Transgenic Centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.) Overexpressing S-Adenosylmethionine Decarboxylase (SAMDC) Gene for Improved Cold Tolerance Through Involvement of H$_2$O$_2$ and NO Signaling

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Centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.) is an important warm-season turfgrass species. Transgenic centipedegrass plants overexpressing *S*-adenosylmethionine decarboxylase from bermudagrass (*CdSAMDC1*) that was induced in response to cold were generated in this study. Higher levels of *CdSAMDC1* transcript and spermidine (Spd) and spermin (Spm) concentrations and enhanced freezing and chilling tolerance were observed in transgenic plants as compared with the wild type (WT). Transgenic plants had higher levels of polyamine oxidase (PAO) activity and H$_2$O$_2$ than WT, which were blocked by pretreatment with methylglyoxal bis (guanylhydrazone) or MGBG, inhibitor of SAMDC, indicating that the increased PAO and H$_2$O$_2$ were a result of expression of *CdSAMDC1*. In addition, transgenic plants had higher levels of nitrate reductase (NR) activity and nitric oxide (NO) concentration. The increased NR activity were blocked by pretreatment with MGBG and ascorbic acid (AsA), scavenger of H$_2$O$_2$, while the increased NO level was blocked by MGBG, AsA, and inhibitors of NR, indicating that the enhanced NR-derived NO was dependent upon H$_2$O$_2$, as a result of expression *CdSAMDC1*. Elevated superoxide dismutase (SOD) and catalase (CAT) activities were observed in transgenic plants than in WT, which were blocked by pretreatment with MGBG, AsA, inhibitors of NR and scavenger of NO, indicating that the increased activities of SOD and CAT depends on expression of *CdSAMDC1*, H$_2$O$_2$, and NR-derived NO. Our results suggest that the elevated cold tolerance was associated with PAO catalyzed production of H$_2$O$_2$, which in turn led to NR-derived NO production and induced antioxidant enzyme activities in transgenic plants.

**Keywords:** antioxidants, centipedegrass, cold tolerance, hydrogen peroxide, nitrate reductase, nitric oxide, polyamines, S-adenosylmethionine decarboxylase (SAMDC)
INTRODUCTION

Polyamines are important plant regulators involving in plant growth, development and adaptation to environmental stresses (Minocha et al., 2014; Shi and Chan, 2014; Liu et al., 2015). Putrescine (Put), spermidine (Spd), and spermine (Spm) are three major plant polyamines. Put is synthesized from arginine, catalyzed by arginine decarboxylase, N-carbamoylputrescine amidohydrolase, and agmatine iminohydrolase sequentially, or from ornithine, catalyzed by ornithine decarboxylase. Spd is synthesized from decarboxylated 5-adenosylmethionine (dcSAM) and Put, catalyzed by Spd synthase, while dcSAM is formed from 5-adenosylmethionine (SAM), catalyzed by SAM decarboxylase (SAMDC). Spm is synthesized from Spd and Put, catalyzed by Spm synthase (SPMS, Supplementary Figure S1; Liu et al., 2015). Polyamines are oxidized to produce H$_2$O$_2$ catalyzed by polyamine oxidase (Liu et al., 2015). H$_2$O$_2$ is demonstrated to induce NITRATE REDUCTASE1 (NIA1) expression which is responsible for nitric oxide (NO) production (Rockel et al., 2002; Bright et al., 2006; Lu et al., 2014). H$_2$O$_2$ and NO are signaling in multiple physiological processes including adaptation to environmental stresses (Desikan et al., 2004; Miller et al., 2008; Zhao et al., 2009; Farnese et al., 2016; Niu and Liao, 2016; Sewelam et al., 2016; Singh et al., 2016).

