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Stress-Inducible Galactinol Synthase of Chickpea (CaGolS) is Implicated in Heat and Oxidative Stress Tolerance Through Reducing Stress-Induced Excessive Reactive Oxygen Species Accumulation

Prafull Salvi, Nitin Uttam Kamble and Manoj Majee*

MM LAB, National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India
*Corresponding author: E-mail, manojmajee@nipgr.ac.in; Fax, +91-11-26741658.
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Raffinose family oligosaccharides (RFOs) participate in various aspects of plant physiology, and galactinol synthase (GolS; EC 2.4.1.123) catalyzes the key step of RFO biosynthesis. Stress-induced accumulation of RFOs, in particular galactinol and raffinose, has been reported in a few plants; however, their precise role and mechanistic insight in stress adaptation remain elusive. In the present study, we have shown that the GolS activity as well as galactinol and raffinose content are significantly increased in response to various abiotic stresses in chickpea. Transcriptional analysis indicated that the *CaGolS1* and *CaGolS2* genes are induced in response to different abiotic stresses. Interestingly, heat and oxidative stress preferentially induce *CaGolS1* over *CaGolS2*. In silco analysis revealed several common yet distinct *cis*-acting regulatory elements in their 5′-upstream regulatory sequences. Further, in vitro biochemical analysis revealed that the CaGolS1 enzyme functions better in stressful conditions than the CaGolS2 enzyme. Finally, Arabidopsis transgenic plants constitutively overexpressing *CaGolS1* or *CaGolS2* exhibit not only significantly increased galactinol but also raffinose content, and display better growth responses than wild-type or vector control plants when exposed to heat and oxidative stress. Further, improved tolerance of transgenic lines is associated with reduced accumulation of reactive oxygen species (ROS) and consequent lipid peroxidation as compared with control plants. Collectively, our data imply that GolS enzyme activity and consequent galactinol and raffinose content are significantly increased in response to stresses to mitigate stress-induced growth inhibition by restricting excessive ROS accumulation and consequent lipid peroxidation in plants.

Keywords: Abiotic stress • Chickpea • Galactinol • Galactinol synthase • RFO • ROS.

Abbreviations: DAB, 3,3′-diaminobenzidine; GC-FID, gas chromatography flame ionization detector; GolS, galactinol synthase; MDA, malondialdehyde; MS, Murashige and Skoog; NBT, nitro blue tetrazolium; RFO, raffinose family oligosaccharides; ROS, reactive oxygen species; TCA, trichloroacetic acid.

Introduction

The biosynthesis of raffinose family oligosaccharides (RFOs) is a highly specialized metabolic event in flowering plants and is implicated in diverse physiological and developmental processes in plants (Saravitz et al. 1987, Peterbauer and Richter 2001, Downie et al. 2003, Sengupta et al. 2015). Galactinol synthase (GolS; EC 2.4.1.123), a member of the glycosyltransferase 8 (GT8) family, is a key regulatory enzyme of RFO biosynthesis that catalyzes the formation of galactinol (1-*O*-α-D galactopyranosyl-L-*myo*-inositol) from UDP-galactose and *myo*-inositol (Bachmann et al. 1994, Loewus and Murthy 2000). Subsequently, this free galactinol supplies an activated galactosyl moiety to generate series of RFOs including raffinose, stachyose, verbascose and ajugose (Lehle and Tanner 1973, Bachmann et al. 1994). These RFOs perform several physiological and developmental functions in plants. For example, RFOs are major transport carbohydrates in members of numerous families, including Cucarbitaceae, Lamiaceae etc. RFOs are also shown to be highly accumulated in the late stage of seed development and have been reported to contribute to seed desiccation tolerance, seed vigor and longevity (Saravitz et al. 1987, Castillo et al. 1990, Downie et al. 2003, Salvi et al. 2016, de Souza Vidigal et al. 2016). In addition, RFOs are known to act as signaling molecules particularly upon pathogen attack and wounding (Kim et al. 2008, Valluru and Van den Ende 2011, ElSayed et al. 2014). Furthermore, RFOs, primarily galactinol and raffinose, are accumulated in many plant species in response to various environmental stresses, suggesting their protective role in surviving in adverse environmental conditions (Taji et al. 2002, Nishizawa et al. 2006, Nishizawa-Yokoi et al. 2008). However, a low RFO concentration in seeds is more desirable for the human diet because these sugars are indigestible in humans and monogastric animals (Wang et al. 2003). Genes encoding RFO biosynthetic enzymes, particularly GolS, have been characterized from various plant species (Lehle and Tanner 1973, Smith et al. 1991, Peterbauer et al. 2002, Taji et al. 2002, Nishizawa et al. 2008, Kim et al. 2011, Pillet et al. 2012, Unda et al. 2012, Zhou et al. 2012). GolS enzymes are usually encoded by multiple genes which are developmentally and

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spatially regulated. Differential expression of two GolS isoforms has been reported in developing lentil seeds (Kannan et al. 2016). In Arabidopsis, GolS enzymes are encoded by seven genes which exhibit differential expression in response to abiotic stresses. *AtGolS1* is induced by heat (Panikulangara et al. 2004, Nishizawa et al. 2006, Nishizawa-Yokoi et al. 2009), salinity and drought stress, while *AtGolS2* is induced only in drought and salinity stress (Taji et al. 2002). Interestingly, *AtGolS3* is induced exclusively under cold stress (Taji et al. 2002). It has also been reported that transcription of cold-inducible *GolS* genes of bugleweed (*Ajuga reptans*) is spatially regulated, resulting in formation of two different pools for storage and transport of RFOs (Sprenger and Keller 2000). An increase in the synthesis of galactinol and raffinose, as a consequence of co-ordinate transcriptional induction of GolS-coding genes in response to various stresses, has also been reported in, for example, *Arabidopsis thaliana*, *Boea hygrometrica*, *Pisum sativum*, *Vitis vinifera* and *Medicago falcate* (Taji et al. 2002, Nishizawa et al. 2008, Pillet et al. 2012, Zhuo et al. 2013, Lahuta et al. 2014). Furthermore, ectopic overexpression of *GolS* results in improved stress tolerance in plants (Taji et al. 2002, Nishizawa et al. 2008, Sun et al. 2013, Gu et al. 2016, Wang et al. 2016). However, the mechanistic insight and precise role of GolS in plant stress tolerance have not been well explored so far.

GolS and RFO metabolism in chickpea seeds have been previously studied, and a significant positive correlation between substrate and product concentration of RFO biosynthesis has been reported. Further, RFO content in chickpea seeds was shown to be affected by genotype as well as by environment (Gangola et al. 2013). In addition, GolS activity was also shown to influence the RFO accumulation in developing chickpea seeds (Gangola et al. 2016, Salvi et al. 2016). Very recently, the role of GolS in maintaining seed vigor and longevity in chickpea has been demonstrated (Salvi et al. 2016); however, the potential role of GolS in abiotic stress tolerance in chickpea has not been studied so far. Interestingly, *myo*-inositol which is utilized to form galactinol is significantly increased under dehydration stress in chickpea. However, the accumulation pattern of RFOs and regulation of GolS, and their precise role in response to abiotic stresses remains unknown in this crop plant. Chickpea is an annual self-pollinated moderately drought-tolerant legume crop plant. Chickpea is considered as a major source of protein-rich food for developing countries; however, unfortunately, the productivity of this grain legume is adversely affected by environmental stresses. Despite being a major crop of importance, research in chickpea is rather limited, possibly because of the lack of an efficient and dependable transformation protocol and non-availability of mutant resources.

