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CONSTITUTIVE OVEREXPRESSION OF THE DELLA-LIKE GENE *ZPR31* FROM *ZYGOPHYLLUM POTANINII* MAXIM SUPPRESSES THE ANTIOXIDANT RESPONSE OF *ARABIDOPSIS THALIANA* UNDER MANNITOL-INDUCED OSMOTIC STRESS

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ABSTRACT

ZpR31, a DELLA-like transcript whose deduced protein shares sequence similarity with GAI1, was previously identified by suppression subtractive hybridization (SSH) as downregulated in mannitol-stressed *Zygophyllum potaninii*. To characterise its function, transgenic Arabidopsis thaliana lines constitutively overexpressing ZpR31 (ZpR31-OE) were generated and, together with wild-type (WT) Col-0 plants, exposed to 200 mM mannitol. After 15 days of treatment, malondialdehyde (MDA) content was markedly higher in two independent ZpR31-OE lines (approximately 23–32 nmol g^{-1}) than in WT (approximately 16.5 nmol g^{-1}), indicating more extensive membrane lipid peroxidation. Superoxide dismutase (SOD) activity increased markedly in stressed WT (from approximately 410 to 515 U g^{-1}), whereas it declined to approximately 210 U g^{-1} in both ZpR31-OE lines. Stem-loop RT-qPCR of six stress-associated microRNAs in ZpR31-OE plants relative to WT revealed transient induction of miR156, miR165, miR398 and miR827 at 24 h, a delayed miR393 peak at 48 h, and sustained miR171 repression. Because miR398 is normally downregulated under oxidative stress to permit induction of Cu/Zn-SOD genes, its induction here suggests a plausible mechanism by which *ZpR31* suppresses the SOD response. These results indicate that *ZpR31* acts as a negative regulator of osmotic-stress tolerance in *Arabidopsis*, consistent with its identification as a stress-downregulated transcript in native *Z. potaninii*.

Keywords: DELLA protein, *ZpR31*, *Zygophyllum potaninii*, *Arabidopsis thaliana*, mannitol stress, superoxide dismutase, microRNA, osmotic stress

INTRODUCTION

DELLA proteins are GRAS-family repressors of gibberellin (GA) signalling that restrain plant growth; when GA is present, the GA-GID1 complex binds DELLA, which is polyubiquitinated by SCF(SLY1/GID2) and degraded by the 26S proteasome [1, 2]. They do not bind DNA directly but act through protein-protein interactions with transcription factors, including regulators of stress and hormone signalling [2]. *Arabidopsis thaliana* has five DELLA proteins (GAI, RGA, RGL1, RGL2 and RGL3), and mutants lacking the N-terminal DELLA domain, such as *gai-1*, cannot be degraded and are GA-insensitive dwarfs [2, 3].

Zygophyllum potaninii Maxim. is a xerophytic, nationally endangered shrub of the Mongolian Gobi [4] that offers an informative resource for drought-tolerance genes. *Zygophyllum* species differ in drought tolerance: under PEG-induced osmotic stress, the most tolerant of three species accumulated the least MDA and reactive oxygen species and showed the highest SOD, POD and CAT activities [5]. In a companion suppression subtractive hybridization (SSH) screen of mannitol-stressed *Z. potaninii*, *ZpR31* was among the transcripts downregulated by the treatment. *ZpR31* encodes a protein with sequence similarity to the DELLA protein GAI1. Stabilised DELLA protein has been reported to enhance abiotic-stress tolerance in *Arabidopsis*, partly through induction of ROS-detoxifying enzymes [6], and DELLA-regulated genes include stress-response genes and the abscisic acid biosynthesis gene *XERICO* [2]; however, DELLA accumulation can also limit tolerance, as *Arabidopsis gai* loss-of-function mutants exhibit greater salt tolerance than wild type, whereas *gid1* triple mutants, which cannot degrade DELLA proteins, are more sensitive [7]. Whether a DELLA-like protein from *Z. potaninii* reproduces this relationship in a heterologous host has not been tested. Here we generated *Arabidopsis* lines overexpressing *ZpR31* and assessed malondialdehyde (MDA) content, superoxide dismutase (SOD) activity and the expression of six stress-associated microRNAs under 200 mM mannitol, in order to test whether *ZpR31* acts as a negative regulator of osmotic-stress tolerance.

MATERIALS AND METHODS

The *ZpR31* coding sequence was amplified from *Z. potaninii* cDNA with *Sal*I/*Eco*RI-adaptor primers, and ligated into the binary vector pRI201-AN (Takara, Japan). The construct was introduced into *Escherichia coli* DH5a and then into *Agrobacterium tumefaciens* GV3101 [8]. ZpR31-overexpressing (ZpR31-OE) *Arabidopsis thaliana* (Col-0) lines were generated by Agrobacterium-mediated floral-dip transformation [9], and transformants were selected on MS medium containing kanamycin (50 µg/mL). Homozygous T3 lines were used for all experiments. Two independent lines (R31-I and R31-II) were used for the MDA and SOD assays, and one representative line for the microRNA analysis.