Polyamines accumulate in plants in response to drought (Li et al., 2015), salinity (Tassoni et al., 2008), and cold stress (Kovác et al., 2010; Chen et al., 2013), while plant tolerance to drought and cold is increased by exogenous application of polyamines (Shi et al., 2013; Peng et al., 2016). Plant tolerance to abiotic stress may be modified by regulation of polyamine synthesis For example, overexpression of ADC, ODC, and SPDS (Roy and Wu, 2001; Kumria and Rajam, 2002; Kasukabe et al., 2004; He et al., 2008) result in enhanced abiotic stress tolerance in transgenic plants, while knock-out or down-regulation of ADC, SPDS, and SPMS genes decreases tolerance (Kasianathan and Wingler, 2004; Yamaguchi et al., 2007; Cuevas et al., 2008). SAMDC is a key enzyme for Spd and Spm formation. Transgenic rice and tobacco plants down-regulating SAMDC expression have decreased Spd and Spm levels along with reduced tolerance to drought, salinity, and cold (Moschou et al., 2008; Chen et al., 2014), while transgenic plants overexpressing SAMDC had enhanced Spd and Spm levels along with elevated tolerance to drought (Waie and Rajam, 2003), salinity (Waie and Rajam, 2003; Hao et al., 2005; Wi et al., 2006), cold (Hao et al., 2005; Wi et al., 2006), and heat (Cheng et al., 2009). However, there is no report to modulate abiotic stress tolerance in turgrass by overexpressing SAMDC gene. Centipedegrass is a warm-season turfgrass species with excellent adaptation to low pH and poor soil, thick sod formation, and uniform and aggressive growth. It is a low maintenance grass and requires infrequent mowing due to its slow-growing habit. Thus it is commonly used in soil conservation, residential lawns, and recreational turf in tropical and subtropical regions and a grazing-purpose grass for low-input grassland systems in Japan (Hanna and Liu, 2003; Hirata et al., 2016). Centipedegrass can be potentially used for phytoremediation due to its capacity to transport heavy metals from roots to shoots and leaves (Li et al., 2016). Low temperature is a major environmental factor limiting the plantation of centipedegrass. Reactive oxygen species (ROS) is accumulated in plants under low temperature conditions when the absorbed light energy cannot be used by CO$_2$ assimilation as a result of inhibition of Calvin–Benson cycle enzymes. These accumulated ROS may result in oxidative damages of photosynthetic apparatus if it could not be scavenged effectively. Antioxidant defense system protects plants against the oxidative damages by scavenging ROS for maintenance of ROS homeostasis in plant cells under stress conditions (Miller et al., 2008). By using gamma-ray radiation, a chilling-tolerant mutant was selected in our laboratory. The mutant maintained higher levels of antioxidants and polyamines during chilling stress compared with the WT, suggested that polyamines and antioxidants are associated with chilling tolerance in centipedegrass (Chen et al., 2013). However, centipedegrass has low genetic diversity (Hanna and Liu, 2003; Harris-Shultz et al., 2012), which limits improvement of centipedegrass. Plantlet generation and Agrobacterium-mediated transformation in centipedegrass have been established in our laboratory (Liu et al., 2008, 2012). The objectives of this study were to increase cold tolerance in centipedegrass by modulating polyamine synthesis through overexpressing a SAMDC gene from bermudagrass and investigate whether H$_2$O$_2$ and NO were involved in the improved cold tolerance in transgenic plants.

MATERIALS AND METHODS

Plant Growth Conditions and Treatments

Centipedegrass plants and a common bermudagrass (Cynodon dactylon) that was used in our previous study (Lu et al., 2008) were planted in 15-cm diameter plastic pots containing a mixture of peat and perlite (3:1, v/v) in a greenhouse for 2 months, with temperature ranging from 25 to 30°C, irrigating daily and fertilizing once a week with 15N–6.6P–12.5K fertilizer. Bermudagrass plants were placed in a growth chamber with a 12-h photoperiod under light of 200 µmol m$^{-2}$ s$^{-1}$ at 6°C for 4 days for cold treatment for analysis of CdSAMDC1 expression, while centipedegrass plants were used for physiological and molecular measurements. For treatment with chemicals, leaf fragments of transgenic plants and WT were placed in deionized water for 1 h to eliminate the potential wound stress, and then placed in beakers containing 1 mM methylglyoxal bis (guanylylhydrzone) or MGBG, 1 mM ascorbic acid (AsA), 1 mM Na$_2$NO$_3$, 100 mM tungstate, or 200 mM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide (PTIO) under light of 80 mmol photons m$^{-2}$ s$^{-1}$ for 12 h, while those treated with deionized water for 12 h under the same condition served as a control. After treatments the leaf fragments were sampled and immediately frozen under liquid N$_2$ for further analysis.