In this study, we analyzed stress-induced up-regulation of CaGolS activity and consequent galactinol and raffinose accumulation in chickpea. Further, GolS-encoding genes (*CaGolS1* and *CaGolS2*) were found to be co-ordinately induced in response to abiotic stresses and encode enzymes with differential activity in stressful conditions. To evaluate the potential role of these two genes in plant stress tolerance, transgenic *A. thaliana* Col-0 plants overexpressing *CaGolS1* and *CaGolS2* were generated and their growth responses were analyzed under various stress conditions. Furthermore, the molecular basis of GolS-mediated improvement of stress tolerance has also been explored.

Results

GolS activity, and galactinol and raffinose content are significantly increased in chickpea in response to heat and oxidative stress

To investigate the role of GolS in abiotic stress tolerance in chickpea, initially GolS activity was examined in chickpea seedlings challenged or not with various stresses including salt, drought, cold, high temperature and oxidative stress. Subsequently, total crude proteins were extracted from each sample and GolS activity was analyzed. As shown in **Fig. 1A**, GolS activity was found to be differentially up-regulated in response to various abiotic stresses. The highest up-regulation of GolS activity was observed in chickpea seedlings challenged with heat stress where approximately 2.5-fold more activity than control was observed. Significant up-regulation (~2-fold) of GolS activity was also observed in response to oxidative stress. A considerable increase in activity was also observed in seedlings challenged with salinity, cold and dehydration stress. These results essentially indicate a possible role for GolS in stress tolerance in chickpea. GolS is known to be a key regulatory enzyme of RFO biosynthesis. Hence, we were also interested to analyze the content of galactinol, raffinose and the substrate molecule *myo*-inositol in chickpea seedlings challenged or not with these stresses.

Galactinol, raffinose and *myo*-inositol content were quantified in these stress-treated and untreated seedlings as described in the Materials and Methods. In normally grown chickpea seedlings, galactinol content was found in the range of 0.09–0.11 mg g^{-1} FW. However, similar to GolS activity, galactinol content was significantly increased up to 0.45 mg g^{-1} FW in seedlings challenged with heat stress. A significant increase in galactinol was also found in response to oxidative stress (0.2–0.3 mg g^{-1} FW). Like galactinol, raffinose content was also significantly increased in response to heat and oxidative stress compared with normally grown seedlings. In response to heat stress, raffinose content was found to reach as high as 1.5 mg g^{-1} FW, while normally grown chickpea seedlings contained 0.2–0.3 mg g^{-1} FW. Interestingly, only *myo*-inositol, but not galactinol and raffinose, content was significantly increased under dehydration stress (**Fig. 1B**).

These results suggest that GolS possibly plays an important role in accumulating RFOs and is likely to play a key regulatory role in abiotic stress tolerance.

CaGolS1 and *CaGolS2* are co-ordinately induced in response to abiotic stresses

In our previous experiment, GolS activity was found to increase under various environmental stress conditions (**Fig. 1A**). To understand the regulation of differential GolS activity in response to various abiotic stresses, we further investigated *CaGolS1* and *CaGolS2* transcript accumulation through

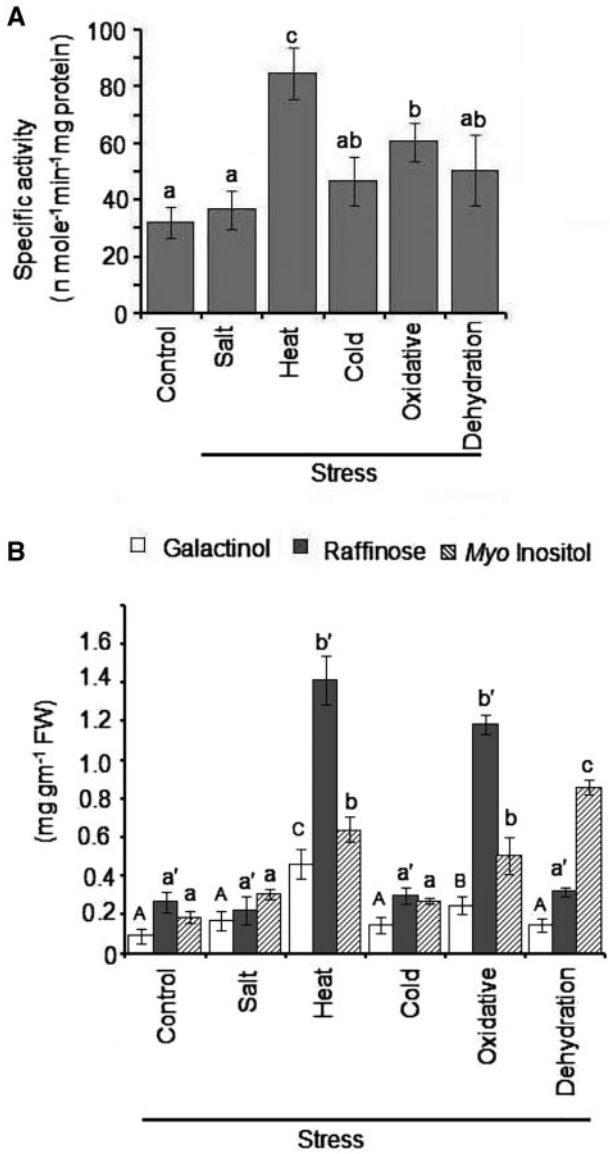

Fig. 1 (A) Galactinol synthase (CaGolS) enzyme activity was assayed in 7-day-old chickpea seedlings under various abiotic stress conditions. A 50 μg aliquot of crude protein was used in the assay. Specific activity was calculated as nmol Pi released mg^{-1} protein min^{-1}. In each case, values are the mean ± SD of three replicates. Significant differences among means ($\alpha = 0.01$) are denoted by different letters. (B) Accumulation of galactinol, raffinose and *myo*-inositol content in chickpea during different stress treatments. Seven-day-old chickpea seedlings were challenged with different abiotic stresses followed by isolation of polar metabolites. Metabolites were derivatized and quantified using GC-FID analysis. Significant differences among means ($\alpha = 0.01$) are denoted by the different letters (*myo*-inositol in lower case letters, galactinol in upper case letters and raffinose in lower case letters with a prime).

