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Overexpression of *Rhizobium rhizogenes* A4-*rolB* enhances osmotic stress resistance in Arabidopsis

Xuefei Chen[1*], Bruno Trevenzoli Favero[1], Fulai Liu[1] and Henrik Lütken[1]

Abstract

Background Functional investigation of individual *Rhizobium rhizogenes* oncogene contributes to a deeper understanding of the mechanisms underlying *R. rhizogenes*-plant transformation, which holds the potential of enhancing plants' abiotic stress resistance. The *root oncogenic locus* (*rol*) *B* gene, a key oncogene of *R. rhizogenes*, was reported to participate in abiotic stress resistance in transformed plants. Yet, the underlying mechanisms remain largely elusive. This study investigated Arabidopsis overexpressing A4-*rolB* (*rolB*-OX) for phenotypic modifications and short-term osmotic stress resistance.

Results Data showed that A4-*rolB* induced pronounced dwarfing phenotypes and weakened root growth in *rolB*-OX as shown by its compact growth and significantly decreased root length and root surface area (59% and 63% of wild type Col-0, respectively) under normal growth conditions. Under polyethylene glycol 6000 (PEG) 10% (w/v)-induced osmotic stress, *rolB*-OX exhibited enhanced resistance to osmotic stress compared with Col-0, as exemplified by less severe leaf wilting, increased total antioxidant capacity (TAC), and a better recovery of stomatal conductance after PEG treatment. Moreover, A4-*rolB* mediated enhancement in abscisic acid (ABA) levels under well-watered conditions, which may have facilitated stress resistance of *rolB*-OX under PEG exposure, probably through inducing TAC. Furthermore, after PEG stress, *rolB*-OX exhibited dramatically up-regulated (3.3–5.7-fold of Col-0) transcript levels of genes encoding plasma membrane intrinsic proteins (*PIPs*) (i.e., *PIP2;5* and *PIP2;7*), which are correlated with an improved plant hydraulic conductivity.

Conclusions This study reports an enhanced osmotic stress resistance in *rolB*-OX, which could be attributed to A4-*rolB*-mediated increase in leaf ABA levels and TAC and improved stomatal regulation. Furthermore, the association between ABA and TAC, and its effect on the osmotic stress resistance caused by *rolB*, was thoroughly discussed in this study. These findings reveal novel physiological effects of A4-*rolB* on plant abiotic stress resistance.

Keywords *Agrobacterium rhizogenes*, Antioxidant defense, Osmotic potential, Polyethylene glycol, Root architecture, Stomatal regulation

Introduction

Rhizobium rhizogenes (syn. *Agrobacterium rhizogenes*) [47, 62], is a soil-born bacterium that, upon infection, transfers and integrates a segment of its root-inducing (Ri) plasmid, known as T-DNA, into the nuclear genome of plant hosts [61]. The transformation can occur naturally in a transient form producing hairy roots from the site of infection and generating entire plants based

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on tissue culture as reviewed by Lütken et al. [37] and Desmet et al. [16]. This *R. rhizogenes*-mediated transformation is excluded from GMO legislation in e.g., the European Union and Japan due to its use of naturally occurring bacterial strains without recombinant DNA [12, 17, 42]. This provides new avenues for developing both ornamental plants and agricultural crops with novel traits, directly applicable to breeders [19, 23, 24, 46]. The Ri-plasmid of agropine-type strains is divided into two regions designated as the left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA), which harbor numerous T-DNA genes, such as the *root oncogenic locus* (*rol*) genes and other less characterized open reading frames (ORFs) [10, 25, 43, 54, 61]. Notably, the T_L-DNA contains the most critical T-DNA genes, i.e. the *rol* genes *rolA*, *rolB*, *rolC,* and *rolD*, which are essential for induction of the hairy root syndrome, while the T_R-DNA primarily comprises the *aux1* and *aux2* genes, which are responsible for auxins biosynthesis as reviewed by Chandra [10].

