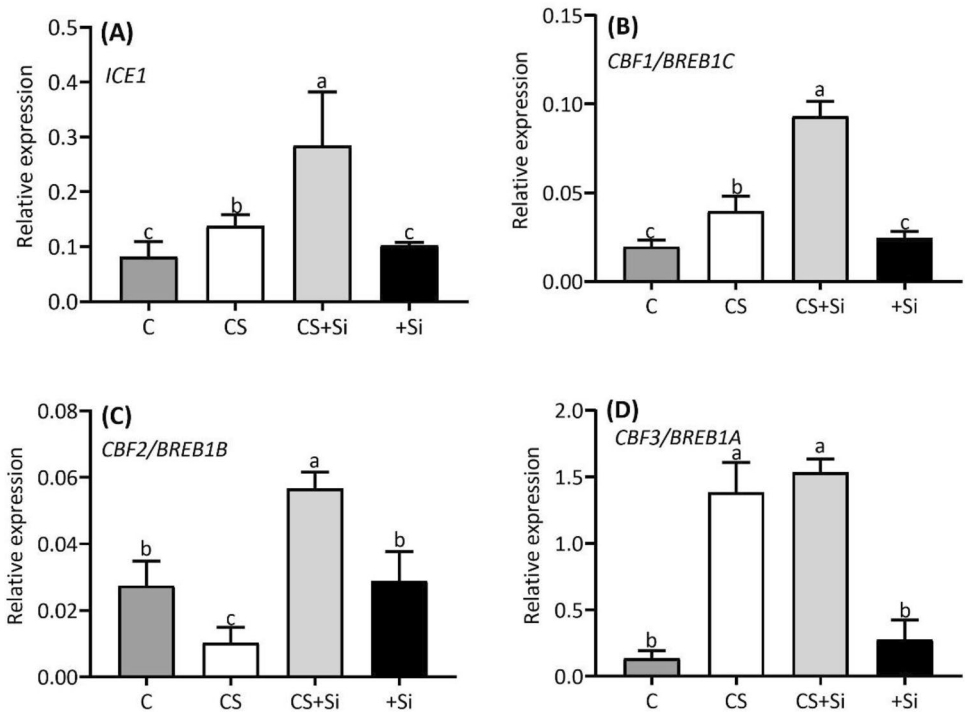

Fig. 6 Expression analysis of cold responsive key genes in in alfalfa seedlings under different treatments. **A** *ICE1* (inducer of CBF expression 1). **B** *ABF1/DREB1C* (abscisic acid responsive element binding factor1/dehydration-responsive element-binding protein 1 C. **C** *ABF2/DREB1B*. **D** *ABF3/DREB1A*. The *MsActin* was used as internal control. The expression level of target genes was calculated following the $2^{-\Delta\Delta Ct}$ method that was mentioned detail in material and method section. Different letters above the bar column show significant differences ($P \leq 0.05$) among the group means with standard error (SE). Abbreviation, C, control; CS, cold stress; + Si, silicon supplementation with control