Cloning of CdSAMDC1

Total RNA was isolated from bermudagrass leaves by using TRIzol reagent (Life Technologies, Carlsbad, CA, United States) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 1 µg of total RNA in a 20-µl
reaction mixture, using M-MLV reverse transcriptase and Oligo (dT)\textsubscript{18} primer. For amplification of \textit{CdSAMDC1}, polymerase chain reaction (PCR) was conducted in a reaction mixture containing the first-strand cDNA as the template, primers \textit{SAMDC\textsubscript{F}} (CCGTCTTCAATGGTGTTTCT) and \textit{SAMDC\textsubscript{R}} (CCCGTCTTACTCATCAAGCACTC), and \textit{KOD-Plus DNA polymerase} (TOYOBO, Osaka, Japan).

**Generation of Transgenic Centipedegrass Plants**

Embryogenic calli were induced from sterilized mature seeds of centipedegrass and cultivated as previously described (Liu et al., 2008). The embryogenic callus was transformed using \textit{Agrobacterium tumefaciens} strain EHA105 harboring pCAMBIA-35S-\textit{CdSAMDC1} construct as described previously (Liu et al., 2012). The calli were placed on callus induction medium without selection pressure for 1 week after co-cultured for 3 days, followed by placing on selection medium containing hygromycin B (50 mg \text{L}^{-1}) for 8-week. The hygromycin-resistant calli were subjected for regeneration on regeneration medium containing hygromycin (50 mg \text{L}^{-1}), illuminated with a 16 h photoperiod (800 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}). The regenerated shoots were transferred to half-strength of MS medium containing sucrose for rooting. The plantlets were transferred to soil in 15-cm plastic pots growing in a greenhouse at temperatures of 30/25°C (day/night) under natural light.

**DNA Blot Hybridization**

One gram of leaves was used for extract genomic DNA using the hexadecyltrimethylammonium bromide (CTAB) method. DNA samples (20 \mu g) were digested overnight with \textit{HindIII}, separated by electrophoresis on 0.8% agarose gel, and transferred to Hybond XL nylon membrane (Amersham, GE Healthcare Limited, Buckinghamshire, United Kingdom), sequentially. The coding sequence of \textit{hpt} was labeled as DNA probes for hybridization using a PCR digoxigenin (DIG) probe synthesis kit (Roche Diagnostics, Basel, Switzerland). The DNA filter was washed sequentially with 2 \times \text{SSC}, 0.1% SDS; 1 \times \text{SSC}, 0.1% SDS for 10 min at room temperature; and 0.5 \times \text{SSC}, 0.1% SDS for 15 min at 65°C after prehybridization and hybridization. Hybridization signals were detected using a Lumivision PRO (TAITEC, Saitama, Japan).