In recent decades, the effects of T-DNA genes have been widely explored by overexpressing specific individual (e.g. *rolB*) in various plants or plant tissues, such as *Rubia cordifolia* and *Arabidopsis thaliana* [7, 59]. The study of individual genes derived from *R. rhizogenes* is crucial for gaining a deeper understanding of the effects of *R. rhizogenes*-mediated transformation on plants, thus providing a theoretical foundation for the broader application of this biotechnological breeding method. The *rolB* gene is regarded as a key functional determinant of *R. rhizogenes* [22, 39, 55]. Its impact on transformed plants has been extensively documented, with the most prominent findings highlighting the significant phenotypic changes it mediates. For instance, *rolB*-overexpressing plants have demonstrated dwarfism in, e.g., *Arabidopsis thaliana* [30] and *Kalanchoë blossfeldiana* [20]; substantial morphological changes in leaves, flowers, and roots have been observed in *rolB* overexpressing species such as *Glycine max*, *Nicotiana tabacum* and walnut (*Juglans hindsii*×*J. regia*) [9, 57, 67]. Moreover, necrosis symptoms in leaves and roots triggered by *rolB* have also been reported by Schmülling et al. [48] and Kodahl et al. [30]. Furthermore, Sharma et al. [50] concluded that *rolB* effectively induces rooting in various fruit tree species, including kiwi, almond, walnut, cherry, grape, and apple. Morphological changes induced by *rolB* suggest its role in modulating phytohormone levels, given the strong correlation between plant hormone regulation and plant morphology [58]. In support of this view, *rolB* was reported to alter the internal concentrations of, and the sensitivity to, various plant hormones, particularly auxin [15, 51, 55]. Moreover, heterologous expression of the naturally found homolog of *rolB* from *Ipomoea batatas* in *Arabidopsis thaliana* increased the concentrations of salicylic acid (SA) and methyl jasmonate (MeJA), as well as abscisic acid (ABA) levels [52]. Furthermore, phytohormone perturbation triggered by *rolB* explains the identification of *rolB* as the most potent activator of plant secondary metabolites (e.g., resveratrol and anthraquinones) [8, 28, 39, 53], as hormone levels regulate metabolic production [31].

Additionally, *rolB* has been widely reported to be closely associated with acquired resistance to biotic stress in plants. For example, it improved resistance to pathogens such as *Fusarium oxysporum* and *Alternaria solani* in tomato (*Solanum lycopersicum*) by elevating levels of lycopene, phenolic compounds, and ascorbic acid [2]. In addition, *rolB* can also trigger increased resistance to abiotic stress, such as salt resistance in cell cultures of *Rubia cordifolia* [8] and *Panax ginseng* [29], and enhanced temperature stress (both high and low temperatures) resistance in *Solanum lycopersicum* [49], as well as enhanced drought tolerance in *Arabidopsis thaliana* [59]. These stress defenses were generally associated with *rolB*-mediated regulation of expression of genes involved in the reduction of reactive oxygen species (ROS), activation of scavenging enzymes, and modulation of hormone homeostasis, etc. [39, 40]. Recent research has partially elucidated the *rolB*-induced changes in phytohormone and stress resistance by demonstrating that *rolB* interacts with TOPLESS repressor proteins, thereby inducing a specific and partial reprogramming of phytohormone signaling and immunity [22]. Nevertheless, the mechanisms underlying the enhancement of plant stress resistance induced by *rolB* is still unclear.

Stomatal closure, influenced by both rhizospheric and phyllospheric conditions, regulates water loss in plants under water stress as reviewed by Kamrani et al. [27]. Stress-induced ABA signals (e.g. drought) might have contributed to reduced water use and improved water use efficiency in plants via restricting stomatal opening according to Liu et al. [35]. Additionally, the hydraulic transport capacity of plants is regulated by 'water channels' in the plasma membrane, known as aquaporins [1, 26]. Extensive research suggests that the expression of genes encoding aquaporins, specifically the plasma membrane intrinsic proteins (*PIPs*), significantly influences plant hydraulic conductivity, promoting water homeostasis in plants [13, 33, 64]. Collectively, these advanced water stress response strategies involve regulating stomatal activity and hydraulic conductance through ABA levels and the expression of *PIPs*.

In this study, Arabidopsis plants overexpressing the A4-*rolB* gene (*rolB*-OX) were examined for phenotypic changes such as root architecture under normal growth conditions. Moreover, polyethylene glycol (PEG) 6000 exposure, which mimics plant water deficit via its

osmotic effects [60], was used to investigate the effects of A4-*rolB* on plant resistance to short-term osmotic stress. We hypothesized that A4-*rolB* exerts an important role in mediating plant response to PEG-induced osmotic stress through modulating endogenous ABA levels, stomatal regulation, and transcript levels of *PIPs*, thus facilitating stress resistance in *rolB*-OX.

Materials and methods

Plant materials and growth conditions

A4-*rolB,* derived from the agropine-type A4 strain of *R. rhizogenes* was overexpressed under the cauliflower mosaic virus (CaMV) *35S* promoter in *Arabidopsis thaliana*. Arabidopsis lines overexpressing A4-*rolB* (*rolB*-OX) were generated by Kodahl et al. [30] by utilizing wild-type Arabidopsis Columbia-0 ecotype (Col-0, stock code N1092) bought from the European Arabidopsis Stock Centre NASC (https://arabidopsis.info/BasicForm).