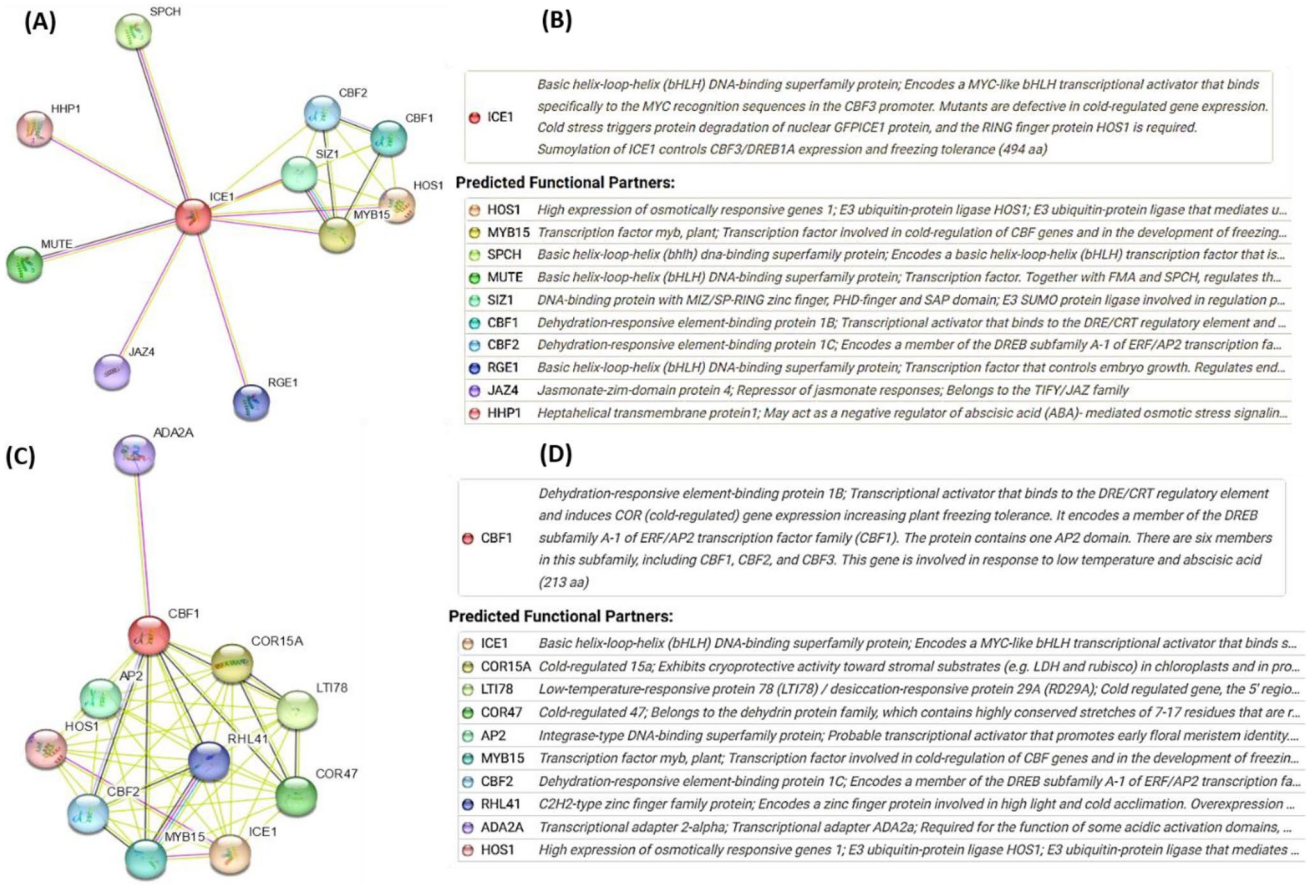

Fig. 7 Interactome analysis of *Medicago ICE1* and *CBF1*candidates. Interactions of candidates and their predicted partners are presented by clusters. The gene networks are detected through STRING network. **A**-**B** Interactome analysis of *ICE1* and its predicted partner. **C**-**D** Interactome analysis of *CBF1* and its predicted partner

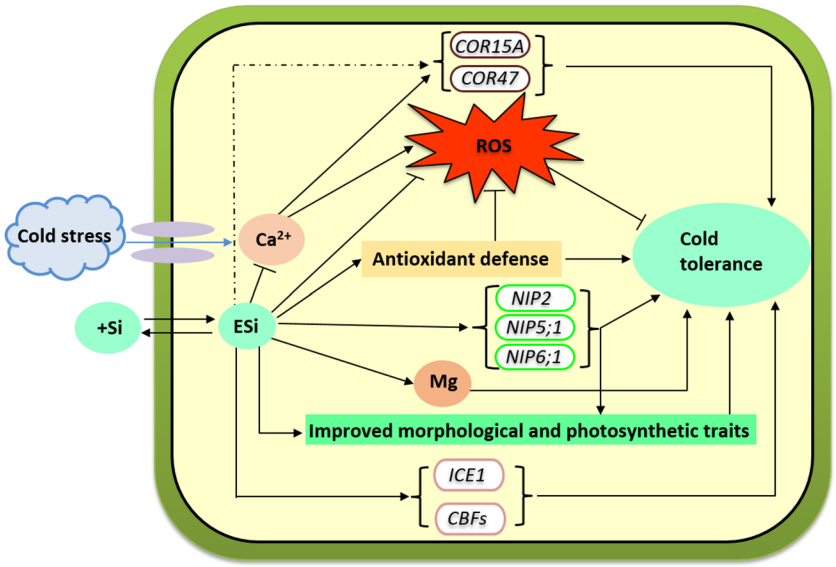

Fig. 8 Working model of the mechanistic insights of Si-mediated CS tolerance in alfalfa. CS induces Ca^{2+} and ROS signals that inhibit cold tolerance. In contrast, exogenous Si supplementation with CS enhances endogenous Si (ESi), and Mg accumulations along with regulating Si transporters, cold-responsive candidate genes, which involved in improving morphological and photosynthetic traits. These alterations lead to enhance alfalfa growth, vigor, and CS tolerance**.** Abbreviation, CS, cold stress; Si, exogenous silicon; ESi, endogenous silicon; Ca^{2+}, calcium; Mg, magnesium; ROS, reactive oxygen species; *COR15A*, cold regulated gene 15A; *COR47*, cold regulated gene 47; NIP, nodulin 26-like intrinsic proteins, *ICE1*, inducer of CBF expression 1; *ABFs*, C-repeat-binding factors