**Real-time Quantitative RT-PCR**

Total RNA was extracted as described above. One \mu g of total RNA was used for synthesis of first-strand cDNA, using the PrimeScript RT reagent Kit With gDNA Eraser (Takara Bio, Inc., Otsu, Shiga, Japan). After diluted for 50-fold, the cDNA was used as template for real-time quantitative RT-PCR (qRT-PCR) analysis in a total of 10 \mu l PCR reaction, containing 15 ng of cDNA, 200 nM each of forward and reverse primers, and 5 \mu l SYBR Premix \textit{Ex Taq} (Takara Bio, Inc., Otsu, Shiga, Japan), with three technical replicates and two biological replicates. A parallel reaction to amplify \textit{actin1} was used to normalize the amount of template. The PCR primers include: \textit{CdSAMDC1} forward primer \textit{ZG5547} (5’-CGCCATCGAACAGCATAGAAAA-3’), reverse primer \textit{ZG5548} (5’-CCCGGGGACAGCAGGAA-3’); \textit{actin1} forward primer \textit{ZG1551} (5’-TCTGCTGTCGTTGACTGTGAGCAGG-3’) and reverse primer \textit{ZG1552} (5’-ACCTGCCCATGAGCAGATCAT-3’). The primers were designed using the software tool Beacon Designer (Premier Biosoft International, Palo Alto, CA, United States), and the primer specificity was validated by melting profiles and showed a single product specific melting temperature. All PCR efficiency was above 95%.

**Determination of Cold Tolerance**

The temperature that resulted in 50% lethal (LT\textsubscript{50}) was calculated using a fitted model plot for evaluation of freezing tolerance (Pennycooke et al., 2008; Guo et al., 2014). Freezing tolerance was also evaluated by survival rate. Stolons were cut into segments with one node, and placed in a beaker incubating in a Programmable Freezer (model: Polystat c1 & k6, Huber Unit, Offenburg, Germany), following freezing treatment by decreasing temperature from 25 to 0°C linearly within 6 h, and maintained at 0, −2, and −3°C for 1 h respectively. After thawing overnight at 0°C, the stolons were planted in soil for vegetative propagation until new shoots were regenerated. The regenerated plants were counted and survival rate were calculated. The experiments were performed three times using 40 segments each line per replicate. For assessment of chilling tolerance, centipedegrass plants were moved into a growth chamber at 6°C with a 12-h photoperiod under light of 200 \mu mol m\textsuperscript{−2} s\textsuperscript{−1} for 30 days as described previously (Chen et al., 2013). Net photosynthetic rate (A) was measured by using a LI-6400P Portable Photosynthesis System (LI-COR, Inc., Lincoln, NE, United States) (Chen et al., 2013).

**Determination of H\textsubscript{2}O\textsubscript{2}**

Leaves were stained in 1 mg ml\textsuperscript{−1} of 3,3-diaminobenzidine (DAB) solution for 1 h, followed by decoloring in boiling ethanol (95%) for 20 min before photography (Orozco-Cárdenas and Ryan, 1999). In another case, H\textsubscript{2}O\textsubscript{2} was determined spectrophotometrically as previously described (Zhou et al., 2006).

**Determination of Polyamines**

Free polyamines were extracted and measured as described previously (Chen et al., 2013). Leaves (0.5 g) were extracted in 5 ml of 5% (v/v) cold perchloric acid (PCA) and incubated on ice for 1 h. The homogenate was centrifuged at 20,000 \times g for 30 min. Aliquots (0.5 ml) of supernatant were mixed with 1 ml of 2 M NaOH and 7 \mu l of benzoyl chloride and incubated at 37°C for 20 min in dark for benzoylation. The benzoylated polyamines were extracted to diethyl ether, resuspended in 1 ml of 2 M NaOH and 7 \mu l of benzoyl chloride and incubated at 37°C for 20 min in dark for benzoylation. The benzoylated polyamines were extracted to diethyl ether, resuspended in 1 ml of mobile phase solution (64% methanol in an isocratic elution), and filtered (4.5 \mu m filter) before HPLC analysis. Twenty \mu l of sample was injected into a Waters chromatographic system (Waters, Milford, MA, United States), supplied with a C18 column (4.6 mm \times 250 mm), and detected at 254 nm using a 2487 dual UV detector (Waters, Milford, MA, United States). Polyamine levels were calculated based on standard curves of...
commercial standards in combination with a recovery of the extraction procedure.