In the Arabidopsis lines overexpressing A4-*rolB*, eight independent lines of second-generation (T2) were generated for preliminary characterization (data not shown). Among these T2 lines, one line (*rolB*-OX-2) was selected for seed collection, which had stable and representative compactness features and was characterized by Kodahl et al. [30]. The third-generation plants (T3) grown from *rolB*-OX-2 seeds were used as experimental materials (termed *rolB*-OX in this study) and the wild-type Arabidopsis Col-0 was utilized as control in this study. Plants of both genotypes were initially germinated on standard ½ MS media and subsequently transplanted to pots filled with soil (peat: vermiculite: perlite 1:1:1) after reaching the four-leaf stage. The grown conditions of the climate chamber were established as follows: photosynthetic active radiation of 120 μmol m^{-2} s^{-1}, with temperatures of 22 °C day/20 °C night, and a photoperiod of 16 h.

For the polyethylene glycol (PEG) 6000 treatments, Arabidopsis seedlings (at the four-leaf stage) were transplanted to a hydroponic system and grown in containers containing nutrient solution [34]. The nutrient solution was changed every five days and was continuously aerated using an air compressor. The pH of the nutrition solution was monitored daily and adjusted to 6.0 with 0.1 M HCl or 0.1 M NaOH. The growth conditions of the chamber were the same as described above for pot-grown plants.

Polyethylene glycol 6000 treatments

After 5 weeks of seed germination, *rolB*-OX and Col-0 plants were subjected to 10% (w/v) PEG 6000 solution (termed PEG treatment in this study) that induced − 0.16 MPa level of short-term osmotic stress [41]. Plants were monitored, respectively, at 0, 2, 4, 6, and 24 h (expressed as P0, P2, P4, P6, and P24), and stressed plants were then transferred back to PEG-free nutrient solution and monitored, respectively, at 2, 4, 6, and 24 h (expressed as R2, R4, R6, and R24) as short-term recovery. The experiment was repeated two times displaced in time.

Root architecture characterization

Root architecture characterization was conducted by harvesting plants (grown in the hydroponic system for five weeks) under well-watered conditions for scanning. Fresh roots from four plants in each line were sampled and scanned using a root scanner (Epson Expression 12000XL, USA). The images were subsequently analyzed using WinRHIZO Pro (Version 2009c, 32-bit) software to measure root length (cm) and root surface area (cm^2).

Abscisic acid concentrations and total antioxidant capacity measurement

ABA concentrations were measured using ELISA (Enzyme-Linked Immuno Sorbent Assay) on fully expanded upper leaves from four plants per line, following the protocol of Asch [4]. The total antioxidant capacity (TAC) [nmol μL^{-1} fresh weight (FW)] was measured using the Total Antioxidant Capacity Assay Kit MAK187 (Sigma), based on the method described by Fraser et al. [21]. Antioxidant levels in the samples were expressed as Trolox equivalents.

Stomatal conductance measurements

During the PEG treatment, stomatal conductance (g_s, mol m^{-2} s^{-1}) was measured. At each sampling time point (P0, 2, 4, 6, 24; R2, 4, 6, 24), g_s was measured on the fully expanded upper leaves with a portable photosynthetic system LI-COR LI-600 (LI-Cor, USA). At each time point, measurements were conducted on one leaf per plant and four plants for each line.

RNA extraction, cDNA synthesis, and quantitative real-time PCR reactions

RNA was extracted from snap-frozen leaf and root tissues of four plants per line using the RNeasy Plant Mini Kit (Qiagen, Germany) for subsequent cDNA synthesis and quantitative real-time PCR analysis. Total RNA (500 ng) underwent DNase I Amplification Grade treatment (Sigma Aldrich, USA) followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Quantitative real-time PCR (qRT-PCR) was conducted with a SYBR FAST qPCR Kit on a Bio-Rad CFX Connect Real-Time System. *AtActin8* was used as a reference gene for normalizing transcript levels in Arabidopsis according to Chen et al. [11]. Relative gene expression levels were calculated using the comparative CT ($2^{-\Delta\Delta CT}$)

method [36]. qRT-PCR was conducted using two technical replicates and four biological replicates. The specific primers utilized for qRT-PCR are detailed in Table S1.

Statistical Analyses

Statistical analyses were performed using one-way ANOVA (Duncan test) in SPSS 21. Differences among genotypes and treatments were considered statistically significant at $p \leq 0.05$. The bar charts were generated using Origin 2020.

Results

Morphological traits under normal growth conditions

Shoot and root morphology of *rolB*-OX were characterized to investigate the effects triggered by the A4-*rolB*. The *rolB*-OX line exhibited a pronounced dwarf phenotype compared with Col-0 (Fig. 1). In addition, the root length and root surface area were both significantly decreased in *rolB*-OX (790.83 ± 218.49 cm, 37.48 ± 9.61 cm^2) in relation to Col-0 (1343.14 ± 242.28 cm, 59.51 ± 11.49 cm^2) (Fig. 2).

