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Poaceae Type II Galactinol Synthase 2 from Antarctic Flowering Plant *Deschampsia antarctica* and Rice Improves Cold and Drought Tolerance by Accumulation of Raffinose Family Oligosaccharides in Transgenic Rice Plants

Li Hua Cui[1,2,6], Mi Young Byun[3,6], Hyeong Geun Oh[1,2], Sung Jin Kim[4], Jungeun Lee[3,5], Hyun Park[3,5], Hyoungseok Lee[3,5,*] and Woo Taek Kim[1,2,*]

[1]Division of Life Science, Department of Systems Biology, Yonsei University, Seoul 03722, Korea
[2]Institute of Life Science and Biotechnology, Yonsei University, Seoul 03722, Korea
[3]Unit of Polar Genomics, Korea Polar Research Institute, Incheon 21990, Korea
[4]Division of Life Sciences, Korea Polar Research Institute, Incheon 21990, Korea
[5]Polar Science, University of Science & Technology, Daejeon 34113, Korea
[6]The first two authors contributed equally to this work.
*Corresponding authors: Hyoungseok Lee, E-mail, soulaid@kopri.re.kr; Fax, +82-32-760-5509; Woo Taek Kim, E-mail, wtkim@yonsei.ac.kr; Fax, +82-2-312-5657.
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***Deschampsia antarctica* is a Poaceae grass that has adapted to and colonized Antarctica. When *D. antarctica* plants were subjected to cold and dehydration stress both in the Antarctic field and in laboratory experiments, galactinol, a precursor of raffinose family oligosaccharides (RFOs) and raffinose were highly accumulated, which was accompanied by upregulation of galactinol synthase (GolS). The Poaceae monocots have a small family of *GolS* genes, which are divided into two distinct groups called types I and II. Type II GolSs are highly expanded in cold-adapted monocot plants. Transgenic rice plants, in which type II *D. antarctica GolS2* (*DaGolS2*) and rice *GolS2* (*OsGolS2*) were constitutively expressed, were markedly tolerant to cold and drought stress as compared to the wild-type rice plants. The RFO contents and GolS enzyme activities were higher in the *DaGolS2*- and *OsGolS2*-overexpressing progeny than in the wild-type plants under both normal and stress conditions. *DaGolS2* and *OsGolS2* overexpressors contained reduced levels of reactive oxygen species (ROS) relative to the wild-type plants after cold and drought treatments. Overall, these results suggest that Poaceae type II GolS2s play a conserved role in *D. antarctica* and rice in response to drought and cold stress by inducing the accumulation of RFO and decreasing ROS levels.**

Keywords: Antarctic flowering plant • *Deschampsia antarctica* • Cold and drought tolerance • Poaceae type II galactinol synthase • Raffinose family oligosaccharides • Transgenic rice plants.

Introduction

Antarctica is a harsh environment with low temperatures, limited water availability, high soil salinity, drastic fluctuations in annual irradiation and a short-day length, all of which severely restrict plant growth and development. Despite these unfavorable conditions, two angiosperm species inhabit Antarctica, and one of these, *Deschampsia antarctica* Desv., is the only Antarctic Poaceae species (Bravo et al. 2001). *Deschampsia antarctica* exhibits specialized morphological traits, such as a distinct leaf anatomy and chloroplast ultrastructure, which affect its photosynthetic characteristics (Gielwanowska et al. 2005, Sáez et al. 2019). In addition, the physiological and biochemical changes accompanied by the expression regulation of diverse genes are related to the plastic responses of *D. antarctica* plants to the Antarctic climate (Lee et al. 2010, Lee et al. 2013). For example, increases in ice recrystallization inhibition protein (*DaIRIP*) expression and recrystallization inhibition (RI) activity in response to freezing stress may contribute to the cryotolerance of *D. antarctica* (John et al. 2009). In addition, *D. antarctica* displayed tolerance to ultraviolet B-induced oxidative stress not only by synthesizing phenolic-type molecules but also by activating both enzymatic and non-enzymatic antioxidant systems (Köhler et al. 2017). Furthermore, non-structural carbohydrates were highly accumulated in *D. antarctica* during growing summer season (January–February), which represents a metabolic adaptation of this plant (Zuñiga et al. 1996). These traits might be linked to its capacity to colonize this harsh environment, suggesting that *D. antarctica* could serve as a model for the study of the genetic and metabolic mechanisms responsible for plant adaption to a variety of abiotic stresses.

Raffinose family oligosaccharides (RFOs), which are α-1,6-galactosyl extensions of sucrose, are plant non-structural carbohydrates that play diverse cellular roles, including protection of embryos from maturation-associated seed desiccation, transport and storage of carbon, and signal transduction (Stevenson et al. 2000, Downie et al. 2003,

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Xue et al. 2007). RFOs are becoming increasingly recognized as crucial molecules during stress responses in plants due to their protective roles in response to adverse environmental conditions (Zuther et al. 2004, Nishizawa-Yokoi et al. 2008, ElSayed et al. 2014, Sengupta et al. 2015). RFOs function as osmolytes to maintain cell turgor and stabilize cellular proteins (Bartels and Sunkar 2005) and as antioxidants to counteract the accumulation of reactive oxygen species (ROS) under stress conditions (Nishizawa et al. 2008, Valluru and Van den Ende 2011).

Galactinol synthase (GolS) is a key enzyme in the biosynthesis of RFOs. GolS catalyzes the formation of galactinol from UDP-galactose and myo-inositol. Galactinol is subsequently converted to series of RFOs, including raffinose and stachyose (Lehle and Tanner 1973, Loewus and Murthy 2000). The *AtGolS* genes in Arabidopsis are upregulated in response to abiotic stresses (Taji et al. 2002), and overexpression of *AtGolS2* in transgenic rice plants improves drought tolerance (Selvaraj et al. 2017). Ectopic expression of chickpea *GolS* (*CaGolS*) in Arabidopsis resulted in heat and oxidative stress-tolerant phenotypes (Salvi et al. 2018). *OsGolS1* is involved in the thermo-tolerance response in rice plants (Hue et al. 2013), and overexpression of wheat *TaGolS* in rice plants conferred enhanced tolerance to chilling injury (Shimosaka and Ozawa 2015). In addition, transgenic Arabidopsis plants that overexpress maize *ZmGolS2* exhibited drought-, salt- and heat-tolerant phenotypes (Gu et al. 2016). These results indicate that GolS plays a critical role in the response to a broad spectrum of abiotic stresses in both monocot and dicot species.

Deschampsia antarctica GolS (*DaGolS*) was previously identified as a stress-related gene that may be involved in the adaptive mechanism of Antarctic hairgrass (Gidekel et al. 2003, Lee et al. 2013). However, there is no biological evidence that the GolS-mediated accumulation of RFOs contributes to the environmental stress tolerance of this plant in Antarctica. To assess the potential role of DaGolS in the response to low temperature, we generated *DaGolS2*-overexpressing transgenic rice plants (*Ubi:DaGolS2*) and analyzed their phenotypes. Our results showed that the *DaGolS2* overexpressors showed markedly enhanced tolerance to both cold and drought stress as compared to the wild-type rice plants. We also identified the rice *GolS2* (*OsGolS2*) gene and found that *OsGolS2*-overexpressing transgenic rice progeny (*Ubi:OsGolS2*) also exhibited enhanced tolerance to drought and cold stress. The levels of galactinol and raffinose were higher in the *DaGolS2* and *OsGolS2* overexpressors than in the wild-type rice plants under both normal and stress conditions. Both the *DaGolS2*- and *OsGolS2*-overexpressing plants contained lower levels of ROS than the wild-type plants after cold and drought treatments. Overall, these results suggest that Poaceae type II GolS2s play a conserved role in *D. antarctica* and rice plants in response to drought and cold stress by inducing the accumulation of galactinol and raffinose and decreasing ROS levels.

Results

Endogenous galactinol and raffinose levels in *D. antarctica* were increased in the Antarctic field and in response to cold and drought stress in the laboratory

RFOs accumulate in the vegetative tissues of various plant species in response to a broad spectrum of abiotic stresses (Sengupta et al. 2015); therefore, we considered the possibility that RFOs play a crucial role in the adaptation of *D. antarctica* to Maritime Antarctica, one of the harshest environments for plant growth. Naturally colonized *D. antarctica* plants were collected from the Antarctic field (field control) near King Sejong Antarctic Station (62°4′29″S; 58°44′18″W) on the Barton Peninsula of King George Island, transplanted and incubated in the laboratory (at 15°C under 16 h light and 8 h dark) for 6 d (L6d; **Fig. 1A**). The plants were transferred back to the Antarctic field and harvested at different time points (8 h, 3 d and 5 d). When the plants were moved from the Antarctic field to the laboratory, the expression of *DaIRIP*, a well-known cold-induced gene in *D. antarctica* (John et al. 2009), declined to the background level. *DaIRIP* transcript levels gradually increased after the plants were transferred back to the Antarctic field (from 8 h to 5 d) (**Fig. 1A**).

The endogenous galactinol and raffinose levels in the leaves of *D. antarctica* in the Antarctic field control were 1.10 ± 0.20 and 12.90 ± 1.20 mg/g FW, respectively (**Fig. 1B**). After a 6-d incubation under laboratory conditions (L6d), galactinol and raffinose contents markedly decreased to 0.002 ± 0.001 and 1.19 ± 0.04 mg/g FW, respectively. The levels of galactinol and raffinose gradually increased after the plants were transferred back to the adverse environment of the Antarctic field. At 8 h (F8h), 3 d (F3d) and 5 d (F5d) after transfer, the galactinol contents were 0.84 ± 0.18, 1.89 ± 0.37 and 5.86 ± 1.30 mg/g FW, respectively, and at these same time points, the raffinose content increased to 2.44 ± 0.17, 9.42 ± 1.96 and 38.67 ± 2.20 mg/g FW, respectively (**Fig. 1B**). The levels of galactinol increased more rapidly than the levels of raffinose (**Fig. 1C**), suggesting stepwise synthesis of RFOs in *D. antarctica* in response to the Antarctic environment. In contrast, the changes in the levels of sucrose, glucose and fructose in response to the different growth conditions were not as great as those of the RFOs (**Fig. 1C, D**).

We next measured the accumulation of RFOs in laboratory-cultured *D. antarctica* plants in response to cold and drought stress. For the cold-stress treatment, *D. antarctica* plants grown at 15°C were transferred to a climate chamber at 4°C for 10 d. For the dehydration treatment, *D. antarctica* plants were incubated at 15°C on filter paper for 4 d. The results showed large increases in the levels of both galactinol and raffinose after cold treatment and dehydration. The low basal level of galactinol (0.59 ± 0.39 mg/g FW; mock control) was increased to 4.32 ± 1.08 mg/g FW after cold stress and to 2.05 ± 0.20 mg/g FW after dehydration (**Fig. 1E–G**). The raffinose content was increased