**Determinations of Polyamine Oxidase (PAO) Activity**

Polyamine oxidase was extracted in 0.1 M phosphate buffer (pH 7.0), and the activity was measured as described previously (Chen et al., 2014). The reaction was initiated and incubated at 30°C for 30 min after addition of 20 µl of 20 mM Spd or Spm into reaction mixture (3 ml) that was consisted of 2.50 ml of 0.1 M phosphate buffer (pH 7.0), 0.1 ml of horseradish peroxidase (250 units), 0.2 ml of coloring solution (25 µl N, N-dimethylaniline and 10 mg 4-aminantipyrine were dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.0) and 0.2 ml enzyme extract or inactivated enzyme (by heating the enzyme for 20 min in a boiling water bath) as control. Absorbance at 550 nm was recorded. One unit of PAO activity was defined as the amount of enzyme required for catalyzing 1 µmol of Spd or Spm oxidation within 1 min.

**Determination of SOD, CAT, and NR Activity**

Leaves were ground in 5 ml of 50 mM phosphate buffer (pH 7.8) and supernatants were recovered for determinations of SOD, CAT, and NR after centrifugation at 13,000 x g for 15 min as previously described previously (Guo et al., 2006; Lu et al., 2014). SOD reaction solution (3 ml), which was consisted of 13 µM methionine, 1.3 µM riboflavin, 63 µM p-nitro blue tetrazolium chloride, and enzyme extract in 50 mM phosphate buffer (pH 7.8), was incubated for 10 min at room temperature under fluorescent light with 80 µmol m−2 s−1. Absorbance at 560 nm was determined with a spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of p-nitro blue tetrazolium chloride (NBT) by 50%. CAT reaction that contained 15 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0) was initiated by adding 50 µl of enzyme extract. The decreased absorbance at 240 nm within 1 min was recorded. One unit of CAT was defined as the amount of enzyme required for catalyzing the conversion of 1 µmol H₂O₂ within 1 min. NR reaction that contained 60 mM KNO₃ and 0.25 mM NADH in 1.6 ml of 50 mM phosphate buffer (pH 7.5) was started by adding 0.4 ml of enzyme extract. After incubation at 25°C for 30 min, 1 ml of 1% sulfanilamide in 1.5 M HCl and 1 ml of 0.01% N-(1-naphthyl)-ethylenediammonium dichloride were immediately added into the reaction solution. Absorbance at 540 nm was recorded after centrifugation, and nitrite production was calculated by comparison with a standard curve of NaNO₂. One unit of NR was defined as the amount of enzyme required for catalyzing the conversion of 1 mmol NO₂ within 1 h.

**Determination of NO**

Nitric oxide content was determined using Griess reagent as described previously (Zhou et al., 2005). Leaves (0.6 g) were ground in a mortar with pestle in 3 ml of 50 mM cool acetic acid buffer containing 4% zinc diacetate (pH 3.6). The homogenates were centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was added by 0.1 g of activated charcoal. The filtrate was collected after vortex and filtration. One ml of filtrate was mixed with 1 ml of the Greiss reagent, followed by incubation for 30 min at room temperature. Absorbance at 540 nm was determined. NO content was calculated based on a standard curve of NaNO₂.

**Determination of Protein Concentration**

Protein concentration in enzyme extracts was determined using Coomassie Brilliant Blue G-250 solution using albumin from bovine serum (BSA) as a standard (Bradford, 1976).

**Statistical Analysis**

The experiments were arranged in a completely randomized design with three pots of plants as replicates. For measurements of A, five leaves in each pot were randomly chosen and used for assay independently. For all the biochemical and physiological measurements, a pooled material from several different plants in each pot was randomly collected and used for assay. Significance of differences in the various parameters was assessed by one-way ANOVA (P < 0.05) using an SPSS program (SPSS, Inc., Chicago, IL, United States).