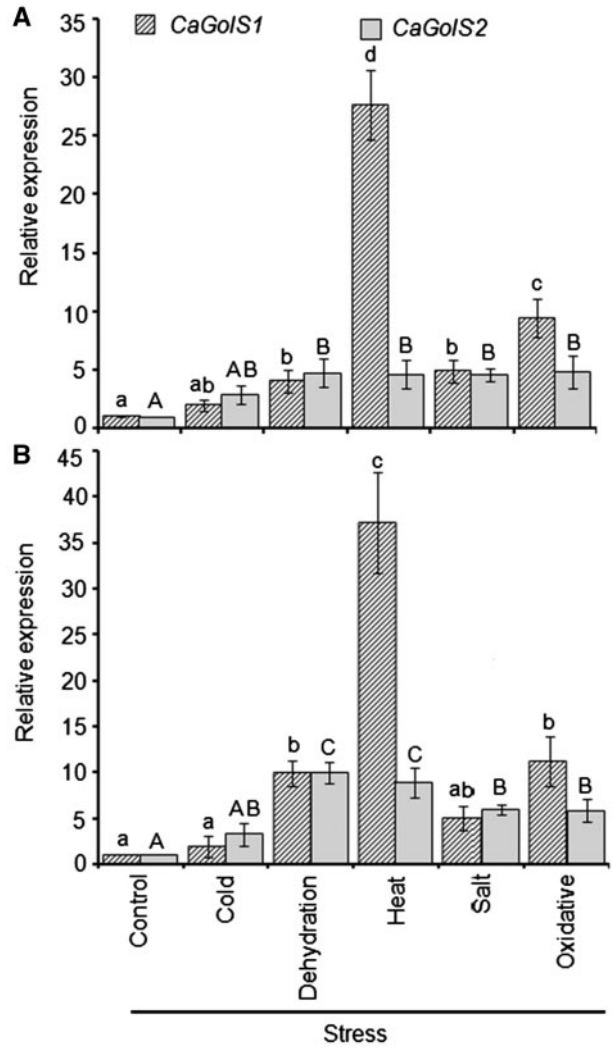

Fig. 2 Quantitative real-time PCR analysis showing relative expression of *CaGolS1* and *CaGolS2* under control and different abiotic stress conditions. The expression of *CaGolS* mRNA was normalized using two endogenous controls, *18S RNA* (A) and *EF1α* (B), and calculated using the ΔΔCT method. Three biological replicates for each sample and three technical replicates for each biological replicate were analyzed by real-time PCR. Error bars indicate the SD. Significant differences among means ($\alpha = 0.01$) are denoted by different letters.

quantitative real-time PCR in chickpea seedlings challenged with cold, salt, dehydration, heat and oxidative stresses. For quantitative real-time PCR analysis, we used two different endogenous controls (*EF1α* and *18S rRNA*) for normalization yet yielded similar results (**Fig. 2A, B**). We observed that *CaGolS1* and *CaGolS2* were induced in response to different abiotic stresses. Increased accumulation of *CaGolS1* and *CaGolS2* transcript was observed under all stress conditions though maximum accumulation was found with heat and oxidative stress (**Fig. 2A, B**). Interestingly under heat and oxidative stress, chickpea seedlings accumulated significantly more *CaGolS1* transcript (25- to 30-fold) than *CaGolS2* (3- to 5-fold), indicating that heat and oxidative stress preferentially induce *CaGolS1* over *CaGolS2*. This result indicates that increased GolS activity is possibly a consequence of transcriptional induction of these two genes under stress conditions.

To understand the molecular basis of differential expression of *CaGolS*(s) in response to abiotic stresses, we analyzed the upstream regulatory regions of these genes. The 5′-upstream

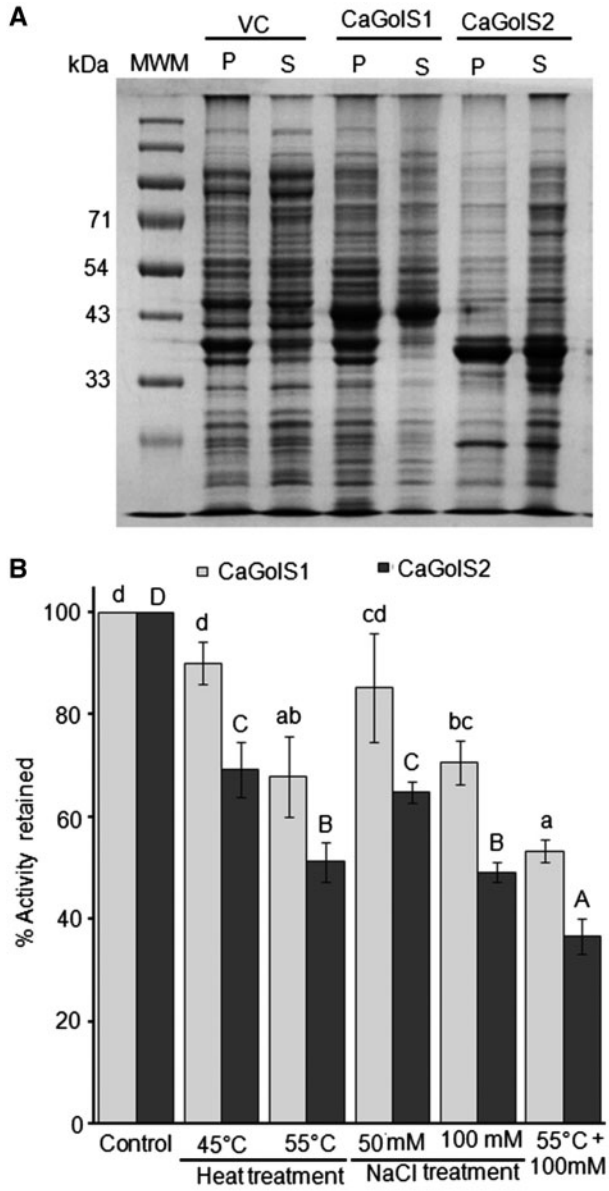

Fig. 3 (A) SDS–PAGE analysis of CaGolS1 and CaGolS2 overexpressed in *E. coli*. The proteins were electrophoresed on a 12% acrylamide gel and stained with Coomassie Brilliant Blue R-250. (VC, pET23b empty vector transformed induced cells; MWM, molecular weight marker; P, pellet fraction; S, soluble fraction). (B) Effect of heat and NaCl on CaGolS1 and CaGolS2 enzyme activity. Approximately 5 µg of purified protein was assayed in each case. CaGolS1 and CaGolS2 purified proteins were pre-incubated under high temperature (45 and 55 °C) and high salt conditions (50 and 100 mM) for 60 min. Error bars indicate the SD from three independent experiments. Significant differences among means ($\alpha = 0.01$) are denoted by different letters.

regulatory regions of *CaGolS1* and *CaGolS2* were retrieved from the chickpea genome database (legumeinfo.org/organism/Cicer/arietinum) and sequences were analyzed using the plantCARE database to identify the *cis*-acting regulatory elements. The nucleotide sequences of the 5′-upstream regions of *CaGolS1* and *CaGolS2* with selected *cis*-regulatory sequences have been presented in Supplementary Fig. S1 and Supplementary Table S1. Sequence analysis revealed that the 5′-upstream regulatory regions of each gene were divergent, suggesting a different transcriptional regulatory mechanism. Interestingly, abiotic stress-responsive *cis*-acting elements such as ABRE (ACGTGG/TC), DRE (ACCGACAT), heat stress-responsive element (AAAAAATTT) and a few other *cis*-regulatory elements such as the E-box element (CANNTG) are found in both the *CaGolS1* and *CaGolS2* promoter. However, the number and position of these abiotic stress-responsive elements of these promoters are different. Interestingly, W box (TTG ACC/T) and defense- and stress-responsive elements (TTAAGA AAAT) are only present in the *CaGolS1* promoter, while pathogen-responsive element/salicylic acid-responsive elements (GA GAAAAATA), elicitor-responsive elements (TAAAATAC) and methyl jasmonic acid-responsive elements (CGTCA) are enriched in the *CaGolS2* promoter region.