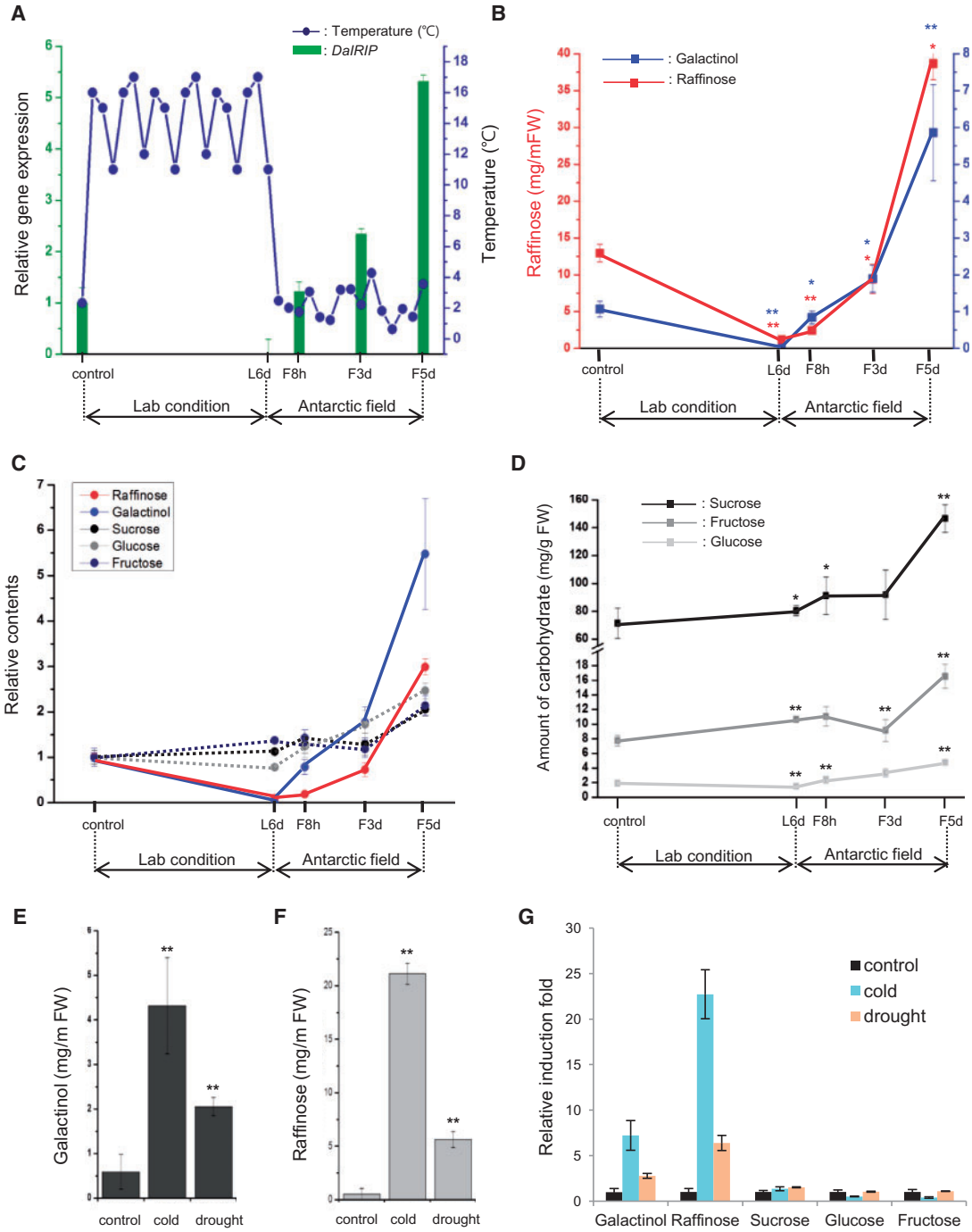

Fig. 1 Amounts of galactinol and raffinose were increased in response to cold and drought stress in *Deschampsia antarctica*. (A) Expression patterns of *DaIRIP* in response to different growth conditions. *Deschampsia antarctica* plants were collected from Antarctica field (control) and incubated in the laboratory conditions for 6 d (L6d), after which the plants were transferred back to Antarctic field for 8 h (F8h), 3 d (F3d) and 5 d (F5d). Total RNAs were isolated from each sample and used for real-time qRT-PCR analysis with a gene-specific primer set (**Supplementary Table S1**). The relative expression of *DaIRIP* was normalized to that of *DaEF1a* that was served as an internal control. Data are means ± SD (*$P < 0.05$, **$P < 0.01$, Students *t*-test) from three biologically independent experiments. Changes in the temperatures in the Antarctic field and laboratory conditions are indicated. (B) Galactinol and raffinose content in *D. antarctica* plants grown in the Antarctic field and laboratory conditions. Data are means ± SD (*$P < 0.05$, **$P < 0.01$, Students *t*-test) from three biologically independent experiments. (C) Relative levels of galactinol, raffinose, sucrose, glucose and fructose in response to different growth conditions in *D. antarctica* plants. Relative sugar levels indicate the measured values of each growth point divided by the value of field control. (D) Amounts of sucrose, glucose and fructose in *D. antarctica* plants grown in the Antarctic field and laboratory conditions. Data are means ± SD (*$P < 0.05$, **$P < 0.01$, Students *t*-test) from three biologically independent experiments. (E, F) Galactinol and raffinose content in the laboratory-cultured *D. antarctica* plants before (control) and after cold (4°C for 10 d) and drought (air dried on the filter paper at 15°C for 4 d) treatments. (G) Relative carbohydrate levels in the laboratory-cultured *D. antarctica* under stress conditions. Relative levels of each carbohydrate indicate the measured values of each stress treatment divided by the value of normal condition.

from 0.53 ± 0.53 mg/g FW in the mock control to 21.12 ± 0.80 mg/g FW after cold stress and to 5.62 ± 0.73 mg/g FW after dehydration (Fig. 1E–G). In contrast, the relative levels of sucrose, glucose and fructose remained unchanged in response to the cold and drought treatments (Fig. 1G). Thus, it appears that the accumulation of RFOs may play a key role in the response of Antarctic hairgrass to abiotic stresses. Overall, these results raised the possibility that the biosynthesis and storage of RFOs are elaborate features of *D. antarctica* plants that have evolved to cope with harsh Antarctic environment.

Identification and characterization of DaGolS1 and DaGolS2 in *D. antarctica*

GolS is a key enzyme that catalyzes an initial step in the RFO biosynthetic pathway. In *D. antarctica*, two homologous *GolS* genes, *DaGolS1* (GenBank accession no. MK286464) and *DaGolS2* (GenBank accession no. MK286465), were identified based on the transcriptome database using TBLASTN (Lee et al. 2013; SRA051881). DaGolS1 and DaGolS2 were predicted to consist of 336 and 326 amino acids, respectively, with 72% identity (Fig. 2A). Both proteins contained a single glycosyltransferase domain and a conserved catalytic DxD motif (Sengupta et al. 2012) (Fig. 2A and Supplementary Fig. S1). The results of the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis revealed that the expression levels of *DaGolS1* and *DaGolS2* were unchanged when the *D. antarctica* plants were moved from the Antarctic field to the laboratory at 6 d (Fig. 2B). However, transcript levels of *DaGolS1* and *DaGolS2* were rapidly upregulated at 8 h after the plants were transferred back to the Antarctic field. Subsequently, the transcripts of these two genes were downregulated to basal levels during prolonged exposure (from 8 h to 5 d) in the Antarctic field (Fig. 2B). These expression profiles of *DaGolS1* and *DaGolS2* were highly comparable to those of *DaCBF4* and *DaCBF7*, which encode transcription factors that turn on a subset of initial cold-responsive genes (Fig. 2B; Byun et al. 2015, Byun et al. 2018). These results suggest that *DaGolS1* and *DaGolS2* play roles in the early stage of the cold stress response in *D. antarctica*. This notion was further supported by the results that *DaGolS1* and *DaGolS2* transcripts were rapidly upregulated in laboratory-cultured *D. antarctica* plants after 8 h in the cold (4°C) (up to 25-fold and 8-fold, respectively) and 4 h of drought (up to 6-fold and 11-fold, respectively) (Fig. 2C, D). Thus, the increased levels of RFOs (Fig. 1) were positively correlated with the rapid transcriptional induction of *DaGolS1* and *DaGolS2* (Fig. 2B–D) under stress conditions.

The subcellular localization of the DaGolSs was investigated via a protoplast transient expression system. Protoplasts were prepared from the mature leaves of *D. antarctica* plants and were transfected with the *35S:DaGolS1-sGFP* or *35S:DaGolS2-sGFP* chimeric constructs. The expressed proteins were visualized by fluorescence microscopy. The results revealed that the fluorescence signals for DaGolS1-sGFP and DaGolS2-sGFP were predominantly located in the cytosolic fraction (Fig. 2E), which suggested that DaGolS1 and DaGolS2 are cytosolic enzymes.

DaGolS2 belongs to the Poaceae type II family of GolSs

Phylogenetic analysis of the full-length GolS sequences from Poaceae, which are important cultivated monocot cereals, showed that these crops possess a small *GolS* gene family, which is divided into two different groups, type I and type II (Fig. 3). For example, *D. antarctica* and rice have two genes (type I: II = 1:1), barley has three genes (type I: II = 1:2) and maize has four genes (type I:II = 3:1). Winter wheat (*Triticum aestivum* L.), a representative freeze-tolerant hexaploid crop, contains six genes in its B genome, most of which were expanded type II *GolS* genes (type I:II = 1:5) (Fig. 3 and Supplementary Fig. S2). The role of one of the wheat *GolS* multigene, *TaGolS1*, which is a type II gene, was identified. Transgenic rice plants overexpressing *TaGolS1* showed increased tolerance to chilling stress (Shimosaka and Ozawa 2015). DaGolS1 is a type I GolS and DaGolS2 is a type II enzyme (Fig. 3). We selected DaGolS2 for our experiment to investigate the in planta role of type II GolS in relation to the cold stress tolerance response of *D. antarctica*.

Generation of *DaGolS2*- and *OsGolS2*-overexpressing transgenic rice plants

Although genetic transformation has been widely used to explore the cellular roles of stress-related genes in diverse plant species, stable gene transformation and regeneration of *D. antarctica* has not yet been established. *Deschampsia antarctica* and rice, a monocot model plant, belong to the same Poaceae family. Thus, to investigate the role of DaGolS2 in planta, *DaGolS2* was constitutively expressed in rice under the control of the maize *Ubiquitin* promoter (*Ubi*), and transgenic rice plants were generated (Fig. 4A). Under normal growth conditions, the *DaGolS2*-overexpressing T4 transgenic rice plants (*Ubi:DaGolS2*) exhibited no detectable morphological difference as compared to the wild-type rice plants (Fig. 4B). Based on the results of a genomic Southern blot analysis, two independent *Ubi:DaGolS2* lines (#1 and #2) were selected, in which *DaGolS2* transcripts were detected by RT-PCR (Fig. 4C, D). Accordingly, GolS activity was also upregulated in the *Ubi:DaGolS2* lines compared to the activity in the wild-type rice plants (Fig. 4E).

To address the biological function of GolS2 in Poaceae, we also characterized *OsGolS2*, a rice homolog of *DaGolS2*. The deduced OsGolS2 comprised 328 amino acids with 79% identity to DaGolS2 (Fig. 4F). OsGolS2 was predominantly localized in the cytosolic fraction of rice leaf protoplasts (Supplementary Fig. S3). *OsGolS2* was rapidly induced by drought and high salinity. However, unlike *DaGolS2*, *OsGolS2* was not induced during 0.5–2 d of cold stress, but it was clearly induced after 5 d of cold treatment in rice plant (Fig. 4G). This late cold-induction pattern of *OsGolS2* in rice was somewhat different from those of *DaGolS1* and *DaGolS2*, whose expressions were increased after 8 h cold treatment in *D. antarctica* (Fig. 2C). *OsGolS2*-overexpressing transgenic rice plants were constructed, and two independent T3 *Ubi:OsGolS2* lines (#1 and #2) were chosen for subsequent phenotypic analysis with respect to the abiotic stress tolerance response (Fig. 4H–K). Increased GolS