Surface-sterilized wild-type (WT) and ZpR31-OE seeds were sown on MS medium [10] with 3% sucrose, stratified (4 °C, 2 d) and germinated (12 h/12 h light/dark, 23 °C). Seven days after germination, seedlings were transferred to MS medium with or without 200 mM mannitol. After 15 days of treatment, MDA content and SOD activity were determined in WT and both ZpR31-OE lines using commercial assay kits (Solarbio, China) according to the manufacturer's instructions ($n = 3$). For microRNA analysis, total RNA was extracted with TRIzol reagent (Ambion) and reverse-transcribed using stem-loop primers for miR156, miR165, miR171, miR393, miR398

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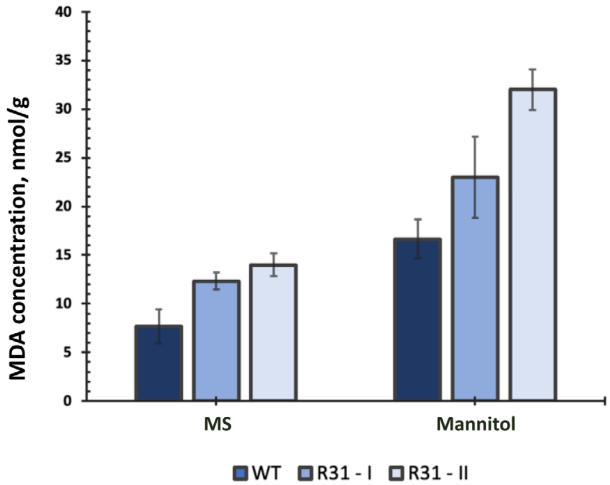


Figure 1. Malondialdehyde (MDA) content in wild-type and two independent ZpR31-overexpressing lines (R31-I, R31-II) under MS and 200 mM mannitol conditions (mean ± SD, n = 3).

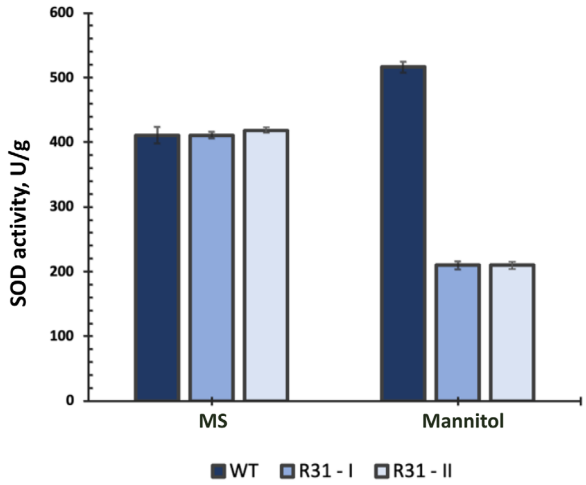


Figure 2. Superoxide dismutase (SOD) activity in wild-type and two independent ZpR31-overexpressing lines under MS and 200 mM mannitol conditions (mean ± SD, n = 3).

and miR827 [11], with *AtActin* as the reference gene. Quantitative PCR (CFX96, Bio-Rad; SYBR Green I) was run in biological and technical triplicate with no-template controls and melt-curve analysis at 24, 48 and 96 h of mannitol treatment. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method, normalised to *AtActin* and expressed relative to WT at each time point. Data are presented as mean ± SD of three biological replicates, and pairwise differences were assessed by Student's *t*-test ($P < 0.05$).

RESULTS

After 15 days of mannitol treatment, MDA content increased to a greater extent in both independent ZpR31-OE lines (R31-I, approximately 12.3 to 23.0; R31-II, approximately 14.0 to 32.0 nmol g^{-1}) than in WT (approximately 7.6 to 16.5 nmol g^{-1}; Fig. 1). SOD activity exhibited the opposite pattern: it was comparable among genotypes under control conditions (approximately 410–420 U g^{-1}) but increased markedly in stressed WT (approximately 515 U g^{-1}), whereas it declined in both ZpR31-OE lines (approximately 210 U g^{-1}; Fig. 2), falling below their respective unstressed baselines.

Stem-loop RT-qPCR of six microRNAs in ZpR31-OE plants relative to WT (Fig. 3) revealed pronounced but transient induction of miR156, miR165, miR398 and miR827 at 24 h of mannitol treatment (up to approximately 28-fold), a delayed maximum for miR393 at 48 h (approximately 6-fold), and sustained repression of miR171 throughout the time course; by 96 h, most differences had returned to near-WT levels.