CaGolS1 and CaGolS2 enzymes respond differently to high temperature and salt

Since heat and oxidative stress preferentially induce *CaGolS1* over *CaGolS2*, and CaGolS1 and CaGolS2 exhibit considerable differences (72% identity) in their amino acid sequences (Salvi et al. 2016) (Supplementary Fig. S2), we were interested to check whether CaGolS1 protein can function better in stressful conditions than CaGolS2. To test this, we have analyzed their enzyme activities in the presence of destabilizing factors such as high temperature and salt. Initially, we overexpressed CaGolS1 and CaGolS2 proteins in *Escherichia coli* and purified the recombinant proteins to near homogeneity through a His trap Ni-NTA column as described previously (**Fig. 3A**; Supplementary Fig. S3) (Salvi et al. 2016). Purified recombinant CaGolS1 and CaGolS2 proteins were used to examine their enzymatic activities under a stressful environment in in vitro conditions. Our results revealed that the catalytic activity of CaGolS1 and CaGolS2 differed noticeably in the presence of high temperature and salt concentration (**Fig. 3B**). As compared with CaGolS2, CaGolS1 was found to retain significantly more activity after the exposure to high temperature (45 and 55 °C), at high salt concentration (50 and 100 mM NaCl) or in the presence of both high temperature and high salt concentration. Even after the combination of 55 °C and 100 mM NaCl treatment, CaGolS1 was able to retain >50% activity while CaGolS2 could retain only about 35% activity (**Fig. 3B**).

Based on this experimental evidence, our data clearly suggest that CaGolS1 activity was considerably less affected by stressful environments while CaGolS2 activity was more affected in similar conditions.

Ectopic expression of CaGolS(s) in plants results in increased galactinol and raffinose content and enhanced tolerance to heat and oxidative stress

In our earlier experiments, we observed that GolS of RFO biosynthesis is significantly up-regulated in response to heat and oxidative stress and consequently galactinol and raffinose content are also markedly increased under such stress conditions. Hence, we were interested in investigating the potential role of

GolS in mitigating the adverse effect of abiotic stresses in plant. To investigate this, *CaGolS1* and *CaGolS2* were constitutively overexpressed in *A. thaliana* using the *Cauliflower mosaic virus* (CaMV) 35S promoter. *CaGolS1* and *CaGolS2* were transformed to *A. thaliana* through the floral dip method, and transformed plants were initially selected for basta resistance. Finally, *CaGolS1* and *CaGolS2* transformed lines were confirmed by the PCR analysis using the respective gene-specific primers, and homozygous lines for *CaGolS1* and *CaGolS2* were subsequently identified in the T_3 generation. Overaccumulation of the respective transcript was also confirmed in several independent homozygous lines in the T_3 generation (Supplementary Fig. S4). Subsequently, galactinol, raffinose and *myo*-inositol contents in these selected transgenic lines were analyzed. Significantly increased accumulation of galactinol as well as raffinose was observed in *CaGolS1* and *CaGolS2* transformed lines compared with the wild type or vector-transformed lines (**Fig. 4A**). Next to evaluate the stress tolerance ability of *CaGolS* overexpression lines, *CaGolS1* and *CaGolS2* transgenic plants along with wild-type and vector control plants at both the seedling and mature stage were subjected to heat and oxidative stress, and growth responses of these transgenic lines and control lines were compared. There was no significant difference detected in the growth pattern of control plants and transgenic lines until they were challenged by various abiotic stresses. Under normal growth conditions, wild-type, vector control, *CaGolS1* and *CaGolS2* transgenic lines were comparable in their overall growth both at the seedling and at the mature stage. However, under both heat stress and oxidative stress, growth of the wild-type and vector control plants was severely inhibited (**Fig. 4B, C**). Under both these stress conditions, control line seedlings turned yellow and exhibited reduced growth. Under heat stress, the root growth of seedlings was significantly reduced. In contrast to this, *CaGolS1*- and *CaGolS2*-overexpressing seedlings remained green and healthy, and root growth was less inhibited. Overall, *CaGolS1* and *CaGolS2* transgenic seedlings showed improved growth as compared with wild-type and vector control lines under heat and oxidative stress conditions. Subsequently, growth performances of these lines were also assessed in soil-grown mature plants. For this purpose, 4-week-old soil-grown mature plants were subjected to heat and oxidative stress as described in the Materials and Methods. As expected, the growth of the wild type and vector control lines was severely affected by heat and oxidative stress. The wild type and vector control lines exhibited chlorotic symptoms in their leaves and stunted shoot growth. In contrast to this, *CaGolS1* and *CaGolS2* transformed lines were relatively less affected as both these transgenic lines showed improved vegetative growth with fewer chlorotic symptom in their leaves. Reproductive growth of these transgenic lines was less affected than the wild type or vector-transformed lines. Interestingly, the *CaGolS1* lines showed a slightly better growth response than *CaGolS2* lines (**Fig. 4B, C**). Galactinol, raffinose and *myo*-inositol contents in these selected transgenic lines were also analyzed after stress treatment. Significantly increased accumulation of galactinol as well as raffinose was observed in *CaGolS1* and *CaGolS2* transformed lines compared with the wild type or vector-transformed lines even after stress treatments (Supplementary Fig. S5)

Improved tolerance of *CaGolS1* and *CaGolS2* transgenic lines is associated with reduced ROS accumulation and lipid peroxidation

Abiotic stresses, particularly heat and oxidative stresses, are known to induce reactive oxygen species (ROS) accumulation, and excess ROS accumulation causes cellular damage. Therefore, restricting excess ROS accumulation during stresses is essential to combat ROS-mediated cellular damage. Interestingly, RFOs, particularly galactinol and raffinose, have been shown to act as antioxidative molecules and have the ability to scavenge hydroxyl radicals (Nishizawa et al. 2008). Therefore, to investigate whether improved stress tolerance of *CaGolS1* and *CaGolS2* overexpression lines is the result of restricting stress-induced excess ROS accumulation and consequent lipid peroxidation, we checked ROS content, in particular hydrogen peroxide (H_2O_2) and superoxide (O_2^-), through 3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining, respectively, before and after heat and oxidative stress. The pattern of DAB and NBT staining in seedlings and mature leaves clearly revealed that under heat and oxidative stress conditions, *CaGolS*-overexpressing lines accumulate significantly less H_2O_2 and O_2^- than control lines (**Fig. 5A, B**). This result was further confirmed by the quantitative analysis of H_2O_2 content in these *CaGolS* transgenic and control lines (**Fig. 5C**).