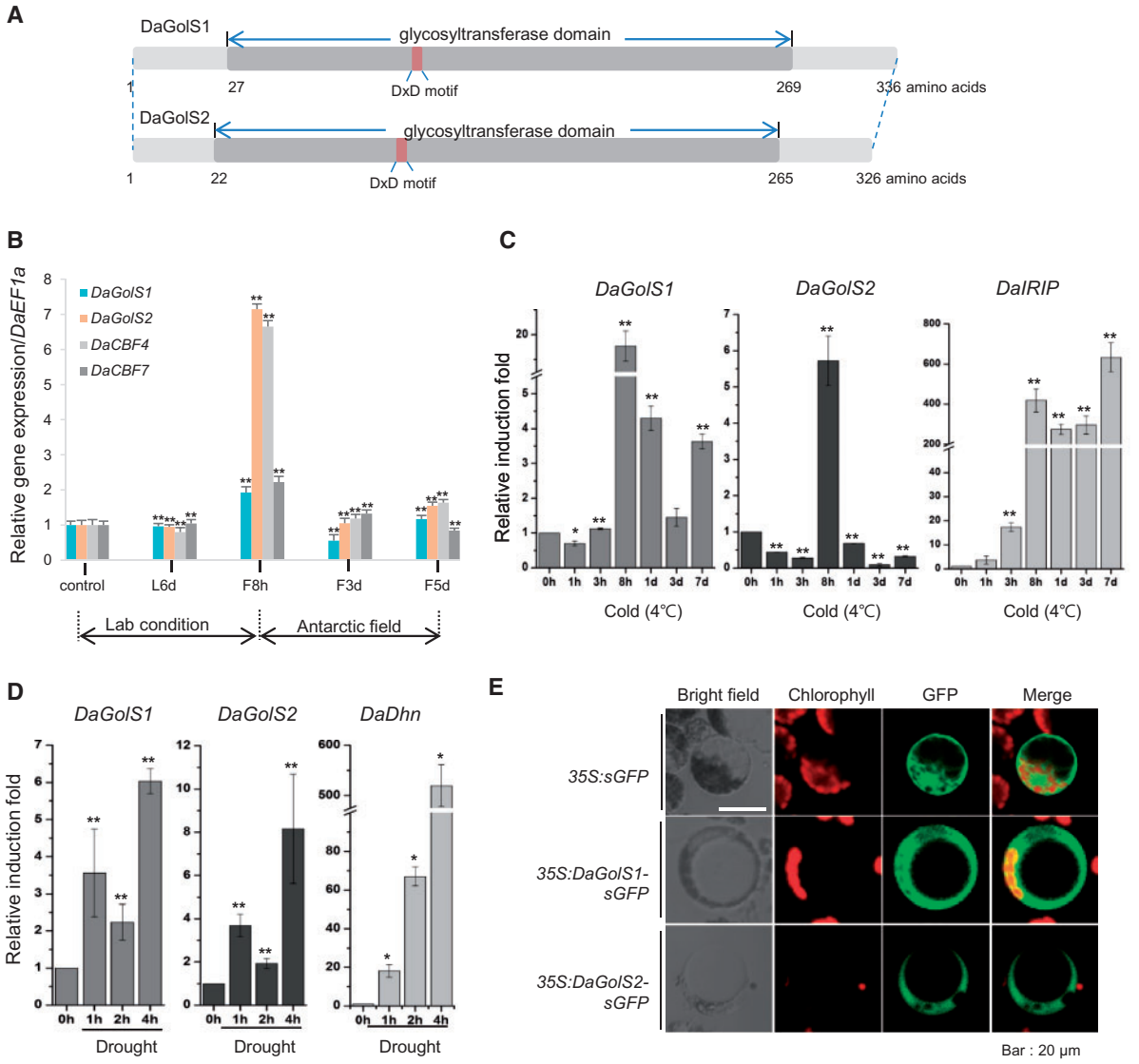

Fig. 2 Identification and characterization of *DaGolS1* and *DaGolS2* in *Deschampsia antarctica*. (A) Schematic structures of predicted DaGolS1 and DaGolS2. The glycosyltransferase domain is shown as a dark gray bar and the DxD catalytic motif is marked as a red bar. (B) Expression patterns of *DaGolS1* and *DaGolS2* in response to the Antarctica field and laboratory conditions in *D. antarctica* plants. Real-time qRT-PCR analysis was performed as described in **Fig. 1A**. *DaCBF4* and *DaCBF7* were used as positive controls for cold stress. Relative expression level of each gene was normalized to that of *DaEF1a*. Data are means ± SD (*$P < 0.05$, **$P < 0.01$, Students *t*-test) of three biologically independent experiments. (C, D) Expression profiles of *DaGolS1* and *DaGolS2* in response to cold and drought stress. Laboratory-cultured 3-week-old *D. antarctica* seedlings were subjected to cold (4°C for 10 d) and dehydration (air dried on the filter paper at 15°C for 4 d) treatments, and total RNAs prepared from the treated tissues were analyzed by qRT-PCR using gene-specific primer sets (**Supplementary Table S1**). *DaIRIP* and *DaDhn* were used as positive controls for cold and drought stress, respectively. Relative expression level of each gene was normalized to that of *DaEF1a*. Data are means ± SD (*$P < 0.05$, **$P < 0.01$, Students *t*-test) of three biologically independent experiments. (E) Subcellular localization of DaGolS1 and DaGolS2. The *35S:DaGolS1-sGFP* and *35S:DaGolS2-sGFP* fusion constructs were transfected into the protoplasts prepared from mature leaves of *D. antarctica*. The fluorescent signals of the expressed proteins were visualized by fluorescence microscopy. sGFP was used as a cytosolic marker protein. Bars = 20 μm.

activity was detected in the *Ubi:OsGolS2* progeny relative to that in the wild-type plants (**Fig. 4L**).

DaGolS2- and *OsGolS2*-overexpressing transgenic rice plants exhibited enhanced tolerance to cold stress relative to the wild-type plants

To examine the cold-tolerant phenotypes of the *DaGolS2* and *OsGolS2* overexpressors, wild-type, T4 *Ubi:DaGolS2* (independent lines #1 and #2) and T3 *Ubi:OsGolS2* (independent lines #1 and #2) rice plants were grown at 28°C for 5 weeks under long-day (16 h light and 8 h dark) conditions, transferred to a cold room at 4°C and incubated under continuous light. After 8 d of low temperature treatment, these plants were transferred back to the growth room at 28°C, allowed to recover and grow for 50 d and their survival was monitored. Under our experimental conditions, most of the wild-type rice plants exhibited discolored leaves, with markedly reduced

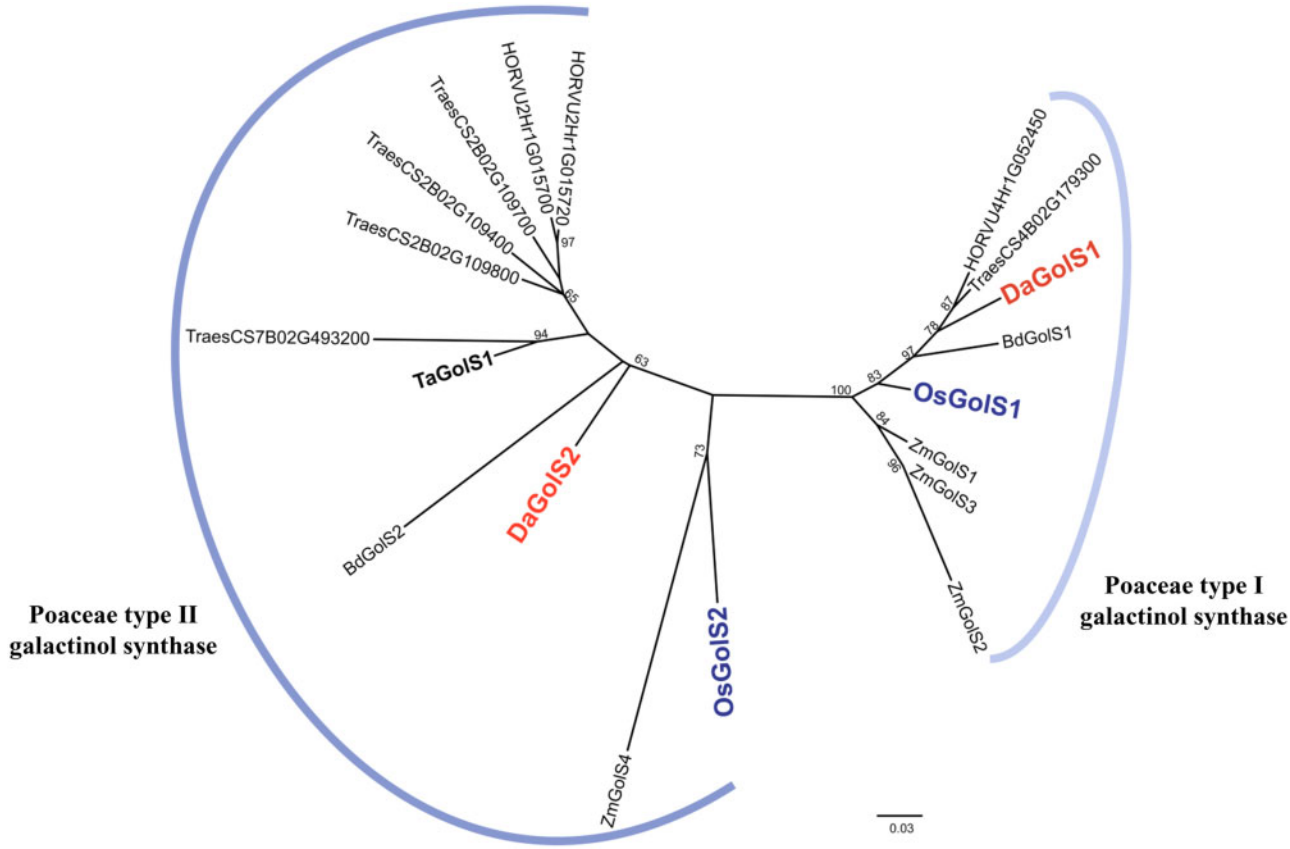

Fig. 3 Phylogenetic analysis of GolS proteins. The full-length amino acid sequences of DaGolS1, DaGolS2 and GolS homologs from Poaceae monocot crops were retrieved from the GenBank database and proofread. Phylogenetic trees were constructed from the data sets by the neighbor-joining method based on the Jones-Taylor-Thornton (JTT) matrix-based model using MEGA7 software. The tree is drawn to scale with branch lengths in the same units, as those of the evolutionary distances were used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method. The *Poaceae* monocot GolS members are divided into two different groups, type I and type II.

turgor, after recovery from the cold stress and were unable to grow (survival rate: 16.8 ± 2.5–20.9 ± 3.8%; **Fig. 5A–D**). In contrast, the *DaGolS2* and *OsGolS2* overexpressors clearly showed healthier morphology and resumed growing (survival rates: 64.0 ± 9.2–84.4 ± 4.0% and 61.0 ± 4.8–63.8 ± 4.4%, respectively) (**Fig. 5A–D**).

To measure the total leaf chlorophyll content (chlorophyll *a* + chlorophyll *b*), mature leaves were detached from the plants of each genotype before and after cold treatment. Before cold treatment, the chlorophyll content of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* plants was indistinguishable (**Fig. 5E, F**). However, the *DaGolS2*- and *OsGolS2*-overexpressing progeny contained higher amounts of chlorophyll than the wild-type rice plants in response to cold temperature. After 1 month of recovery from cold treatment (4°C), the chlorophyll content of the wild-type leaves was 1.5 ± 0.7–2.0 ± 0.4 mg/g DW, whereas the chlorophyll contents of the *Ubi:DaGolS2* and *Ubi:OsGolS2* plants were 10.4 ± 1.3–10.8 ± 0.6 and 8.5 ± 0.9–10.3 ± 1.5 mg/g DW, respectively (**Fig. 5E, F**).

To quantify the cellular response to cold stress, an electrolyte leakage assay was conducted using 8-day-old seedlings. Wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* whole seedlings were incubated at 4°C for 0, 5 and 10 d. Seedlings were then soaked in 35 ml of distilled water overnight at 25°C, and electrolyte leakage was measured with a conductivity meter. As shown in **Fig. 5G**, the *Ubi:DaGolS2* and *Ubi:OsGolS2* seedlings showed lower ion leakage rates (12.4 ± 0.7–12.5 ± 0.7% and 12.5 ± 0.7–12.7 ± 0.7%, respectively, at 5 d; 15.4 ± 1.0–15.8 ± 1.2% and 14.4 ± 2.4–16.5 ± 2.0%, respectively, at 10 d) than the wild-type seedlings (14.7 ± 1.1% at 5 d and 25.6 ± 1.7% at 10 d) in response to prolonged cold stress. These results showed that overexpression of *DaGolS2* and *OsGolS2* resulted in increased tolerance to low temperature treatment, suggesting that DaGolS2 and OsGolS2 play positive roles in the cold stress response in rice plants.

DaGolS2- and *OsGolS2*-overexpressing transgenic rice plants exhibited enhanced tolerance to drought stress when compared to the wild-type plants

Wild-type, T4 *Ubi:DaGolS2* and T3 *Ubi:OsGolS2* plants were grown at 28°C for 5 weeks under long-day (16 h light and 8 h dark) conditions. The plants were then subjected to drought stress by withholding water for 9–10 d. After dehydration, the plants were re-watered, allowed to grow for 3–4 weeks to recover and their growth was monitored. After recovery from drought stress, 28.0 ± 4.0–32.0 ± 2.8% of the *Ubi:DaGolS2* plants and 38.1 ± 5.7–51.0 ± 8.1% of the *Ubi:OsGolS2* plants resumed growing (**Fig. 6A–D**). In contrast, most of the wild-type plants

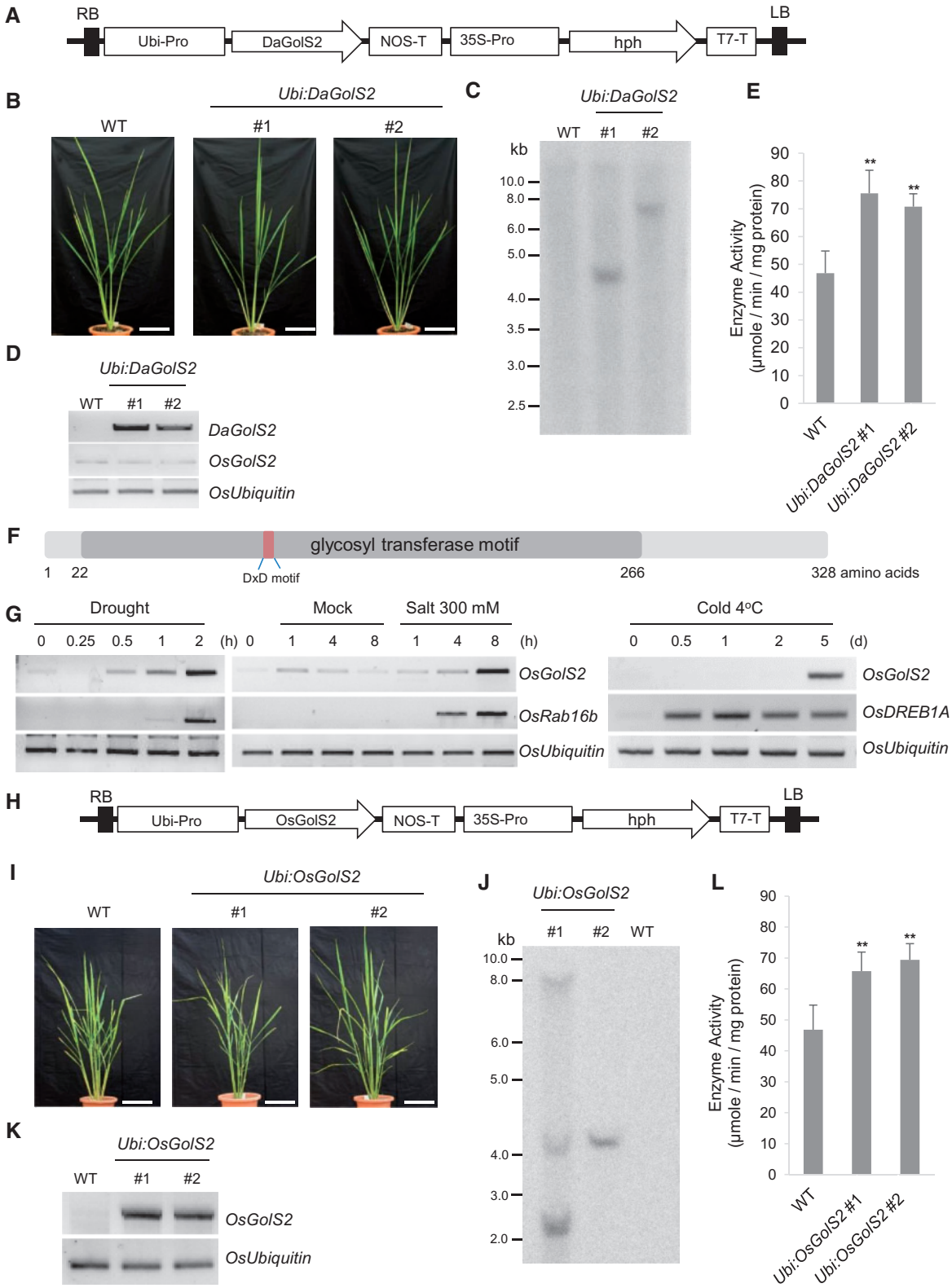

Fig. 4 Molecular characterizations of *DaGolS2*- and *OsGolS2*-overexpressing transgenic rice plants. (A) Schematic representation of a *DaGolS2*-overexpressing binary vector construct. RB, right border; pUbi, maize ubiquitin promoter; NOS-T, NOS terminator; 35S-Pro, CaMV 35S promoter; *hph*, hygromycin phosphotransferase; T7-T, T7 terminator; LB, left border. (B) Morphology of 2-month-old wild-type (WT) and T4 *Ubi:DaGolS2* (independent lines #1 and #2) transgenic rice plants grown under long-day condition (16 h light and 8 h dark). Bars = 20 cm. (C) Genomic Southern blot analysis. Total leaf genomic DNA was extracted from the wild-type and T4 *Ubi:DaGolS2* (lines #1 and #2) rice plants. The DNA was digested with *Hin*dIII and hybridized to a ^{32}P-labeled hygromycin B phosphotransferase (*hph*) probe. (D) RT-PCR analysis of the wild-type and T4 *Ubi:DaGolS2*

were severely wilted, with discolored leaves after irrigation and their survival rates were as low as 8.8 ± 2.0–11.0 ± 2.0% (Fig. 6A–D).