**RESULTS**

**Characterization of CdSAMDC1**

A cDNA sequence of CdSAMDC1 (GenBank accession number JX878505) with 1212-bp length was cloned from bermudagrass leaves. It encodes a peptide of 403 amino acids with 43.8 kDa and isoelectric point (pI) of 4.72. Sequence blast showed that CdSAMDC1 was most homologous (94%) to a CaSMADC2 (ADC45378) in Cleistogenes songorica in amino acid sequence, and 84 and 83% identical to ZmSAMDC4 in maize (CAA69075) and OsSAMDC1 in
Polyamine Synthesis Was Improved in Transgenic Plants

Transgenic centipedegrass plants were molecularly detected. DNA hybridization signals by using hpt fragment as probe were shown in transgenic lines 23 and 24 with a unique integration pattern, while no signal was shown in the WT plants (Figure 2B). Compared to WT plants, CdsAMDC1 transcript was detected in transgenic plants (Figure 2C). The results indicated that the transgene was integrated into centipedegrass genomes with one copy and CdsAMDC1 was expressed in transgenic centipedegrass plants. Free polyamines were measured in transgenic centipedegrass in comparison with WT. Put level showed no difference between WT and transgenic plants. Compared to WT, Spd level was 2.3 to 2.9-fold higher and Spd level and 1.6 to 1.8-fold higher in transgenic lines (Figure 2D).

Transgenic Plants Had Enhanced Cold Tolerance

Freezing tolerance was evaluated using LT50 and survival rate. Compared to a −3.2°C of LT50 in WT plants, transgenic lines had lower level of LT50 (−5.2°C) under non-acclimated condition. After 7 days of cold acclimation, LT50 decreased to −5.8°C in WT and −6.5°C in transgenic lines, respectively (Figure 3A). In consistence, WT had a 40% of survival rate, while transgenic lines had 59–61% of survival rate under non-acclimated condition.
Analysis of freezing and chilling tolerance in transgenic plants in comparison with the WT. Plants were treated for 7 days at for cold acclimation (A), followed by measuring ion leakage to calculate the temperature that resulted in 50% lethal (LT_{50}, A) and survival rate after freezing treatment at –3°C (B), while those were untreated by low temperature as non-acclimated control (NA). Net photosynthetic rate (A, C) were determined and photography (D) was taken after 30 days of chilling treatment at 6°C, followed by detaching the second leaf for taking photography (E) and H$_2$O$_2$ staining (F). Means of three independent samples and standard errors are presented; the same letter above the column indicates no significant difference at $P < 0.05$.

After cold acclimation, WT had a 64% of survival rate, while transgenic lines had a 79% of survival rate (Figure 3B). Chilling tolerance was also evaluated after 30 days of chilling treatment. WT and transgenic plants had similar $A$ under non-stressed condition. Chilling treatment resulted in decrease in $A$, while significantly higher levels of $A$ were observed in transgenic plants than in WT (Figure 3C). More brown or dead leaves were observed in WT than in transgenic plants after chilling treatment (Figure 3D). Color and H$_2$O$_2$ of the second leaf from the top were compared. Chilling treatment resulted in leaf browning in WT, but the leaf was maintained green in transgenic plants (Figure 3E). DAB staining showed that more H$_2$O$_2$ were accumulated in the second leaf in WT than in transgenic plants after chilling treatment (Figure 3F).

**Transgenic Plants Had Higher Antioxidant Enzyme Activities**

Antioxidant enzymes protect plants against low temperature induced oxidative damage on photosynthetic apparatus and membrane system (Chen et al., 2013; Lu et al., 2013). Antioxidant enzyme activity was measured. Compared to WT, 37 to 39% and 2.1- to 2.2-fold higher activities of SOD and CAT were observed in transgenic plants under control condition. SOD and CAT activities were increased after 7 days of cold acclimation, and higher activities were still maintained in transgenic plant than in WT (Figures 4A,B).