Under heat and stress conditions, the H_2O_2 content of the seedlings was found to increase irrespective of genotypes; however, this increase was much higher and significant in the case of the wild type and vector control lines, while it was less pronounced in overexpression lines. Under heat and oxidative stress conditions, the wild type or vector control accumulated in the range of 0.5–0.6 μmol g^{-1} FW. Noticeably, *CaGolS1* and *CaGolS2* overexpression lines limited the H_2O_2 content in the range of 0.3–0.45 μmol g^{-1} FW (**Fig. 5C**). Further, the malondialdehyde (MDA) content was also analyzed in these genotypes. As expected, under heat and oxidative stress conditions, wild-type and vector control plants accumulated a significantly higher MDA content (6–8 μmol g^{-1} FW) than *CaGolS1* and *CaGolS2* overexpression lines, as MDA accumulation of these *CaGolS1* and *CaGolS2* was found to be limited to 4–5 μmol g^{-1} FW (**Fig. 5D**), indicating that *CaGolS* lines underwent less lipid peroxidation.

Overall, our data clearly showed that *CaGolS1*- and *CaGolS2*-transformed lines accumulate increased galactinol and raffinose content and exhibit improved tolerance to heat and oxidative stress by limiting stress-induced excess ROS accumulation and consequent lipid peroxidation.

Discussion

GolS is a plant-specific enzyme which catalyzes the key regulatory step of RFO biosynthesis in plants. RFOs play a diverse yet significant role in higher plants including seed desiccation

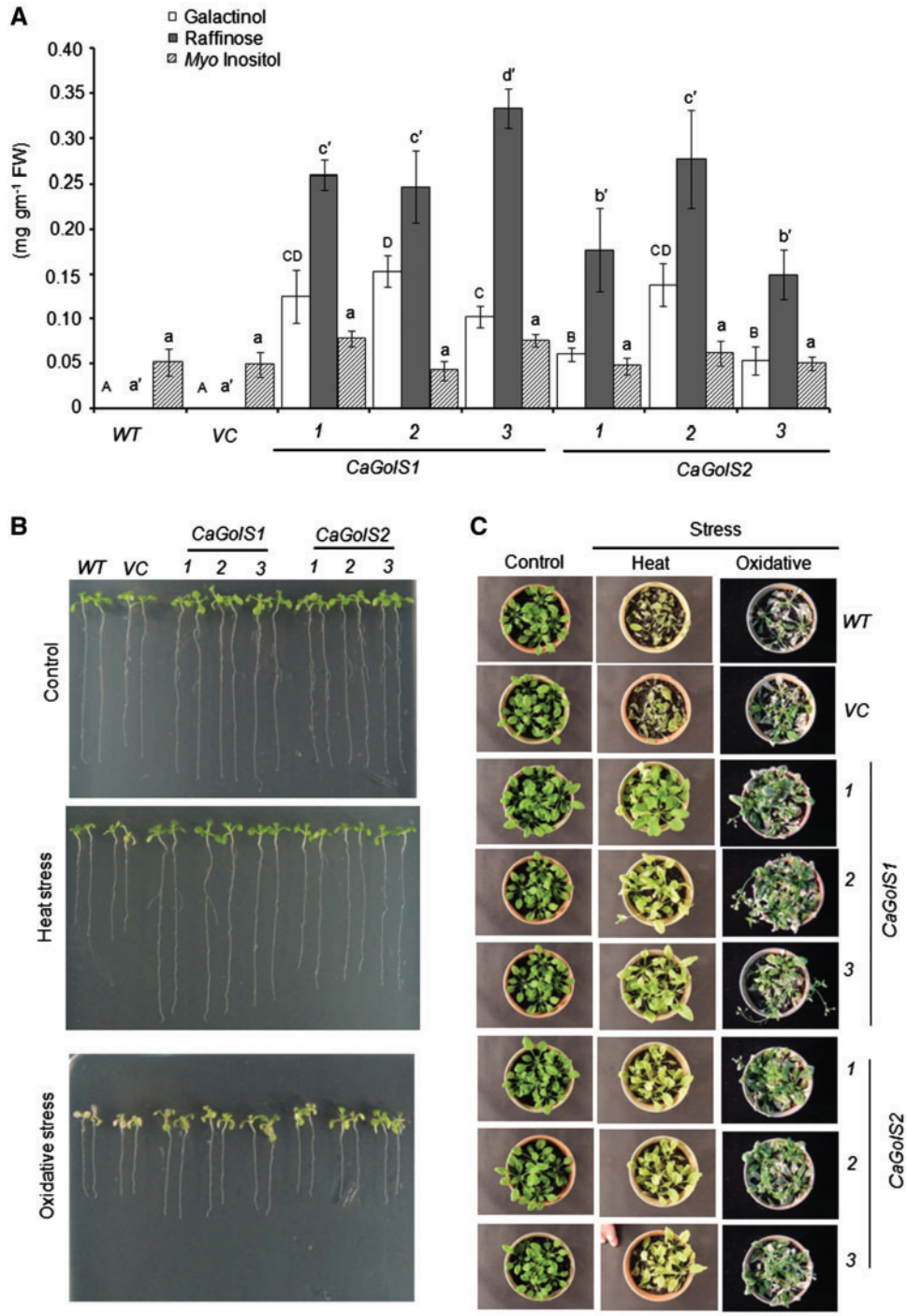

Fig. 4 (A) Galactinol, raffinose and *myo*-inositol contents of transgenic Arabidopsis lines overexpressing *CaGolS1* and *CaGolS2*. Two-week-old seedlings of wild-type, vector control and three independent transgenic lines of *CaGolS1* and *CaGolS2* were used for polar metabolite extraction and GC-FID analysis (WT, wild-type Arabidopsis Col-0; VC, pEG201 vector control; 1, line 4; 2, line 5; and 3, line 6 of 35S:*CaGolS1*; and 1, line 5; 2, line 6; and 3, line 7 of 35S:*CaGolS2*). Data are means ± SD of three biological repeats. Error bars indicate the SD. Significant differences among means ($\alpha = 0.01$) are denoted by different letters. (B) Abiotic stress tolerance in transgenic seedlings overexpressing *CaGolS1* and *CaGolS2*. (A) Images showing 10-day-old seedlings grown on 1/2 MS under control conditions. Seven-day-old Arabidopsis seedling of WT, VC, 35S:*CaGolS1* and 35S:*CaGolS2* lines were transferred to 1/2 MS plates subjected to different abiotic stresses (heat stress, 38 °C; and oxidative stress, 2 μM paraquat) and grown in culture room conditions. The phenotype of seedlings was monitored and photographs were taken. (C) Growth pattern of Arabidopsis mature plants overexpressing *CaGolS1* and *CaGolS2* under heat and oxidative stress. Four-week-old transgenic plants, WT and VC were subjected to heat and oxidative stress. For heat stress, plants were subjected to high temperature stress (38 °C) for 3 d and the growth phenotype of plants was monitored and photographed after 5 d. These experiments were repeated at least three times with similar results, and data from one representative experiment are shown. For oxidative stress, 4-week-old plants were sprayed with 10 μM paraquat alternatively with 0.05% Triton X-100 for 10 d. Stress survivability and growth were monitored and photographed after 7 d. These experiments were repeated at least three times with similar results, and data from one representative experiment are shown.