After recovery from drought treatment, the *DaGolS2*- and *OsGolS2*-overexpressing transgenic leaves contained much higher amounts of chlorophyll (9.6 ± 2.5–10.7 ± 1.7 and 11.3 ± 2.1–11.6 ± 1.1 mg/g DW, respectively) than the leaves of the wild-type plants (1.4 ± 0.4–2.8 ± 1.4 mg/g DW; Fig. 6E, F). In addition, the detached transgenic leaves lost their water content more slowly than the wild-type leaves. After a 5-h incubation at room temperature, the wild-type leaves retained 48.9 ± 4.9% of their fresh weight. In contrast, 61.7 ± 1.8–65.1 ± 3.9% and 62.8 ± 2.1–63.0 ± 2.1% of the fresh weight was still retained in the *Ubi:DaGolS2* and *Ubi:OsGolS2* leaves, respectively (Fig. 6G). Thus, constitutive expression of *DaGolS2* and *OsGolS2* resulted in enhanced tolerance to dehydration stress in rice. Overall, the results of the phenotypic analyses presented in Figs. 5, 6 indicated that DaGolS2 and OsGolS2 are positive factors in the responses to both cold and drought stress.

The improved tolerance of the *DaGolS2*- and *OsGolS2*-overexpressing transgenic rice plants in response to cold and drought stresses was associated with decreased levels of ROS

Many abiotic stresses induce ROS accumulation, and excessive ROS eventually cause cell damage (Blokhina et al. 2003, Peshev et al. 2013). RFOs have been shown to act as antioxidant molecules and scavenge hydroxyl radials (Nishizawa et al. 2008). To investigate whether the enhanced tolerance of the *Ubi:DaGolS2* and *Ubi:OsGolS2* plants in response to cold and drought stress was caused by effective restriction of stress-induced ROS accumulation, we evaluated the phenotypes of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* plants under MV-induced oxidative stress. In the absence of MV, the growth patterns of the wild-type and transgenic progeny were highly similar. However, in the presence of 2 μM MV, the wild-type seedlings showed more developmental anomalies than the transgenic rice plants (Fig. 7A). These differences became even more pronounced at a higher concentration of MV (5 μM). The total chlorophyll contents (chlorophyll *a* + chlorophyll *b*) in each sample were measured in the presence of different concentrations (0, 2 and 5 μM) of MV. Before MV treatment, the total leaf chlorophyll contents of the wild-type and *DaGolS2*- or *OsGolS2*-overexpressing plants were indistinguishable. However, after treatment with 2–5 μM MV, the wild-type rice plants contained much lower chlorophyll contents (4.7 ± 1.3–7.4 ± 1.9 mg/g DW) than the *Ubi:DaGolS2* and *Ubi:OsGolS2* transgenic rice plants (11.3 ± 1.1–19.7 ± 1.0 mg/g DW) (Supplementary Fig. S4).

Although the H_2O_2 and malondialdehyde (MDA) contents of both the wild-type and transgenic seedlings were upregulated under stress conditions, the accumulation of ROS was clearly lower in the *GolS2*-overexpression lines than in the wild-type. The H_2O_2 levels in the wild-type plants were 266–291 μmol/g FW under cold stress and 285–461 μmol/g FW under drought stress, while those of the *DaGolS2*- and *OsGolS2*-overexpressing lines were lower at 153–267 and 199–352 μmol/g FW in response to the cold and drought treatments, respectively (Fig. 7B). It is worth noting that the H_2O_2 levels in the wild-type and transgenic plants appeared to be comparable after 16 d of cold treatment. This suggests that cellular amounts of H_2O_2 are saturated in response to the prolonged cold stress in transgenic rice plants. Further, the cellular levels of MDA in the wild-type rice plants were as high as 20–23 and 18–37 μmol/g FW under cold and drought stress, respectively (Fig. 7C). The levels of MDA in the *Ubi:DaGolS2* and *Ubi:OsGolS2* plants were 14–18 μmol/g FW under cold stress and 13–30 μmol/g FW under drought stress.

Analysis of ROS-related gene expression showed that *SODcc2* (LOC_Os07g46990), which encodes rice superoxide dismutase (Xiong et al. 2018), was upregulated by cold and drought treatments in both *DaGolS2*- and *OsGolS2*-overexpressors as compared to the wild-type rice plants (Fig. 7D). In addition, the expression levels of rice catalase *CATB* (LOC_Os06g51150) (Xiong et al. 2018) were markedly higher after 10 d of cold treatment and 4 d of drought stress in the transgenic plants than in the wild-type plants. However, we also noticed that the *CATB* expression was similar in the wild-type and transgenic rice plants after 16 d of cold- and 2 d of drought-treatments, suggesting that the expression of ROS-related genes is fluctuated in response to different environmental conditions in the transgenic lines. Collectively, these results suggested that the *DaGolS2*- and *OsGolS2*-overexpressing progeny exhibited

Fig. 4 Continued
(lines #1 and #2) rice plants to examine the overexpression of *DaGolS2* and endogenous transcript level of *OsGolS2*. *OsUbiquitin* was used as a loading control. (E) GolS enzyme activity assay. Crude extracts (50 μg protein) of the wild-type and *Ubi:DaGolS2* (lines #1 and #2) rice seedlings were used in each assay. Activity was calculated as μmol Pi released per mg protein per min. Data are means ± SD (**$P < 0.01$, Students *t*-test) of three biologically independent experiments. (F) Schematic structure of deduced OsGolS2. The glycosyltransferase and DxD motifs are indicated as dark gray and red bars, respectively. (G) Expression patterns of *OsGolS2* in response to drought (0, 0.25, 0.5, 1 and 2 h), high salinity (300 mM for 0, 1, 4 and 8 h) and low temperature (4°C for 0, 0.5, 1, 2 and 5 d) stress in rice plants. *OsRab16b* was used as a positive control for drought and salt treatments, whereas *OsDREB1A* was used as a positive control for cold stress. (H) Schematic representation of an *OsGolS2*-overexpressing binary vector construct. RB, right border; pUbi, maize ubiquitin promoter; NOS-T, NOS terminator; 35S-Pro, CaMV 35S promoter; *hph*, hygromycin phosphotransferase; T7-T, T7 terminator; LB, left border. (I) Morphology of 2-month-old WT and T3 *Ubi:OsGolS2* (independent lines #1 and #2) rice plants grown under long-day condition. Bars = 20 cm. (J) Genomic Southern blot analysis. Total leaf genomic DNA was extracted from the wild-type and T3 *Ubi:OsGolS2* (lines #1 and #2) rice plants, digested with *Hin*dIII, and hybridized to a ^{32}P-labeled hygromycin B phosphotransferase (*hph*) probe. (K) RT-PCR analysis of the wild-type and T3 *Ubi:OsGolS2* (lines #1 and #2) rice plants to examine the expression of *OsGolS2* transcript. *OsUbiquitin* was used as a loading control. (L) GolS enzyme activity assay. Crude extracts (50 μg protein) of the wild-type and *Ubi:OsGolS2* (lines #1 and #2) rice seedlings were used in each assay. Activity was calculated as μmol Pi released per mg protein per min. Data are means ± SD (**$P < 0.01$, Students *t*-test) of three biologically independent experiments.

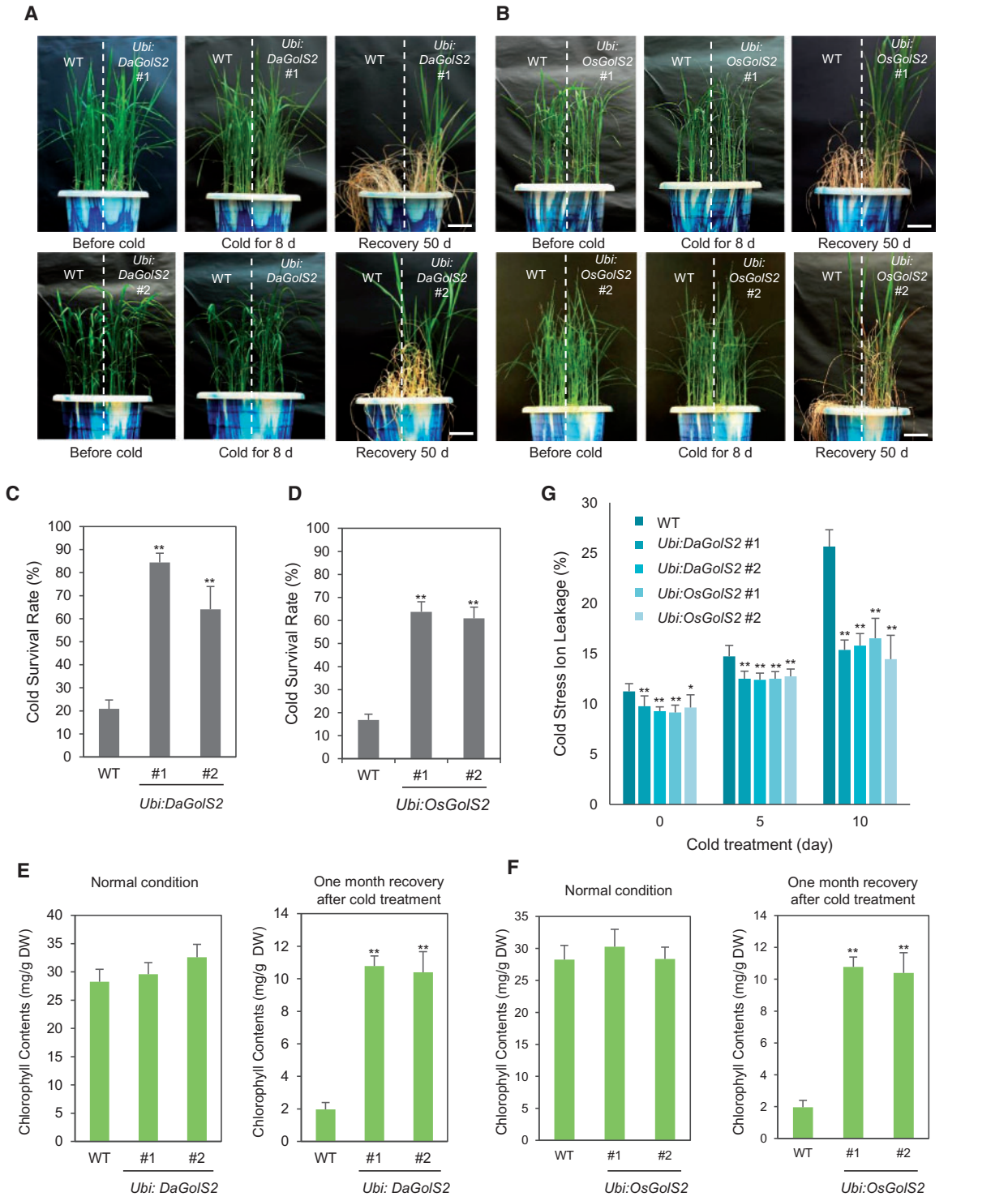

Fig. 5 Increased tolerance of *DaGolS2*- and *OsGolS2*-overexpressing transgenic rice plants in response to cold stress. (A, B) Cold stress phenotypes of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* transgenic rice plants. Light-grown, 5-week-old wild-type, T4 *Ubi:DaGolS2* (lines #1 and #2) and T3 *Ubi:OsGolS2* (lines #1 and #2) rice plants were transferred to a cold room at 4°C for 8 d, after which the plants were recovered at 28°C for 50 d. Bars = 15 cm. (C, D) Survival rates of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* plants in response to cold stress. Data are means ± SE ($n \geq 6$ biologically independent experiments; >30 plants were used in each assay, $^{**}P < 0.01$, Students *t*-test). (E, F) Total leaf chlorophyll content of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* plants before and after cold treatments. The amounts of leaf chlorophyll (chlorophyll *a* + chlorophyll *b*) of mock- (before cold) and cold-treated plants were determined 1 month after recovery from cold stress. Data are means ± SE ($n \geq 3$ biologically independent experiments; >10 plants were used in each assay, $^{**}P < 0.01$, Students *t*-test). (G). Electrolyte leakage analysis of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* rice plants in response to cold stress. Electrolyte leakage analysis was conducted using 8-day-old wild-type, T4 *Ubi:DaGolS2* (lines #1 and #2) and T3 *Ubi:OsGolS2* (lines #1 and #2) seedlings at different time points before and after cold (4°C) treatment (0, 5 and 10 d). Data are means ± SD ($n = 3$ biologically independent experiments; >12 plants of each genotype were used in each experiment, $^{**}P < 0.01$, Students *t*-test).