regulating endogenous Si, Mg, and S, except Ca^{2+} in alfalfa seedlings. It is not surprising that Si supplementation may decline the response of Ca^{2+} or can show an antagonistic response. It has been reported that Si supplementation in plant-growing media where + Si significantly decline Ca^{2+} uptake under stress response [12]. However, our data suggested that the potential increase of endogenous Si, Mg in root and shoot helps to enhance CS tolerance in alfalfa. This interaction of Si for enhancing endogenous Si potentially improved the translocation of other micronutrients that enhanced chilling tolerance in maize [11]. We noticed that Si helps to uptake Mg in alfalfa roots and shoots. Recently, it has been validated that Mg supplementation was also found to be improved which leads to maintaining chlorophyll content, plant morphology, and photosynthetic attributes in CS tobacco [41]. Our current finding is consistent with the previous report that revealed Si can interact with Mg and suggested that Si enhances Mg uptake in several plant species [42]. Sulfur-assisted plant defense has been documented under mineral stress [43]. In contrast, this study indicates that S accumulation is not affected by Si supplementation under CS. Our data is supported by the finding of the Si study in forage crops, where S accumulation was unaffected by Si supply [44]. Taken together, our data outputs reveal that exogenous Si enhanced endogenous Si, along with Mg accumulation under CS, which leads to enhanced plant morphology, photosynthesis, and plant vigor against CS in alfalfa.

4.4 Molecular Insights of Cold Stress Tolerance in Alfalfa

Plants use diverse molecular mechanisms to cope with multiple abiotic stress tolerance. A combination of CS and Si treatment significantly increased endogenous Si accumulation in alfalfa roots and shoots. This might coincide with the upregulation of Si-transporters genes (*NIP2*, *NIP5;*1 and *NIP6*;1) in alfalfa. It has been found that the upregulation of *Lsi1* increased Si accumulation in rice leaves and roots [45]. *Lsi1* belongs to the Nodulin 26-like intrinsic proteins (*NIPs*) subfamily in the aquaporin (AQP) that functions as an influx transporter for Si [46]. Thus, it is clear that the Si transporter is involved in linking transport systems that influence Si accumulation in plants. In alfalfa, upregulation of several cold-regulated genes (*COR15A*, *COR47*, and *KIN1*) were observed by CS and combined stress (CS + Si), but the expression was higher in combined treatment. These data imply that Si enhanced the gene expression because their expression is correlated positively with the alfalfa CS tolerance. In *Arabidopsis thaliana,* the *COR15a* was reported to be involved in freezing tolerance [47]. More recently, *COR47* is a stress-protective gene because it has been identified using a transcriptome approach in cold-tolerant chickpea genotypes [48]. Similarly, these genes are also strongly involved in CS tolerance in alfalfa, as well as these genes responded to CS and Si.

ICE1 is a key regulator of the cold-induced transcriptome that is involved in cold tolerance. The consistent repression pattern of *ICE1* and C-repeat-binding factors (*CBFs*) genes under CS in alfalfa indicated that *ICE1* plays an inducer of *CBF1/DREB1C*, *CBF2/DREB1B*, and *CBF3/DREB1A* that lead to CS tolerance. *ICE1* improved freezing tolerance in transgenic *Arabidopsis thaliana* [19]. The overexpression of *ICE1* was reported to confer cold stress tolerance in transgenic zoysia grass [20]. Taken together our results reveal that the special activity of *ICE1* and *CBF1* genes provided cold stress defense in alfalfa seedlings. These findings were confirmed by the in silico characterization in which *ICE1* and *CBF1* showed the common localization in the nucleus as expected. The interaction of these candidate genes was supported by the interactome analysis, in which *ICE1* exhibited a share gene network with *CBF1*, *CBF1*, and other CS-responsive genes. The *CBF1* showed a relationship with *ICE1*, *CBF2*, *COR15A*, *COR47*, and low-temperature responsive other candidates and transcription factors in plants. The shared gene networks of the cold-responsive genes validate their potential function and interaction in plants in response to low temperatures.

5 Conclusion

This study reveal the new mechanistic insights of Si-mediated CS tolerance in alfalfa seedlings (Fig. 8). Exogenous supplementation of Si enhances endogenous silicon (E-Si) that leads to induce the activity of antioxidant enzymes (SOD, CAT, and APX), which protected alfalfa plant cells from ROS-induced oxidative damages. This study further indicates that E-Si enhanced Si transporter genes (*NIP2, NIP5;1,* and *NIP6;1*), and cold-responsive several candidate genes including *COR15A*, *COR47*, *ICE1*, and *CBFs*, which were involved in cold stress tolerance in alfalfa seedlings. Consequently, these data open a new avenue for research into CS tolerance and help to deliver elite genetic materials for alfalfa forage breeding program.

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Author Contributions MAR, K-WL, and AHK conceived the research idea. K-WL and S-HL investigated the research. YS, MMH, and MSJ analysed the data, HSP provided technical assistance. MAR wrote the original draft. MHS, DS, FJC, and AHK helped in writing, reviewing, and editing the original draft. MAR and K-WL finally revised the article. All authors read the article, and approved the final version.

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Data Availability Yes.

Declarations

Ethical Approval This study was approved by the Institute ethics board.

Consent to Participate Not applicable.

Consent for Publication Yes.

Competing Interests The authors declare no competing interests.

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