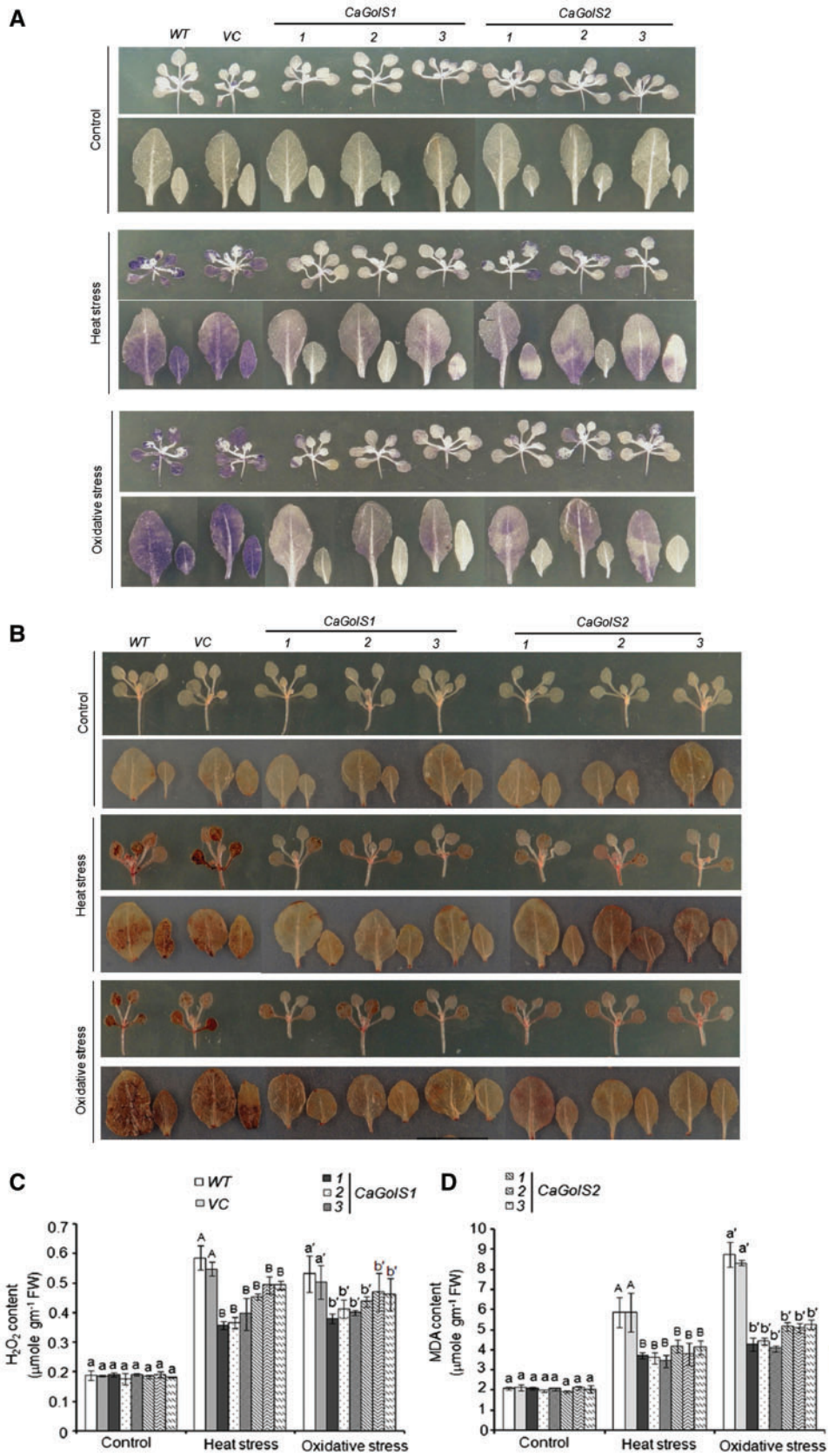

Fig. 5 Comparison of temporal accumulation of reactive oxygen species in transgenic lines and control plants treated with heat and oxidative stress. (A, B) Heat- and oxidative stress-treated seedlings or mature leaves of wild-type and transgenic plants (10-day-old and 6-week-old plants, respectively) were stained with NBT (A) and DAB (B) as described in the Materials and Methods for in situ localization of superoxide anions and H_2O_2. The presence of blue formazan precipitate and dark brown DAB precipitate indicates higher O_2^- or H_2O_2 accumulation, respectively.

tolerance, seed vigor, longevity, storage, transport of carbohydrates and also protection against abiotic and biotic stress (Kim et al. 2008, Unda et al. 2012, Gojlo et al. 2015, Santos et al. 2015, Gu et al. 2016, Salvi et al. 2016, Wang et al. 2016). Previous studies revealed that chickpea possesses two *GolS* genes, and GolS enzyme activity primarily regulates RFO accumulation in developing chickpea seeds (Gangola et al. 2013, Gangola et al. 2016, Salvi et al. 2016). Chickpea plants are known to adapt well in stress conditions particularly water-limited environments, and are a rich source of resistant genes for tolerance of various stresses (Turner et al. 2001). In our present study in chickpea, we showed that GolS activity is significantly up-regulated in response to heat and oxidative stress. Further analysis revealed that galactinol and raffinose content are also markedly increased upon such stresses. It is likely that such increased RFO accumulation during stress conditions is one of several adaptive mechanisms employed in chickpea to combat stresses. Previous studies also indicate that free *myo*-inositol which is utilized to form galactinol also plays a key and regulatory role in RFO synthesis and accumulation in plants (Karner et al. 2004). Furthermore, the initial concentration of substrates (*myo*-inositol and sucrose) and RFO biosynthetic enzyme activities were shown to influence the RFO concentration significantly in chickpea seeds (Gangola et al. 2016). Interestingly, in chickpea, stress-induced accumulation of *myo*-inositol has previously been reported, and inositol metabolism was shown to be implicated in diverse physiological processes including stress tolerance in chickpea (Boominathan et al. 2004, Kaur et al. 2008, Saxena et al. 2013). The increased accumulation of *myo*-inositol and increased GolS activity in chickpea due to stress correlate well with higher accumulation of RFOs under stress conditions. Therefore, inositol and RFO metabolism are likely to be co-ordinately regulated in chickpea, and together play an important role to protect against abiotic stresses. Further studies using high and low RFO chickpea genotypes and determination of their stress tolerance ability would reveal the precise role and contribution of RFO in stress tolerance in chickpea. In our study, we also demonstrated that stress-induced up-regulation of enzyme activity and consequent increase in galactinol and raffinose content in chickpea seedlings are apparently controlled by the co-ordinated transcriptional induction of GolS-coding genes, as both the genes are found to be induced upon abiotic stresses. Furthermore, heat and oxidative stresses preferentially induce *CaGolS1* over *CaGolS2* and thus the former is likely to be primarily responsible for RFO accumulation during heat and oxidative stress. Despite the fact that both promoters contain abiotic stress-responsive elements, *CaGolS1* displayed higher induction under heat and oxidative stress than *CaGolS2*. The different numbers and positions of these abiotic stress-responsive elements or some unknown mechanism are likely to be responsible for higher induction of *CaGolS1* during heat and oxidative stress conditions.