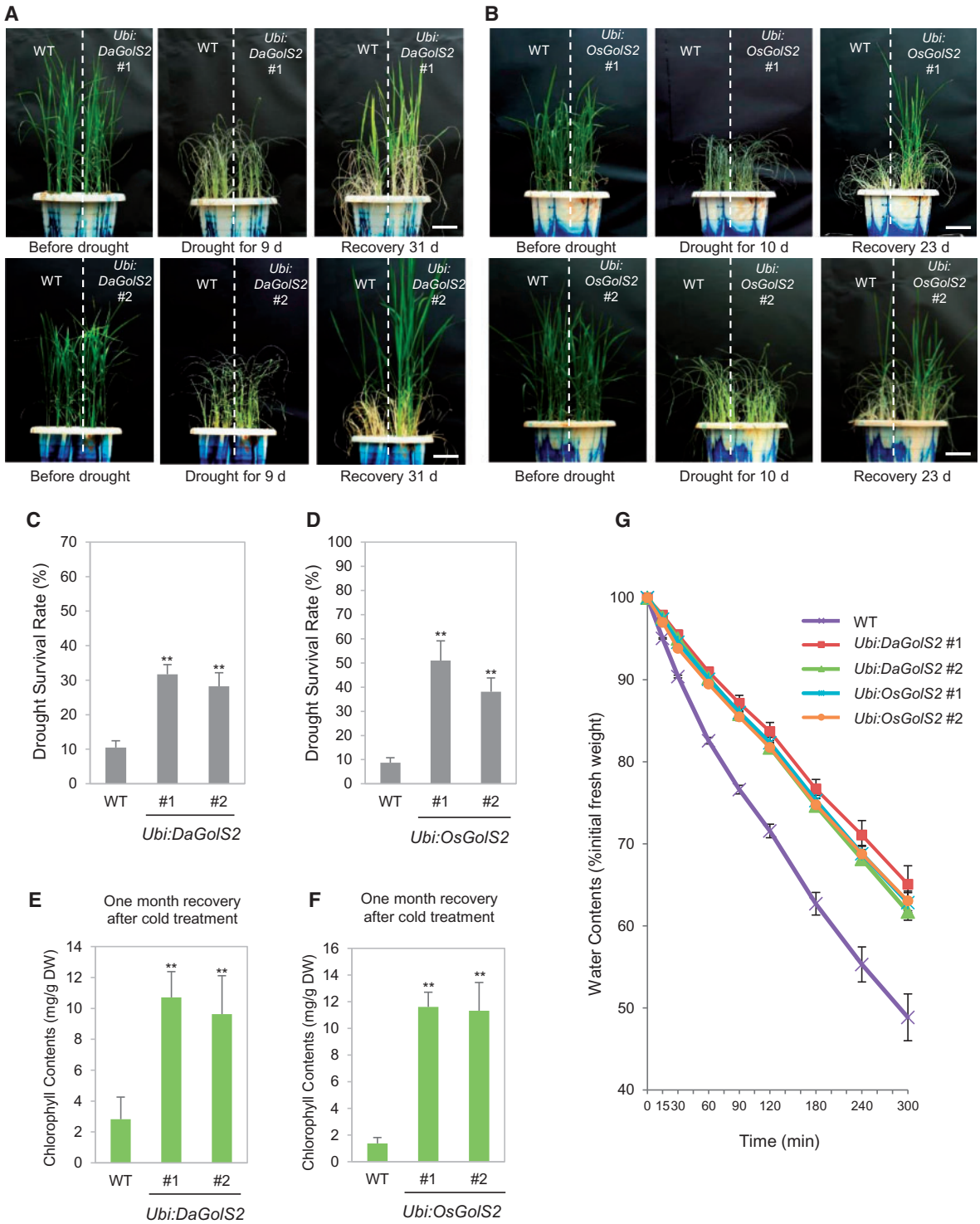

Fig. 6 Increased tolerance of *DaGolS2*- and *OsGolS2*-overexpressing transgenic rice plants in response to drought stress. (A, B) Drought stress phenotypes of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* transgenic rice plants. Light-grown, 5-week-old wild-type, T4 *Ubi:DaGolS2* (lines #1 and #2) and T3 *Ubi:OsGolS2* (lines #1 and #2) rice plants were grown at 28°C without watering for 9–10 d. Dehydration-treated plants were re-watered and their growth patterns were monitored for 23 d after re-watering. Bars = 15 cm. (C, D) Survival rates of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* rice plants in response to drought stress. Data are means ± SE ($n \geq 6$ biologically independent experiments; >30 plants were used in each assay, $^{**}P < 0.01$, Students *t*-test). (E, F) Total leaf chlorophyll content of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* plants after drought treatment. The amounts of leaf chlorophyll (chlorophyll *a* + chlorophyll *b*) of drought-treated plants were determined 1 month after recovery from the stress. Data are means ± SE ($n \geq 3$ biologically independent experiments; >10 plants were used in each assay, $^{**}P < 0.01$, Students *t*-test). (G) Water loss rates of detached leaves. The leaves of 5-week-old wild-type, T4 *Ubi:DaGolS2* (lines #1 and #2) and T3 *Ubi:OsGolS2* (lines #1 and #2) rice plants were detached, and their fresh weights were measured at the indicated time points. The rate of water loss was calculated as the percentage of initial fresh weight of the detached leaves. Data are means ± SD ($n = 3$ biologically independent experiments; >6 plants of each genotype were used in each experiment).

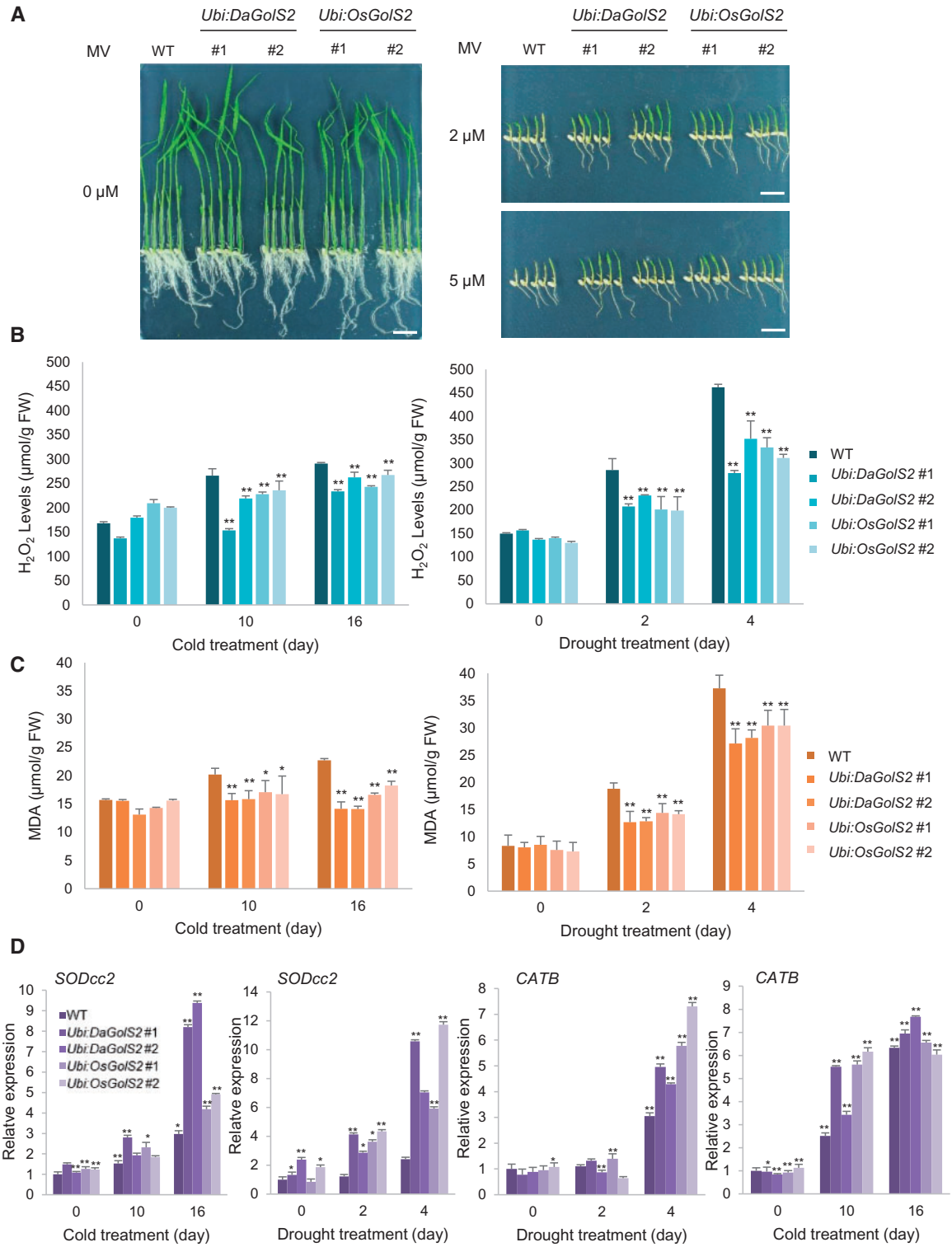

Fig. 7 Accumulation of ROS in the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* rice plants under normal and stress conditions. (A) Oxidative stress phenotypes of the wild-type, *Ubi:DaGolS2* (independent lines #1 and #2) and *Ubi:OsGolS2* (independent lines #1 and #2) plants. Sterilized seeds were germinated on half-strength MS medium for 2 d, after which they were transferred to the medium supplemented with 0, 2 and 5 μM of MV. Representative photos were taken at 9 d after germination. Bars = 1 cm. (B, C) Quantitative analysis of H_2O_2 (B) and MDA (C) in the wild-type and *Ubi:DaGolS2* and *Ubi:OsGolS2* transgenic seedlings, which were subjected to cold and drought stress. Data are means ± SE ($n = 3$ biologically independent experiments; 60 plants were used in each assay, $^{**}P < 0.01$, Students *t*-test). (D) Expression levels of ROS-related genes in *DaGolS2*- and *OsGolS2*-overexpressors in response to cold and drought stress. The relative expression levels of *SODcc2* and *CATB* were determined by qRT-PCR using gene-specific primer sets (**Supplementary Table S1**) and normalized to that of *OsUbiquitin*. Data are the means ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$, Students *t*-test) of three technically independent experiments.

improved tolerance to cold and drought stresses by more effectively restricting stress-induced ROS accumulation.