**Transgenic Plants Had Higher PAO Activity and H$_2$O$_2$ Level**

Spermidine and Spm are catabolized to produce H$_2$O$_2$ in plants catalyzed by PAO, while H$_2$O$_2$ is signaling in induction of cold responsive genes including those encoding antioxidant enzymes (Wan et al., 2009; Guo et al., 2014). Higher activity of PAO was observed in transgenic plants as compared with WT when either Spd or Spm was used as substrate (Figures 5A,B), and H$_2$O$_2$ level was higher in transgenic plants than in WT (Figure 5C). Feeding with MGBG, inhibitor of SAMDC, decreased PAO activity and H$_2$O$_2$ level in transgenic plants (Figure 5), indicating that the increased PAO activity and H$_2$O$_2$ level in transgenic plants were resulted from expression of CdSAMDC1.
NR Dependent NO Production Is Involved in Elevated Antioxidant Enzyme Activities in Transgenic Plants

Hydrogen peroxide has been shown to induce expression of NIR1 and lead to increased NR activity and accumulated NO in plants, while NO induces expression of antioxidant enzyme genes (Wan et al., 2009; Zhang et al., 2009; Lu et al., 2014). Higher levels of NR activities and NO were observed in transgenic plants compared with WT, which were blocked by pretreatment with AsA, an antioxidant to scavenge H$_2$O$_2$, or MGBG (Figures 6A,B), while pretreatment with NaN$_2$ and tungstate, inhibitors of NR, inhibited the elevated NO in transgenic plants. The results indicated that the elevated NR and NO were associated with accumulation of H$_2$O$_2$ as a result of expression of CdSAMDC1, while the elevated NO was dependent upon NR.

To assess whether the elevated PAO activity was affected by NR dependent NO, PAO activity was measured after plants were treated by NR inhibitors and NO scavenger. The results showed that PAO activity was not altered by pretreatments with NaN$_2$, tungstate, and PTIO, a scavenger of NO, when either Spd or Spm was used as substrate (Figure 7).

Activities of SOD and CAT were measured after pretreatment with MGBG, AsA, NaN$_2$, tungstate, and PTIO to understand whether H$_2$O$_2$ and NO signaling were involved in the enhanced antioxidant enzyme activity in transgenic plants (Figure 4). Pretreatments with MGBG and AsA blocked the elevated activities of SOD and CAT in transgenic plants. Similarly, the elevated activities were also blocked by pretreatment with PTIO, NaN$_2$ and tungstate (Figures 8A,B). The results indicated that the elevated SOD and CAT activities were associated with accumulated NO as a result of H$_2$O$_2$-dependent induction of NR.

DISCUSSION

A SAMDC gene, CdSAMDC1, showing induced expression by cold was isolated from bermudagrass in this study. CdSAMDC1 was highly identical to ZmSAMDC4, 1 in maize and OsSAMDC1 in rice. OsSAMDC1 (Os04g42095) and OsSAMDC4 (Os09g25625) are responsive to chilling among six SAMDCs in rice (Yamaguchi et al., 2004). Two transgenic centipedegrass lines overexpressing CdSAMDC1 were generated in this study, with elevated levels of Spd in two lines and Spm in one line. Transgenic centipedegrass lines had higher survival rate and lower LT$_{50}$ than WT under both cold acclimation and non-acclimation conditions, suggesting they had enhanced freezing tolerance. In addition, higher levels of A and less accumulation of ROS and dead leaves were observed in transgenic lines after 30 days of
Polyamine oxidase (PAO) catalyzes Spd and Spm oxidation to produce H$_2$O$_2$, while H$_2$O$_2$ is signaling in plant adaptation to stress and expression of cold responsive genes including those encoding antioxidant enzymes (Wan et al., 2009; Guo et al., 2014; Farnese et al., 2016; Niu and Liao, 2016; Sewelam et al., 2016; Singh et al., 2016). Compared to WT, higher activities of antioxidant enzymes and PAO were observed in transgenic centipedegrass plants. The elevated PAO activity was blocked by inhibitor of SAMDC, while the elevated antioxidant enzyme activities were blocked by inhibitor of SAMDC and scavenger of H$_2$O$_2$ in transgenic plants. The results suggest that the elevated PAO was associated with Spd and Spm synthesis, and the elevated antioxidant enzyme activities were dependent upon PAO-deprived H$_2$O$_2$. This case is supported by our previous observation that Spd and Spm induce PAO activity in tobacco (Guo et al., 2014). Higher antioxidant enzyme activities and transcripts were observed in transgenic tobacco plants overexpressing wheat oxalate oxidase which catalyzes oxalate oxidation to produce H$_2$O$_2$ (Wan et al., 2009; Lu et al., 2014). Antioxidant enzymes function to maintain homeostasis of ROS in plant cell by scavenging the accumulated ROS under stresses. Chilling-tolerant cultivars of centipedegrass, rice and Stylosanthes guianensis have higher antioxidant enzyme activities than sensitive cultivars (Guo et al., 2006; Chen et al., 2013; Lu et al., 2013). Nevertheless, the higher activities of antioxidant enzymes in transgenic centipedegrass were associated with the enhanced cold tolerance.