DISCUSSION

Overexpression of *ZpR31* impaired, rather than enhanced, the osmotic-stress response measures examined here: lipid peroxidation increased, and the stress-induced SOD response observed in WT was largely absent in ZpR31-OE lines. Under oxidative stress, miR398 is normally downregulated to permit induction of the Cu/Zn-SOD genes *CSD1* and *CSD2* [12]; its induction here, rather than repression, represents a plausible post-transcriptional mechanism by which *ZpR31* suppresses the SOD response documented in Fig. 2. The remaining microRNAs point to broader hormonal crosstalk downstream of DELLA-like signalling: miR156 targets *SPL*

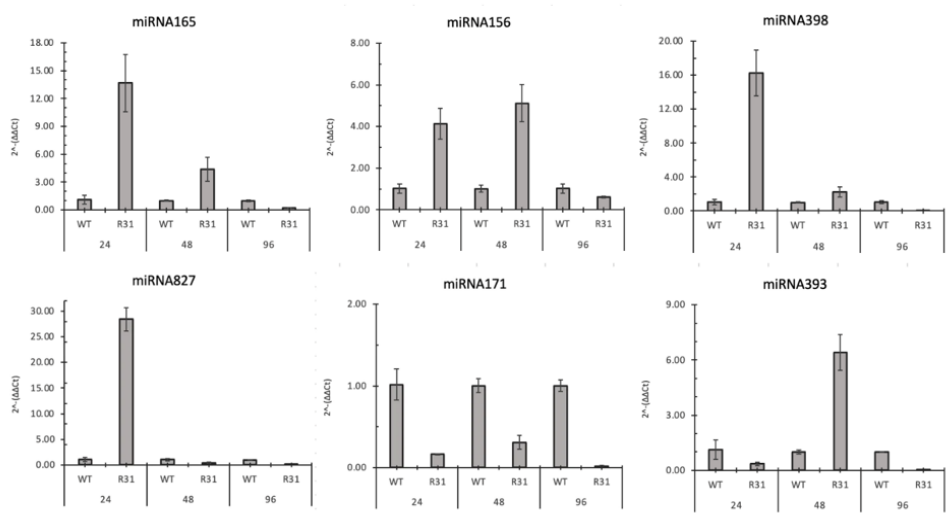


Figure 3. Relative expression ($2^{-\Delta\Delta Ct}$) of six microRNAs in ZpR31-overexpressing Arabidopsis relative to wild-type at 24, 48 and 96 h of 200 mM mannitol treatment (mean ± SD; WT = 1 at each time point).

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transcription factors linked to the GA/DELLA pathway [13], miR165/166 targets HD-ZIP III genes [14], miR393 targets the auxin receptors *TIR1*/*AFB2*/*AFB3* [15], miR827 targets the phosphate-signalling gene *NLA* [16], and miR171 targets GRAS-family SCL genes related to DELLA itself [17].

The deduced ZpR31 protein is similar to GAI-type DELLA proteins, which restrain growth by interacting with transcription factors and other regulators rather than by binding DNA, and whose interaction partners include regulators of stress and hormone responses [2]. Because such partners largely do not discriminate between the different DELLA proteins within a species, and DELLA functional diversity appears to reflect expression pattern more than partner specificity [2], a *Z. potaninii* DELLA-like protein expressed from the 35S promoter could plausibly engage *Arabidopsis* partners and alter hormone- and stress-responsive gene expression, although this was not tested here. Given that DELLA-regulated genes include stress-response genes and the abscisic acid biosynthesis gene *XERICO* [2], the changes in MDA, SOD and microRNA expression may reflect broader changes in hormone signaling rather than a single pathway.

DELLA proteins are more often reported as positive regulators of abiotic-stress tolerance, for example in drought and ROS control [6], but negative effects have also been described: under salt stress in *Arabidopsis*, *gai* loss-of-function mutants exhibited enhanced tolerance, whereas *gid1* triple mutants, which cannot degrade DELLA proteins, showed reduced fresh weight and chlorophyll content [7]. The *ZpR31* result is therefore consistent with published evidence that DELLA accumulation can restrict stress tolerance in a context-dependent manner, although that work concerned salt stress and *Arabidopsis* DELLA mutants rather than osmotic stress and a DELLA-like protein from another species.

CONCLUSION

Constitutive overexpression of *ZpR31* in *Arabidopsis* increased lipid peroxidation and suppressed the stress-induced SOD response under mannitol treatment and altered the expression of six stress-associated microRNAs, most notably inducing miR398. These results support *a role for ZpR31* as a negative regulator of osmotic-stress tolerance, consistent with its downregulation in mannitol-stressed *Z. potaninii* and implicate the miR398-*CSD1*/*CSD2* axis as a candidate mechanism for its effect on antioxidant capacity.

AUTHOR CONTRIBUTIONS

Conceptualization: B.G., B.J., O.D.; Methodology: B.G., B.J., O.D.; Investigation: A.U., M.G., B.J., S.L.; Formal analysis: A.U., M.G.; Writing – original draft: A.U., M.G.; Writing – review & editing: B.G. All authors have read and agreed to the published version of the manuscript.

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