Ectopic expression of *DaGolS2* or *OsGolS2* in rice plants results in increased galactinol and raffinose content and GolS activity under both normal and stress conditions

To further assess the role of GolS2 in mitigating the adverse effects of abiotic stress, we next measured the sugar content in the wild-type and transgenic rice plants before and after stress treatment. In the absence of stress, the wild-type, *Ubi:DaGolS2* (independent lines #1 and #2) and *Ubi:OsGolS2* (independent lines #1 and #2) plants contained 0.63 ± 0.32 mg/g FW, 4.08 ± 0.63 and 2.33 ± 0.28 mg/g FW and 2.36 ± 0.45 and 2.01 ± 0.27 mg/g FW of galactinol, respectively and 0.33 ± 0.44 mg/g FW, 2.07 ± 0.29 and 1.85 ± 0.20 mg/g FW and 1.09 ± 0.20 and 1.53 ± 0.40 mg/g FW of raffinose, respectively (**Fig. 8A, B**). Thus, the *DaGolS2* and *OsGolS2* overexpressors contained higher levels of galactinol and raffinose than the wild-type plants under normal growth conditions. After stress treatment, the accumulation of galactinol and raffinose was more evident in the *Ubi:DaGolS2* and *Ubi:OsGolS2* lines than in the wild-type rice plants. As shown in **Fig. 8A**, under cold stress, the galactinol contents of the *Ubi:DaGolS2* and *Ubi:OsGolS2* lines were increased to 8.19 ± 1.46 and 9.14 ± 0.81 mg/g FW and 7.14 ± 2.04 and 5.97 ± 1.01 mg/g FW, respectively, while the galactinol content in the wild-type plants was 2.38 ± 0.77 mg/g FW. In response to low temperature stress, the raffinose content in the *Ubi:DaGolS2* and *Ubi:OsGolS2* lines was 8.99 ± 0.91 and 7.21 ± 0.81 mg/g FW and 6.95 ± 0.61 and 7.29 ± 0.73 mg/g FW, respectively, while that in the wild-type plants was as low as 4.96 ± 0.22 mg/g FW (**Fig. 8B**). The levels of galactinol and raffinose in the *Ubi:DaGolS2* and *Ubi:OsGolS2* lines were also upregulated under dehydration stress as compared to the levels in the wild-type plants. After drought treatment, the amounts of galactinol were 15.97 ± 2.25 mg/g FW in wild-type, 31.29 ± 2.20 and 29.31 ± 1.51 mg/g FW in *Ubi:DaGolS2* and 25.95 ± 1.60 and 25.32 ± 0.96 mg/g FW in *Ubi:OsGolS2*, and the amounts of raffinose were 3.94 ± 0.78 mg/g FW in wild-type, 8.47 ± 0.98 and 6.68 ± 0.38 mg/g FW in *Ubi:DaGolS2* and 6.29 ± 0.70 and 9.98 ± 0.49 mg/g FW in *Ubi:OsGolS2* (**Fig. 8A, B**). Thus, ectopic expression of *DaGolS2* and *OsGolS2* in rice plants induced the accumulation of galactinol and raffinose under both normal and stress conditions. In contrast to these differences in RFO levels, there were less differences between the wild-type and transgenic lines in the levels of sucrose, glucose and fructose before and after stress treatment (**Fig. 8C–E**). To verify whether the increased amounts of galactinol and raffinose were caused by upregulation of GolS activity, GolS enzyme activities were measured in the wild-type and transgenic rice plants. As shown in **Fig. 8F**, higher levels of GolS activity were detected in the *DaGolS2*- and *OsGolS2*-overexpressing rice plants (independent transgenic lines #1 and #2) than in the wild-type plant before and after stress treatment. These results suggested that the elevated levels of galactinol and raffinose are responsible, at least in part, for the tolerant phenotypes of the *DaGolS2*- and *OsGolS2*-overexpressors in response to cold and drought stress.

Discussion

Higher plants have developed complicated physiological and biochemical processes to cope with unfavorable growth conditions brought by the multitude of environmental factors (Seki et al. 2007). *Deschampsia antarctica* exhibits a combination of morphological and physiological adaptive traits that allows these plants to endure the harsh Antarctic climate (Cavieres et al. 2016). RFOs are plant non-structural carbohydrates and play protective roles in response to a broad spectrum of abiotic stresses (Sengupta et al. 2015). Although several studies showed high accumulation of carbohydrates during growing season and seed maturation in *D. antarctica* (Zuñiga et al. 1996, Zúñiga-Feest et al. 2003), a link between cellular roles of carbohydrates, especially RFOs, and abiotic stress tolerance response remains elusive in an Antarctic hairgrass.

In this study, we first monitored changes in the endogenous levels of RFOs in naturally grown and laboratory-cultured *D. antarctica* plants. The results showed that endogenous galactinol and raffinose levels were markedly increased when the plants were subjected to cold and dehydration treatments in both Antarctic field and laboratory conditions (**Fig. 1**). We next identified two homologous *DaGolS1* and *DaGolS2* genes, which encode enzymes that catalyze the first committed step of RFOs biosynthesis. Both genes were rapidly upregulated under cold and drought stresses, and when plants were transferred to the Antarctic field after 6 d of incubation in the laboratory condition (**Fig. 2**). These early inductions of *DaGolSs* were reminiscent of those of *DaCBF4* and *DaCBF7*, which encode transcription factors that trigger a subset of early cold-responsive genes (Byun et al. 2015, Byun et al. 2018). Previously, the *AtGolS* genes were rapidly induced by 10-h treatment of cold, and were reported as a C-repeat binding factor regulon during cold acclimation in Arabidopsis (Fowler and Thomashow 2002, Taji et al. 2002). In Poaceae cool-season species, 6 *GolS* genes of *Festuca arundinacea*, 3 *GolS* genes of *Lolium perenne* and *TaGolS3* of *T. aestivum* showed early induction as a part of the cold acclimation response (Wang et al. 2016a, Wang et al. 2016b). These reports suggest that GolS has an important function in cold tolerance in plants especially for cold-adapted species. However, *GolS* transcription patterns are different in the case of cold-sensitive crops. Rice *OsGolS2* in this study was induced by 1 h drought treatment, while the induction timing was delayed for the cold stress to 5 d (**Fig. 4G**). This prominent response of *GolS* to the drought was also observed in maize. The transcripts of homologous *ZmGolS1*, *ZmGolS2* and *ZmGolS3* accumulated in maize Hi-II callus cells upon dehydration and heat treatments, but showed no significant response by cold stress (Zhao et al. 2004). Together with results showing no considerable phenotypic difference between *Ubi:DaGolS2* and *Ubi:OsGolS2* rice plants under cold and drought treatment (**Figs. 5, 6**), we could assume that the transcriptional regulation systems for *GolS* have differentiated into various ways specific to the regional climate as the adaptation strategy of plant species. In fact, the upstream sequences of *DaGolS2* and *OsGolS2* contain different combination of *cis*-elements putatively responding to various environmental stresses (**Supplementary Fig. S5**). Based on these

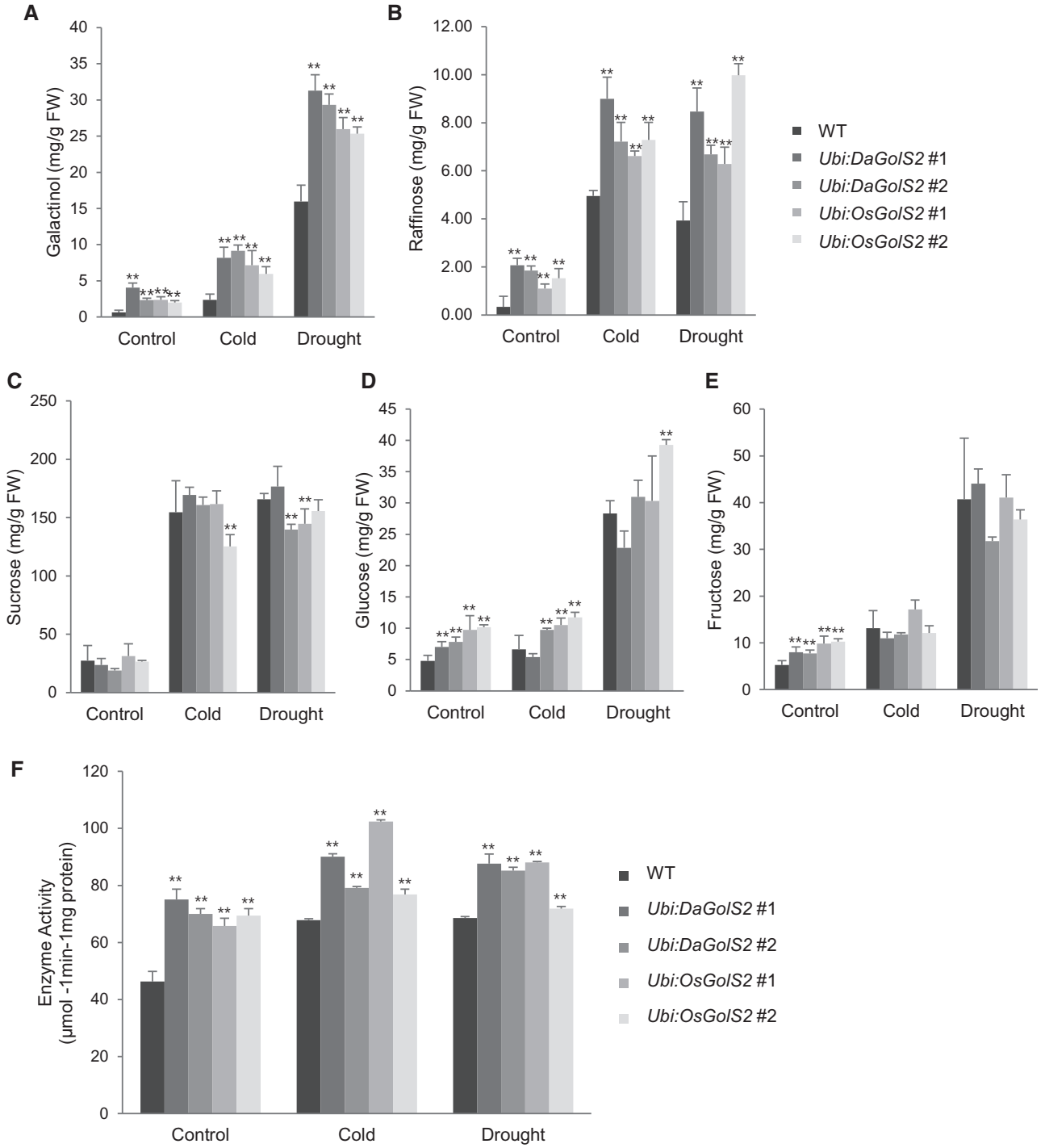

Fig. 8 Carbohydrate content and GolS enzyme activities in the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* rice plants under normal and stress conditions. (A–E) Amounts of galactinol (A), raffinose (B), sucrose (C), glucose (D) and fructose (E) were determined in the mock-, cold (4°C for 10 d)- and drought (air dried for 4 d)-treated wild-type, *Ubi:DaGolS2* (independent transgenic lines #1 and #2) and *Ubi:OsGolS2* (independent transgenic lines #1 and #2) rice plants. Data are means ± SD ($n \geq 3$ biologically independent experiments; >10 plants were used in each assay, $^{**}P < 0.01$, Students *t*-test). (F) Determination of GolS enzyme activities in the wild-type, *Ubi:DaGolS2* (independent transgenic lines #1 and #2) and *Ubi:OsGolS2* (independent transgenic lines #1 and #2) rice plants before and after cold and drought treatments. Crude extracts (50 μg protein) of the wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* rice seedlings were used in each assay. Activity was calculated as μmol Pi released per mg protein per min. Data are means ± SD ($^{**}P < 0.01$, Students *t*-test) of three biologically independent experiments.

results, we speculated that rapid induction of *GolS* under cold condition and RFOs biosynthesis are critical strategies for *D. antarctica* plants to survive and flourish in harsh Antarctic environments.

GolS, a member of glycosyltransferase 8 (GT8) family, is a key enzyme of RFOs biosynthesis that is a specialized metabolic event in higher plants (Yin et al. 2010). Among large members of GT8 family, GolS is the only group that is responsive to a variety of abiotic stress (Sengupta et al. 2012). Expanded numbers of GolS and differential expression profiles of individual *GolS* genes in plants suggest an evolutionary need of RFOs to effectively deal with adverse environments (Taji et al. 2002, Downie et al. 2003, Gu et al. 2016). The cladogram of GolS-like sequences shows a distinct separation of monocot and dicot sequences, suggesting that GolSs were originated before the diversification of monocot and dicot plants (Yin et al. 2010, Sengupta et al. 2012; **Supplementary Fig. S2**). The monocot GolSs are further classified to type I and type II enzymes (**Fig. 3**). The distinct characteristic of type II GolS was highly expanded numbers of the protein in *Triticeae* subfamily, which includes a representative chilling stress-tolerant crop. Winter wheat (*T. aestivum* L.) has a greater number of GolS members belonging to type II than those of type I, while chilling sensitive maize contains lower number of type II GolS than type I (**Supplementary Fig. S2 and Table S3**). The distinct numbers of each type of GolSs might be associated with their functional divergence for the specific requirements of plants to maintain their growth and development under different environmental and physiological situations.

Although the glycosyltransferase domain is highly conserved in GolSs (Sengupta et al. 2012), there is a distinct property in the catalytic DxD motif between monocot and dicot GolSs: two Asp residues in the DxD motif are involved in the interaction with the Mn^{2+} ion, whereas Ala for monocot GolS and Gly for dicot GolS participate, respectively, in ribose binding (**Supplementary Fig. S1**). In addition, the Ser^{84} residue, a predicted phosphorylation site in monocot type II GolS, is substituted to the Asp residue in monocot type I and dicot GolS, while a putative phosphorylation site Thr^{205} in monocot type I and dicot GolS is replaced by Lys or Ala in monocot type II GolS (**Supplementary Fig. S1**). These indicate that monocot type I GolS is more closely correlated with dicot GolS than monocot type II GolS. It is also worth noting that a predicted phosphorylation site Ser^{324} is well conserved in type II DaGolS2, but not in type I DaGolS1 (**Supplementary Fig. S1**), suggesting the unique alternation in the Antarctic DaGolS enzymes. These variations in the putative phosphorylation sites of GolSs might be related to specific protein modification, which, in turn, fine-tunes the cellular function of GolSs in response to different developmental and environmental cues (Zhang et al. 2018). Overall, these results imply that each GolS isoform may have evolved to perform the distinct and diverse roles in planta to overcome the sessility and to survive in the continuously fluctuating environment.