Nitrate reductase-dependent NO was demonstrated to be involved in H$_2$O$_2$ induced antioxidant enzyme activity in transgenic plants in this study. Our data showed that higher levels of NR activity and NO were observed in transgenic plants, which was blocked by inhibitor of SAMDC and scavenger of H$_2$O$_2$, suggesting that the elevated NR-deprived NO was dependent upon polyamine synthesis and H$_2$O$_2$. It was consistent with the case in bermudagrass and transgenic tobacco plants, in which H$_2$O$_2$ induces NRI expression and results in higher NR activity and NO level (Lu et al., 2009, 2014). In addition, the increased activities of antioxidant enzymes in transgenic centipedegrass were blocked by inhibitors of NR and scavenger of NO, suggesting that the elevated antioxidant enzyme activities were dependent upon NR-deprived NO. Likely, the NR-dependent NO is involved in H$_2$O$_2$-induced antioxidant enzyme activities in transgenic tobacco and bermudagrass (Lu et al., 2009, 2014; Zhang et al., 2009). Exogenous treatment with H$_2$O$_2$ increased the NR-dependent NO level; either H$_2$O$_2$ or NO increases...
antioxidant enzyme activities in bermudagrass. The H$_2$O$_2$-induced antioxidant enzyme activities depend upon NO, but NO-induced antioxidant enzyme activities is not dependent upon H$_2$O$_2$ (Lu et al., 2009). Thus our results suggest that the improved Spd and Spm synthesis resulted in accumulation of PAO-deprived H$_2$O$_2$, which in turn led to higher levels of NR-dependent NO that increased antioxidant enzyme activities in transgenic centipedegrass plants.

In summary, overexpression of CdSAMDC1 improved Spd and Spm synthesis in transgenic centipedegrass, which induced PAO activity for production of H$_2$O$_2$. The elevated H$_2$O$_2$ increased NR activity for production of NO, which in turn led to enhanced antioxidant enzyme activities and cold tolerance in transgenic plants.

**AUTHOR CONTRIBUTIONS**

JL and ML conducted most of the experiments of transgenic plants. CZ generated transgenic plants. PZ determined gene expression in bermudagrass. JC cloned CdSAMDC1 from bermudagrass. ZG and SL designed experiments and wrote manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01655/full#supplementary-material

**FIGURE S1** | Polyamine biosynthetic and oxidation pathway for Put, Spd, and Spm in plants. ADC, arginine decarboxylase; CPA, N-carbamoylputrescine amidohydrolase; ADI, agmatine deiminase; ODC, ornithine decarboxylase; CdPAO, cinnamate oxidase; SAMS, S-adenosylmethionine synthetase; SAMDC, SAM decarboxylase; dCdSAM, decarboxylated S-adenosylmethionine; SPDS, spermidine synthase; SPMS, spermine synthase; PAO, polyamine oxidase.

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