Similar to our study, stress-specific induction of a *GolS* member has been reported in other plant species (Taji et al. 2002, Nishizawa et al. 2006, Nishizawa et al. 2008, dos Santos et al. 2011, Pillet et al. 2012). Interestingly, in the presence of destabilizing agents such as heat and salt, the CaGolS1 protein isoform is catalytically more efficient and active than CaGolS2. This overall higher activity of CaGolS1 in stressful environments suggests that the CaGolS1 enzyme is likely to have evolved to function better in stress physiological conditions. A similar observation was also reported earlier in chickpea where the rate-limiting enzyme MIPS2 (CaMIPS2) in the inositol biosynthetic pathway is structurally more stable and thus catalytically more efficient and robust towards destabilizing agents (Kaur et al. 2008). Based on previous findings and our results, it becomes evident that up-regulation of GolS enzymes leads to an overall increase in RFO accumulation in response to stresses and thus GolS is considered as a potential target to manipulate RFO accumulation in plants. In our study, we observed that ectopic overexpression of *CaGolS* not only increases the galactinol content but also increases the raffinose content. Our functional analysis further revealed that both *CaGolS1* and *CaGolS2* have the ability to improve tolerance to heat and oxidative stress when constitutively overexpressed in *A. thaliana*. Improvement of such stress tolerance is correlated well with increased galactinol and raffinose content in transgenic plants.

It is very well established that abiotic stresses lead to accumulation of excessive ROS which cause cellular damage and subsequent adverse effects on plant growth, development and productivity. It has previously been reported that galactinol and raffinose have the ability to scavenge hydroxyl radicals and can also act as osmoprotectants, and therefore RFOs are likely to play a protective role to restrict excess ROS accumulation. In our study, we observed that improved tolerance to abiotic stresses of *CaGolS* transgenic lines is associated with a reduced level of ROS and consequently reduced lipid peroxidation. Therefore, it is clearly evident that *CaGolS* transgenic lines which accumulate increased galactinol and raffinose content could attain efficient ROS-scavenging ability and improve tolerance to various abiotic stresses. Apart from this, increased galactinol and raffinose can be utilized as an extra source for energy and/or stabilize macromolecules and membranes during stress conditions.

Taken together, our results strongly suggest that GolS(s) are significantly up-regulated to produce RFO in chickpea in response to various abiotic stresses and play an important role in protection against these stresses by restricting excess ROS and consequent cellular damage.

Fig. 5 Continued

The stained samples were then bleached in acetic acid : glycerol : ethanol (1 : 1 : 3, by vol.) prior to taking pictures. The representative picture shown here as captured by using a Nikon Coolpix 510 digital camera. Quantitative analysis of (C) H_2O_2 content and (D) MDA content in seedlings of wild-type (WT), vector control (VC), *CaGolS1* and *CaGolS2* transformed lines subjected to heat and oxidative stress. Data are means ± SD of three biological repeats. Significant differences among means ($\alpha = 0.01$) are denoted by different letters. For (C) and (D), control is shown in lower case letters, heat stress in upper case letters and oxidative stress in lower case letters with a prime.

Therefore, overexpression of GolS in crop plants can be exploited to increase tolerance against abiotic stresses, though targeted overexpression of GolS in the vegetative organs should be the preferred choice, as high concentrations of RFOs in seeds are not desirable for the human diet.

Materials and Methods

Plant material and growth conditions

Cicer arietinum L. cv BGD72 was used in this study and plants were grown as described previously (Kaur et al. 2008). All transgenic plants were generated in the *A. thaliana* ecotype Columbia (Col-0) background. Arabidopsis plants were grown on agar plates or in soil in pots in an Institute growth facility center (Conviron) maintained at 22 ± 2°C with a 16/8 h light (200 μmol m^{-2} s^{-1})/dark cycle after stratification for 3 d at 4 °C (Salvi et al. 2016).

Plasmid construction and transgenic plant generation

Isolation and cloning of *CaGolS1* (accession No. KU189226) and *CaGolS2* (accession No. KU214571) have been described in our previous article (Salvi et al. 2016). *pJET 1.2:CaGolS1* and *pJET:CaGolS2* clones were used for further subcloning in order to make various plasmid constructs in this study.

To obtain the CaGolS-overexpressing transgenic lines, full-length cDNA fragments of *CaGolS1* (1,020 bp) and *CaGolS2* (978 bp) first were subcloned into the entry vector pEDT (Invitrogen) using *pJET 1.2:CaGolS1* and *pJET 1.2:CaGolS2* (Salvi et al. 2016). The resultant positive clones were screened by colony PCR and confirmed by sequencing. Confirmed clones were recombined with the gateway destination vector pEG201 (Invitrogen) using the LR clonase reaction mix (Invitrogen) according to the manufacturer's instructions. The insert in recombinant plasmid pEG201 was verified by restriction digestion and sequencing. The confirmed constructs were initially transformed to *Agrobacterium* strain GV3101 and finally transformed to Arabidopsis using the floral dip method (Clough and Bent 1998). Transformants were selected based on their ability to survive after basta spray (120 mg l^{-1}). Resistant seedlings (green with true leaves) were then transferred to a fresh pot and grown under the conditions described above. T_3 or T_4 homozygous progeny of these transgenic lines were used for all experiments.

Gene expression analysis by quantitative real-time reverse transcription–PCR

Total RNA was extracted from stress-treated 7-day old chickpea seedlings using TRI reagent (Sigma). After DNase I treatment (Ambion/ABI), cDNA was prepared using a cDNA synthesizing kit according to the manufacturer's instructions (Verso, Thermo Scientific). Real-time PCR was performed in triplicate using a SYBR Green Real-Time PCR Master Mix (Agilent) on an ABI Step one real-time PCR using specific primer pairs for *CaGolS* transcripts. Quantification was performed using the ΔΔCT method, and the data were normalized through the quantity of two different endogenous control genes *18S rRNA* and *EF1α*. The sequences of all gene-specific primers are listed in Supplementary Table S2. All reactions were performed in triplicate with three biological replicates.

Metabolite profiling by gas chromatography flame ionization detector (GC-FID)

For GC analysis, standard solutions of *myo*-inositol, galactinol and raffinose of five different concentrations were derivatized. All reagents, solvents and standards used in this study were of GC grade (Sigma). The retention time for each sugar was identified by comparing the peak areas of the spiked samples injected under the same experimental conditions. The same sample was injected three times to evaluate the reproducibility of the retention time. For each sugar, a standard graph was plotted with a known concentration of standards, and linear equations were defined.

Polar metabolites were extracted from stress-treated chickpea seedlings as described by Panikulangara et al. (2004) and dried in a lyophilizer and additionally in a vacuum desiccator at 40–50 °C for 20 min if required before derivatization.

For derivatization, the method described by Agarrwal et al. (2014) was followed. The lyophilized sample was first suspended in 80 μl of methoxyamine hydrochloride (20 mg ml^{-1} pyridine), kept in an incubator shaker at 30 °C for 90 min followed by a 30 min treatment at 37 °C with 80 μl of MSTFA [*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide]. Derivatized samples were centrifuged at 14,000×*g* for 5 min and transferred to a fresh glass vial.