Increasing evidence indicates that GolSs from different kinds of species participate in the response to abiotic stress (Hue et al. 2013, Shimosaka and Ozawa 2015, Gu et al. 2016, Selvaraj et al. 2017). Both *DaGolS2*- and *OsGolS2*-overexpressors displayed markedly enhanced tolerance to cold and drought treatments, as evidenced by stable growth performance, higher leaf chlorophyll content, less leaf electrolyte leakage and lower leaf water loss rate, which resulted in higher survivals as compared to the wild-type rice plants (**Figs. 5**, **6**). Consistent with these phenotypic properties, *DaGolS2*- and *OsGolS2*-overexpressors accumulated higher amounts of galactinol and raffinose, but not sucrose, glucose and fructose, under both normal and stress conditions in comparison with the wild-type rice plants (**Fig. 8**). These results are in agreement with the view that galactinol and raffinose levels were rapidly elevated when naturally grown and laboratory-cultured *D. antarctica* plants were subjected to cold and drought stress conditions (**Figs. 1**, **2**). The cell-free degradation assay using bacterially expressed 6xMyc-DaGolS2 recombinant protein suggested that the DaGolS2 protein stability was unchanged in response to drought and cold treatment as compared to the normal conditions (**Supplementary Fig. S6**). Thus, it was highly plausible that higher amounts of galactinol and raffinose in the *DaGolS2*- and *OsGolS2*-overexpressors were a consequence of increased GolS activity rather than protein stability. Thus, our results provide reasonable evidence that Poaceae type II DaGolS2 and OsGolS2 improve tolerance to abiotic stresses through accumulation of RFOs in *D. antarctica* and rice plants.

Imposition of environmental stress gives rise to excess concentration of ROS, which is associated with oxidative damage at the cellular level (Blokhina et al. 2003, Salvi et al. 2018). Hydroxyl radical is one of the most deleterious forms of ROS and potentially reacts with most, if not all, cellular molecules. Small soluble sugars are widely regarded to be related to oxidative stress and ROS signaling, and there is a close correlation between sugar structure and hydroxyl radical scavenging capacity (Peshev et al. 2013). Recently, RFOs have been proposed to fulfill important roles in oxidative stress protection in plants (Nishizawa et al. 2008, Van den Ende and Valluru 2009, Peshev et al. 2013). RFOs serve as compatible solutes under stress conditions to maintain cell turgor and stabilize cellular proteins, and also acts as antioxidants to counteract the accumulation of ROS (Bartels and Sunkar 2005, Nishizawa et al. 2008, Valluru and Van den Ende 2011). Consistent with these notions, *DaGolS2*- and *OsGolS2*-overexpressors showed enhanced tolerance to MV treatment, and the levels of H_2O_2 and MDA were reduced in transgenic lines under the stress conditions as compared to those of wild-type rice plants (**Fig. 7**). Thus, it is highly plausible that increased levels of galactinol and raffinose in *Ubi:DaGolS2* and *Ubi:OsGolS2* transgenic lines efficiently counteracted the accumulation of ROS and attained improved tolerance to cold and drought stress.

In conclusion, our results showed that ectopic expression of Poaceae type II DaGolS2 and OsGolS2 increased the tolerance of transgenic rice plants to cold and drought stress by inducing the accumulation of RFOs and decreasing ROS. Therefore, under extreme environmental conditions, inducing the *DaGolS* genes for efficient production of RFOs to stabilize cellular proteins and membranes and to counteract ROS accumulation would be an efficient survival strategy for *D. antarctica* in Maritime Antarctica.

Materials and Methods

Plants materials and growth conditions

Deschampsia antarctica was collected near the King Sejong Antarctic Station (62°14′29″S; 58°44′18″W) on the Barton Peninsula of King George Island in January 2007. The plants were transplanted, cultured in vitro in a tissue culture medium [half-strength Murashige and Skoog (MS) (Duchefa Biochemie, Haarlem, The Netherlands), 2% sucrose and 0.8% phytoagar, pH 5.7] under a 16-h- light/8-h-dark photoperiod with a light intensity of 150 μmol/m^2/s at 15°C, and transferred to new medium every 3 weeks.

Dry seeds of rice (*Oryza sativa* L.) japonica variety Dong-jin were washed with 70% ethanol and subsequently sterilized with 0.4% NaClO solution. Sterilized seeds were germinated and grown on half-strength MS medium supplemented with 3% sucrose and 0.75% phytoagar, pH 5.7, for 10–12 d. Germinated seedlings were transplanted to soil and grown at 25–30°C under long-day (16 h light and 8 h dark) conditions in a greenhouse.

Stress treatments and real-time qRT-PCR

For low-temperature treatment, *D. antarctica* plants grown at 15°C were transferred to a climate chamber at 4°C for various time periods (1 h–7 d). For dehydration treatment, plants were transferred to filter paper, dried at 15°C and collected at different time points (1, 2 and 4 h) after the imposition of stress. All the samplings for expression analysis were conducted at the same time to avoid variation by circadian rhythm.

Total RNAs were extracted from mature leaves of *D. antarctica* and rice plants using a RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of RNAs were determined by spectroscopic measurements using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The first-strand cDNA was synthesized from 2 μg of total RNA using TOPscript reverse transcriptase (Enzynomics, Daejeon, South Korea) and oligo (dT) primers. Real-time qRT-PCR analysis was performed in 20 μl reaction mixtures [1 μl of 1:10 diluted cDNA template, 2 μM of each primer and 10 μl of TB Green™ Premix Ex Taq (TaKaRa, Kyoto, Japan)]. The amplification procedure was as follows: 5 min of denaturation and enzyme activation at 95°C; followed by 40 cycles at 95°C for 10 s, 55°C for 10 s and 72°C for 15 s. The *DaEF1a* gene was used as an internal control. The DNA sequences of primers used for PCR amplification are listed in **Supplementary Table S1**.

Measurement of leaf galactinol and raffinose content

Mature leaf tissues (100 mg) of *D. antarctica* and rice plants was ground to a fine powder with liquid N_2 using a mortar and pestle and then homogenized with 1 ml of 80% (v/v) methanol. The homogenates were boiled for 10 min at 90°C and centrifuged at 10,000×*g* for 5 min. The supernatant was collected and dried using dry nitrogen flush at room temperature overnight. Samples were resuspended in 1 ml of HPLC grade distilled water and filtered with 0.2-μm filter. Sugars were analyzed by HPLC (Ultimate 3000, Dionex, USA) with the Suga-pak (Waters, USA) column and an RI detector (Shodex, RI-101, Japan). Peaks for galactinol and raffinose were identified and calculated by comparison with authentic standards.

Subcellular localization assay

The 3′ ends of *DaGolS1* and *DaGolS2* coding regions were tagged with synthetic green fluorescent protein (*sGFP*) in-frame and inserted into the pBI221 binary vector that contains the 35S CaMV promoter. The *35S:DaGolS1-sGFP*, *35S:DaGolS2-sGFP* and *35S:OsGolS2-sGFP* constructs were expressed in *D. antarctica* or rice protoplast by using the polyethylene glycol-mediated DNA transfer method. Fluorescent signals were visualized by fluorescence microscopy (BX51, Olympus, Japan). sGFP were used as a cytosolic marker.

Phylogenetic analysis

The amino acid sequences of DaGolS1, DaGolS2 and GolS homologs from *Poaceae* monocot crops, eudicots and gymnosperm were retrieved from the GenBank database and proofread. Phylogenetic trees were constructed from the data sets by the neighbor-joining method based on the JTT matrix-based model using MEGA7 software (Kumar et al. 2016). All positions with <95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position. Supports for internal branches were tested by bootstrap analyses of 1,000 replications. The GenBank accession numbers of GolS homologs from Antarctic hairgrass (*D. antarctica*), *Oryzeae*, *Leersia perrieri*, *Triticeae*, *Aegilops tauschii*, barley (*Hordeum vulgare*), maize (*Zea mays*), great millet (*Sorghum bicolor*), foxtail millet (*Setaria italic*), *Brachypodium distachyon*, *Musaceae* (*Musa acuminate*), eudicots (*Arabidopsis thaliana*, *Brassica napus*, *Glycine max*) and gymnosperms (*Selaginella moellendorffii*) are presented in **Supplementary Table S2**.

Generation of *DaGolS2*- and *OsGolS2*-overexpressing transgenic rice plants

Full-length coding regions of *D. antarctica DaGolS2* (GenBank accession No. MK286465) and rice *OsGolS2* (GenBank accession No. XP_015645380.1) were ligated into the pGA2897 binary vector plasmids that contained the maize *Ubiquitin* promoter (*Ubi*). The *Ubi:DaGolS2* and *Ubi:OsGolS2* recombinant constructs were transformed into the *Agrobacterium tumefaciens* strain LBA4404 via electroporation as previously reported by Park et al. (2016). All procedures of rice transformation were conducted according to the established protocol as recently described by Cui et al. (2018). Generated transgenic rice T0 plants were transplanted in the soil under the greenhouse conditions and further propagated in the paddy field conditions. The harvested transgenic seeds of individual plant were germinated in the half-strength MS medium supplemented with hygromycin B (40 mg/l) to select the homozygous T2 generation. Homozygous T4 *DaGolS2*-overexpressing (independent lines #1 and #2) and T3 *OsGolS2*-overexpressing (independent lines #1 and #2) transgenic rice progeny were used for our phenotypic analysis.

Protein extraction and GolS enzyme activity assay

Light-grown 10-day-old rice seedlings were ground to a fine powder and suspended in an extraction buffer (100 mM HEPES buffer pH 7.5, 1 mM β-mercaptoethanol and protease inhibitor cocktail). The homogenates were centrifuged at 13,200 rpm for 15 min at 4°C and the supernatants were used for determination of GolS activity. The GolS activity assay was followed by Salvi et al. (2018) with slight modifications. Cell-free protein (50 μg) crude extracts (60 mM myo-inositol, 2 mM dithiothreitol, 50 mM HEPES buffer pH 7.0, 4 mM $MnCl_2$, 20 μg of bovine serum albumin and 4 mM UDP-galactose) were incubated at 32°C for 30 min and placed in the boiling water for 2 min to terminate enzyme reaction. Hydrolysis of UDP was carried out with 500 μl of distilled water, 0.3 unit of potato apyrase and 150 μl of apyrase reaction mixture (250 mM Tris–HCl, pH 7.5, 25 mM KCl, 7.5 mM $CaCl_2$, 0.5 mM ethylenediaminetetraacetic acid and 50 mM glucose). After incubation for 10 min at 37°C, the apyrase reaction was stopped by addition of 60 μl of 75% trichloroacetic acid (TCA). The reaction mixtures were then cooled on ice for 10 min, centrifuged at 5,000 rpm for 10 min and amount of Pi in the supernatant was determined according to the protocol by Fiske and Subbarow (1925). The amount of UDP produced by GolS was determined using a pre-constructed standard curve of Pi.

Stress treatments of wild-type, *Ubi:DaGolS2* and *Ubi:OsGolS2* rice plants

For low-temperature stress treatment, 5-week-old rice plants grown at 28°C in the same pot under the long-day (16 h-light and 8 h-dark) conditions were transferred to cold room at 4°C. After 8 d cold treatment, the plants were recovered at 28°C for 50 d and their growth patterns were monitored as recently described (Cui et al. 2018). Plants that resumed growing with green and healthy leaves were regarded as having survived and the survival rates were determined at 50 d of recovery. Data were obtained from at least six biological independent experiments. Electrolyte leakage analysis was conducted using 8-day-old whole seedlings at different time points of cold treatments (0, 5 and 10 d) at 4°C. The cold stress-treated seedlings of wild-type and transgenic rice plants were soaked in a test tube containing 35 ml of distilled water on an orbital shaker (200 rpm) at room temperature for overnight. The electrolyte conductivity of each sample was determined before and after autoclaving by using conductivity meter (Orion Star A212, Thermo Scientific, USA) by the method of Min et al. (2016).

For water deficit treatment, 5-week-old wild-type, T4 *Ubi:DaGolS2* (independent lines #1 and #2) and T3 *Ubi:OsGolS2* (independent lines #1 and #2) plants were grown in the same pot withholding water for 9–10 d until the leaves were wilted. The plants were then re-watered and their growth profiles were

monitored at different time points of stress recovery. Plants that resumed growing with green and healthy leaves were regarded as having survived. The survival rates and total leaf chlorophyll content were determined at 3–4 weeks of recovery. Data were obtained from at least six biological independent experiments. To measure the leaf water loss rate, detached leaves from 6-week-old wild-type and transgenic rice plants were placed on the filter paper and weighed at the different time intervals (15, 30, 60, 90, 120, 180, 240 and 300 min) at room temperature. The water loss rate was calculated as the percentage of initial fresh weight.

For methyl viologen treatment, the wild-type and T4 *Ubi:DaGolS2* and T3 *Ubi:OsGolS2* transgenic rice seeds were germinated on half-strength MS medium for 2 d at 28°C under the long-day (16 h light and 8 h dark) conditions. Germinated seedlings were transferred to half-strength MS medium supplemented with 0, 2 and 5 μM methyl viologen (Sigma-Aldrich, MO, USA) and incubated for another 7 d in the same growth condition. The chlorophyll content of each genotype was determined at 9 d after germination. More than 100 seeds were used in each assay and three biologically independent assays were performed.

Measurement of total leaf chlorophyll content

Total leaf chlorophyll (chlorophyll *a* + chlorophyll *b*) content of the wild-type and transgenic rice plants was measured before and after stress treatment as described by Min et al. (2016) with slight modifications. The amounts of chlorophyll *a* + chlorophyll *b* were measured at 664.2 and 648.6 nm, respectively, by ELISA microplate reader (VERSAmax, Molecular Devices, USA) and normalized by dry weight of leaves of each genotype.