The GC analysis was carried out with a Shimadzu GC-2010 system coupled to a flame ionization detector (FID). The GC system was equipped with a Phenomenex Rxi-1ms GC column (0.25 mm × 30 m × 0.25 μm, Restek Corporation) with nitrogen as carrier gas. Samples were injected in the split mode with a split ratio of 20 : 1. The injector and the FID were operated at 300 °C. The GC was operated with temperature programming (hold at 230°C for 1 min, 230–260 °C at 7 °C min^{-1} followed by a 1 min hold, 260–290 °C at 3 °C min^{-1} and then a 13 min hold) and the amount of sugars present in the sample was calculated based on computer integration of the peak areas in the GC chromatograms.

Protein extraction and GolS assay

Stress-treated chickpea seedlings were ground in liquid nitrogen and suspended in extraction buffer [100 mM HEPES buffer pH 7.5, 1 mM β-mercaptoethanol and a protease inhibitor cocktail (Sigma)] (Smith et al. 1991, Nishizawa-Yokoi et al. 2008) The homogenate was centrifuged for 15 min at 13,000×*g* and the supernatant was used for determination of GolS activity. Protein concentration was estimated using BIO-RAD protein estimation (Bradford 1976). GolS assay was done according to Ribeiro et al. (2000). The reactions containing crude protein (50 μg) along with reaction mixture [60 mM *myo*-inositol, 2 mM dithiothreitol (DTT), 50 mM HEPES buffer (pH 7.0), 4 mM $MnCl_2$, 20 μg of bovine serum albumin (BSA) and 4 mM UDP-galactose] were allowed to proceed for 1 h at 32 °C. After boiling for 2 min to stop the reaction, hydrolysis of UDP-galactose was carried out in Apyrase reaction mixture by incubating it at 37 °C for 10 min. Inorganic phosphate release was detected by addition of Fiske Subbarow reducer (Fiske and Subbarow 1925).

Bacterial overexpression and biochemical analysis

Bacterial overexpression and purification of CaGolS1 and CaGolS2 were carried out as described previously (Salvi et al. 2016). Both recombinant CaGolS1 and CaGolS2 purified proteins were subjected to heat treatment (45 or 55 °C) or salt treatment (50 or 100 mM NaCl) or both for 1 h before being used for GolS assay.

Stress treatments to chickpea

Seven-day-old chickpea seedlings were challenged with various stress treatments. Salt (150 mM NaCl solution), heat (37 °C, in water), dehydration (no water), cold (4 °C, in water) and oxidative stresses (100 μM paraquat solution) with control (in water) were imposed for 5 h (Boominathan et al. 2004). After treatments, the samples were harvested for RNA and protein extraction.

Stress treatment of Arabidopsis seedlings

To study the stress tolerance in the seedling stage, 7-day-old seedlings of the wild type, the 35S vector control and independent homozygous lines overexpressing 35S:*CaGolS1* and 35S:*CaGolS2* grown on half-strength Murashige and Skoog (1/2 MS) medium were transferred to 1/2 MS plates supplemented or not with paraquat (methyl viologen; 2 μM). For heat stress, 7-day-old seedlings were kept at 38 °C for 16 h and then transferred back to normal growth conditions. The growth pattern and stress survivability were assessed for the next 2–3 d and they were photographed. To avoid a size-dependent effect, seedlings or plants of similar size were used and all stress experiments were performed with at least three technical replicates, each containing >20 seedlings per line, and repeated at least three times

Stress treatment to Arabidopsis mature plants

Surface-sterilized transgenic, wild-type Col-0 and vector control seeds were grown for 7 d in 1/2 MS solid medium and transplanted into pots containing

agro peat and vermiculite (3 : 1). For heat stress, 4-week-old plants were kept at 38 °C for 3 d and then transferred back to normal growth conditions in the plant growth chamber (Guan et al. 2013). The plants that survived or continued to grow were photographed. For oxidative stress treatment, 4-week-old plants were sprayed alternatively for 10 d with a solution of 10 μM paraquat and 0.05% Triton X-100 (Verma et al. 2013). The growth pattern and stress survivability were assessed and they were photographed after 7 d. To reduce the effects of possible temperature and/or light variation by position effect, positions of pots were randomly moved within the growth chamber twice a week.

Histochemical detection of ROS (H_2O_2 and O_2^-)

Ten-day-old seedlings or 6-week-old mature leaves of wild-type and transgenic lines were subjected to oxidative stress (2 μM paraquat) or high temperature (38 °C) for 48 h. Subsequently, in situ detection of H_2O_2 was done by treating tissue samples with freshly prepared 1 mg ml^{-1} DAB solution in 50 mM Tris acetate buffer (pH 3.8) as described by Gong et al. (2015) and then incubated at 25°C for 24 h in the dark. For detection of superoxide, NBT was dissolved 10 mM potassium phosphate buffer (pH 7.8) containing 10 mM NaN_3 (Shi et al. 2013). Tissue samples were infiltrated under vacuum with staining solution and incubated for 18 h at room temperature. Stained samples were then bleached in acetic acid : glycerol : ethanol (1 : 1 : 3, by vol.) and photographed.

Estimation of MDA and H_2O_2 content

To calculate MDA content, we used the method described by Heath and Packer (1968). For this, 100 mg of seedlings were ground and homogenized with 2 ml of 0.25% thiobarbituric acid dissolved in 10% trichloroacetic acid (TCA) and heated to 95 °C for 30 min. The reaction mixture was quickly cooled in an ice bath for 10 min and centrifuged at 13,000 × *g* for 30 min. The absorbance of the supernatant was measured at 532 and 600 nm. The non-specific absorbance of the reaction mix at 600 nm was deducted from the absorbance at 532 nm, and the difference was used to calculate the MDA concentration of samples using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Measurement of H_2O_2 wascarried out according to the method described by Saxena et al. (2013). Briefly, H_2O_2 was extracted by homogenizing 100 mg of leaf tissue with 2 ml of 0.1% TCA and was centrifuged at 13,000 g for 20 min at 4°C. A 0.5 ml aliquot of supernatant was mixed with 0.5 ml of 10 mM potassium phosphate buffer of pH 7 and 1 ml of 1 M potassium iodide, and the mixture was then incubated in the dark for 1 h. The optical absorption of the supernatant was measured spectrophotometrically at 390 nm. The H_2O_2 content was determined using a standard curve plotted from known concentrations of H_2O_2.

In silco promoter analysis

The 1.5 kb 5′-upstream regions from the translational start site of *CaGolS1* and *CaGolS2* genes were examined for the presence of *cis*-regulatory elements using an online tool (the plantCARE database). This bioinformatics tools was used to identify some important *cis*-regulatory elements associated with stress expression, seed expression and hormonal regulation.

Statistical analysis

To determine the statistical significance, data were subjected to one-way analysis of variance (ANOVA) using Duncan's multiple range test (DMRT) with SPSS 23.0 statistical software. All data presented in this study were expressed as means ± SD. Letters in the figures show the result of DMRT ($\alpha = 0.01$); different letters refer to significant differences between means.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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