MDA and H_2O_2 determination assay

To determine the MDA level, thiobarbituric acid (TBA) reactive substances test was performed according to Heath and Packer (1968) with modifications as described by Park et al. (2016). Mock- or stress-treated seedling shoots (100 mg) of the wild-type and transgenic rice plants were homogenized with 1 ml of 0.25% TBA (Sigma-Aldrich) and dissolved in 10% TCA (Sigma-Aldrich). The mixtures were heated in boiling water for 30 min, quickly cooled on ice for 10 min and subsequently centrifuged at 13,200 rpm for 30 min. The absorbance of the supernatant was measured at 532 and 600 nm by ELISA microplate reader and normalized by fresh weight of leaves of each genotype.

To determine the H_2O_2 level, mock- or stress-treated seedling shoots (100 mg) of the wild-type and transgenic rice were homogenized with 1 ml of solution containing 0.1% TCA, 0.5 M KI and 2.5 mM potassium phosphate buffer, pH 7.0. The mixtures were then incubated at 4°C under the dark condition for 10 min. The homogenate samples were centrifuged at 13,200 rpm for 15 min at 4°C and the supernatant from each sample was taken to measure the absorbance at 390 nm.

Cell-free protein degradation assay

In vitro cell-free protein degradation assay was performed as described by Seo et al. (2016) with a slight modification. Briefly, the 6xMyc-DaGolS2 recombinant protein was incubated with a cell-free crude extract (50 μg total proteins) of rice and *D. antarctica* seedlings, which had been treated by normal and cold/drought stress conditions for 0, 1.5 and 3 h. The reactions were terminated by adding 2× SDS sample buffer. The protein degradation patterns were analyzed by immunoblotting with anti-Myc (Applied Biological Materials) antibody.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References

Bartels, D. and Sunkar, R. (2005) Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.* 24: 23–58.

Blokhina, O., Virolainen, E. and Fagerstedt, K.V. (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.* 91: 179–194.

Bravo, L.A., Ulloa, N., Zuñiga, G.E., Casanova, A., Corcuera, L.J. and Alberdi, M. (2001) Cold resistance in Antarctic angiosperms. *Physiol. Plant.* 111: 55–65.

Byun, M.Y., Lee, J., Cui, L.H., Kang, Y., Oh, T.K. and Park, H. (2015) Constitutive expression of DaCBF7, an Antarctic vascular plant *Deschampsia antarctica* CBF homolog, resulted in improved cold tolerance in transgenic rice plants. *Plant Sci.* 236: 61–74.

Byun, M.Y., Cui, L.H., Lee, J., Park, H., Lee, A., Kim, W.T., et al. (2018) Identification of rice genes associated with enhanced cold tolerance by comparative transcriptome analysis with two transgenic rice plants overexpressing DaCBF4 or DaCBF7, isolated from Antarctic flowering plant *Deschampsia antarctica*. *Front. Plant Sci.* 9: 601.

Cavieres, L.A., Saez, P., Sanhueza, C., Sierra-Almeida, A., Rabert, C., Corcuera, L.J., et al. (2016) Ecophysiological traits of Antarctic vascular plants: their importance in the responses to climate change. *Plant Ecol.* 217: 343–358.

Cui, L.H., Min, H.J., Byun, M.Y., Oh, H.G. and Kim, W.T. (2018) OsDIRP1, a putative RING E3 ligase, plays an opposite role in drought and cold stress responses as a negative and positive factor, respectively, in rice (*Oryza sativa* L.). *Front. Plant Sci.* 9: 1797.

Downie, B., Gurusinghe, S., Dahal, P., Thacker, R.R., Snyder, J.C., Nonogaki, H., et al. (2003) Expression of a GALACTINOL SYNTHASE gene in tomato seeds is up-regulated before maturation desiccation and again after imbibition whenever radicle protrusion is prevented. *Plant Physiol.* 131: 1347–1359.

ElSayed, A.I., Rafudeen, M.S. and Golldack, D. (2014) Physiological aspects of raffinose family oligosaccharides in plants: protection against abiotic stress. *Plant Biol. J.* 16: 1–8.

Fiske, C.H. and Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* 66: 375–400.

Fowler, S. and Thomashow, M.F. (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* 14: 1675–1690.

Gidekel, M., Destefano-Beltran, L., GarcíA, P., Mujica, L., Leal, P., Cuba, M., et al. (2003) Identification and characterization of three novel cold acclimation-responsive genes from the extremophile hair grass *Deschampsia antarctica* Desv. *Extremophiles* 7: 459–469.

Gielwanowska, I., Szczuka, E., Bednara, J. and Górecki, R. (2005) Anatomical features and ultrastructure of *Deschampsia antarctica* (Poaceae) leaves from different growing habitats. *Ann. Bot.* 96: 1109–1119.

Gu, L., Zhang, Y., Zhang, M., Li, T., Dirk, L.M.A., Downie, B., et al. (2016) ZmGOLS2, a target of transcription factor ZmDREB2A, offers similar protection against abiotic stress as ZmDREB2A. *Plant Mol. Biol.* 90: 157–170.

Heath, R.L. and Packer, L. (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys*. 125: 189–198.

Hue, N.T., Tran, H.T.T., Phan, T., Nakamura, J., Iwata, T., Harano, K., et al. (2013) Hsp90 and reactive oxygen species regulate thermotolerance of rice seedlings via induction of heat shock factor A2 (OsHSFA2) and galactinol synthase 1. *Agric. Sci.* 04: 154–164.

John, U.P., Polotnianka, R.M., Sivakumaran, K.A., Chew, O., Mackin, L., Kuiper, M.J., et al. (2009) Ice recrystallization inhibition proteins (IRIPs) and freeze tolerance in the cryophilic Antarctic hair grass Deschampsia antarctica E. Desv. *Plant Cell Environ.* 32: 336–348.

Köhler, H., Contreras, R.A., Pizarro, M., Cortés-Antíquera, R. and Zúñiga, G.E. (2017) Antioxidant responses induced by UVB radiation in *Deschampsia antarctica* Desv. *Front. Plant Sci*. 8: 921.

Kumar, S., Stecher, G. and Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870–1874.

Lee, H., Kim, J.H., Park, M., Kim, I.C., Yim, J.H. and Lee, H.K. (2010) Reference genes validation for qPCR normalization in *Deschampsia antarctica* during abiotic stresses. *Antarct. Sci*. 22: 477–484.

Lee, J., Noh, E.K., Choi, H.S., Shin, S.C., Park, H. and Lee, H. (2013) Transcriptome sequencing of the Antarctic vascular plant *Deschampsia antarctica* Desv. under abiotic stress. *Planta* 237: 823–836.

Lehle, L. and Tanner, W. (1973) The function of myo-inositol in the biosynthesis of raffinose. Purification and characterization of galactinol: sucrose 6-galactosyltransferase from *Vicia faba* seeds. *Eur. J. Biochem.* 38: 103–110.

Loewus, F.A. and Murthy, P.P.N. (2000) *myo-Inositol* metabolism in plants. *Plant Sci*. 150: 1–19.

Min, H.J., Jung, Y.J., Kang, B.G. and Kim, W.T. (2016) CaPUB1, a hot pepper u-box E3 ubiquitin ligase, confers enhanced cold stress tolerance and decreased drought stress tolerance in transgenic rice (*Oryza sativa* L.). *Mol. Cells* 39: 250–257.

Nishizawa, A., Yabuta, Y. and Shigeoka, S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.* 147: 1251–1263.

Nishizawa-Yokoi, A., Yabuta, Y. and Shigeoka, S. (2008) The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plant cells from oxidative damage. *Plant Signal. Behav*. 3: 1016–1018.

Park, K.Y., Kim, E.Y., Seo, Y.S. and Kim, W.T. (2016) Constitutive expression of CaPLA1 conferred enhanced growth and grain yield in transgenic rice plants. *Plant Mol. Biol.* 90: 517–532.

Peshev, D., Vergauwen, R., Moglia, A., Hideg, É. and Van den Ende, W. (2013) Towards understanding vacuolar antioxidant mechanisms: a role for fructans? *J. Exp. Bot*. 64: 1025–1038.

Sáez, P.L., Rivera, B.K., Ramírez, C.F., Vallejos, V., Cavieres, L.A., Corcuera, L.J., et al. (2019) Effects of temperature and water availability on light energy utilization in photosynthetic processes of *Deschampsia antarctica*. *Physiol. Plant.* 165: 511–523.

Salvi, P., Kamble, N.U. and Majee, M. (2018) Stress-inducible galactinol synthase of chickpea (CaGolS) is implicated in heat and oxidative stress tolerance through reducing stress-induced excessive reactive oxygen species accumulation. *Plant Cell Physiol*. 59: 155–166.

Selvaraj, M.G., Ishizaki, T., Valencia, M., Ogawa, S., Dedicova, B., Ogata, T., et al. (2017) Overexpression of an *Arabidopsis thaliana* galactinol synthase gene improves drought tolerance in transgenic rice and increased grain yield in the field. *Plant Biotechnol. J.* 15: 1465–1477.

Seki, M., Umezawa, T., Urano, K. and Shinozaki, K. (2007) Regulatory metabolic networks in drought stress responses. *Curr. Opin. Plant Biol.* 10: 296–302.

Sengupta, S., Mukherjee, S., Parween, S. and Majumder, A.L. (2012) Galactinol synthase across evolutionary diverse taxa: functional preference for higher plants? *FEBS Lett.* 586: 1488–1496.

Sengupta, S., Mukherjee, S., Basak, P. and Majumder, A.L. (2015) Significance of galactinol and raffinose family oligosaccharide synthesis in plants. *Front. Plant Sci*. 6: 656.

Seo, D.H., Ahn, M.Y., Park, K.Y., Kim, E.Y. and Kim, W.T. (2016) The N-terminal UND motif of Arabidopsis U-box E3 ligase PUB18 is critical for the negative regulation of ABA-mediated stomatal movement and determination of its ubiquitination specificity to Exo70B1. *Plant Cell* 28: 2952–2973.

Shimosaka, E. and Ozawa, K. (2015) Overexpression of cold-inducible wheat galactinol synthase confers tolerance to chilling stress in transgenic rice. *Breed. Sci.* 65: 363–371.

Stevenson, J.M., Perera, I.Y., Heilmann, I., Persson, S. and Boss, W.F. (2000) Inositol signaling and plant growth. *Trends Plant Sci*. 5: 252–258.

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., et al. (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.* 29: 417–426.

Valluru, R. and Van den Ende, W. (2011) Myo-inositol and beyond-emerging networks under stress. *Plant Sci.* 181: 387–400.

Van den Ende, W. and Valluru, R. (2009) Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging. *J. Exp. Bot*. 60: 9–18.

Wang, Y., Dai, Y., Tao, X., Wang, J.Z., Cheng, H.Y., Yang, H., et al. (2016a) Heat shock factor genes of tall fescue and perennial ryegrass in response to temperature stress by RNA-seq analysis. *Front. Plant Sci*. 6: 1226.

Wang, Y., Liu, H., Wang, S., Li, H. and Xin, Q. (2016b) Overexpression of a common wheat gene *GALACTINOL SYNTHASE3* enhances tolerance to zinc in *Arabidopsis* and rice through the modulation of reactive oxygen species production. *Plant Mol. Biol. Rep.* 34: 794–806.

Xiong, H., Yu, J., Miao, J., Li, J., Zhang, H., Wang, X., et al. (2018) Natural variation in OsLG3 increases drought tolerance in rice by inducing ROS scavenging. *Plant Physiol.* 178: 451–467.

Xue, H., Chen, X. and Li, G. (2007) Involvement of phospholipid signaling in plant growth and hormone effects. *Curr. Opin. Plant Biol.* 10: 483–489.

Yin, Y., Chen, H., Hahn, M.G., Mohnen, D. and Xu, Y. (2010) Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 8. *Plant Physiol.* 153: 1729–1746.

Zhang, M., Su, J., Zhang, Y., Xu, J. and Zhang, S. (2018) Conveying endogenous and exogenous signals: MAPK cascades in plant growth and defense. *Curr. Opin. Plant Biol.* 45: 1–10.

Zhao, T.-Y., Martin, D., Meeley, R.B. and Downie, B. (2004) Expression of the maize *GALACTINOL SYNTHASE* gene family: (II) Kernel abscission, environmental stress and *myo*-inositol influences accumulation of transcript in developing seeds and callus cells. *Physiol. Plant.* 121: 647–655.

Zuñiga, G.E., Alberdi, M. and Corcuera, L.J. (1996) Non-structural carbohydrates in *Deschampsia antarctica* Desv. from South Shetland Islands, Maritime Antarctic. *Environ. Exp. Bot.* 36: 393–399.

Zúñiga-Feest, A., Inostroza, P., Vega, M., Bravo, L.A. and Corcuera, L.J. (2003) Sugars and enzyme activity in the grass *Deschampsia antarctica*. *Antartic. Sci.* 15: 483–491.

Zuther, E., Büchel, K., Hundertmark, M., Stitt, M., Hincha, D.K. and Heyer, A. G. (2004) The role of raffinose in the cold acclimation of *Arabidopsis thaliana*. *FEBS Lett.* 576: 169–173.