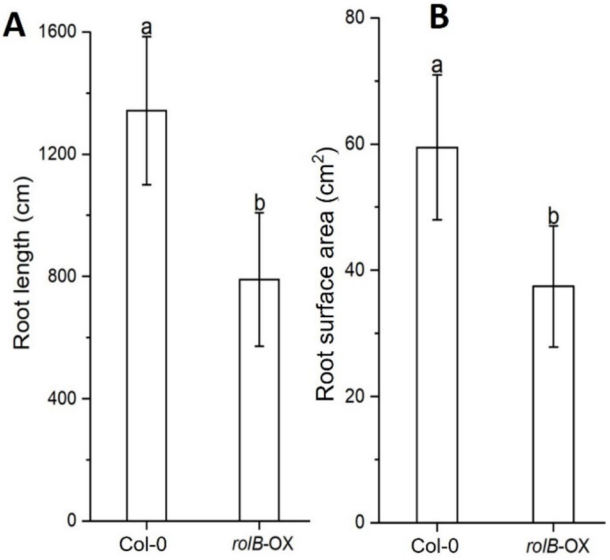

Fig. 2 Root morphology of Arabidopsis (five-week-old). (**A**) Root length. (**B**) Root surface area. Values are mean ± SD ($n = 4$). *rolB*-OX, Arabidopsis plants overexpressing A4-*rolB*. Col-0, wild-type Arabidopsis Columbia-0 ecotype. Different letters on the top of each column indicate statistical significance between genotypes by Duncan test at $p \leq 0.05$ level

Leaf phenotype under PEG treatment

Regarding leaf phenotypes, *rolB*-OX showed no obvious osmotic stress symptoms after 24 h of polyethylene glycol (PEG) treatment (at P24) compared to the Col-0 Arabidopsis. Specifically, the *rolB*-OX plants showed slight wilting only on the leaf edges (highlighted in the red circle), whereas the Col-0 plants exhibited a serious wilting phenotype (Fig. 3A).

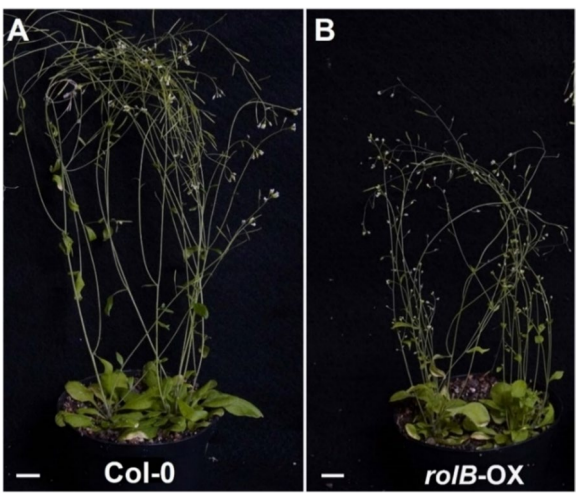

Fig. 1 Phenotypes of Arabidopsis plants (six-week-old). (**A**) Col-0, wild-type Arabidopsis Columbia-0 ecotype. (**B**) *rolB*-OX, Arabidopsis overexpressing A4-*rolB*. Bar = 2 cm

Leaf ABA concentrations during PEG treatment

Leaf ABA concentrations of the *rolB*-OX line were determined to investigate the effects of A4-*rolB* gene on ABA levels before and after PEG stress. Under PEG-free conditions, the average leaf ABA concentration in *rolB*-OX was about 1.88-fold higher than in Col-0. As PEG treatment progressed, the ABA concentration of the *rolB*-OX line remained relatively stable during the whole PEG treatment period compared to Col-0 (Fig. 3B and Table S2). However, the ABA concentrations of Col-0 peaked at P24 (2.20 ± 0.23 μg g^{-1} FW) and fluctuated sharply throughout the period of PEG stress and recovery (Fig. 3B).

Stomatal conductance during PEG treatment

Stomatal conductance (g_s) in *rolB*-OX was determined to investigate the role of A4-*rolB* in regulating stomatal aperture before and after PEG stress. Under PEG-free conditions, the average g_s was slightly lower in *rolB*-OX (0.33 ± 0.04 mol m^{-2} s^{-1}) than in Col-0 (0.36 ± 0.04 mol m^{-2} s^{-1}) (Table S2). As PEG treatment progressed, g_s strongly decreased in both lines within the first 2 h and continued to decline in Col-0 line thereafter even upon the recovery period (Fig. 3C). However, g_s of

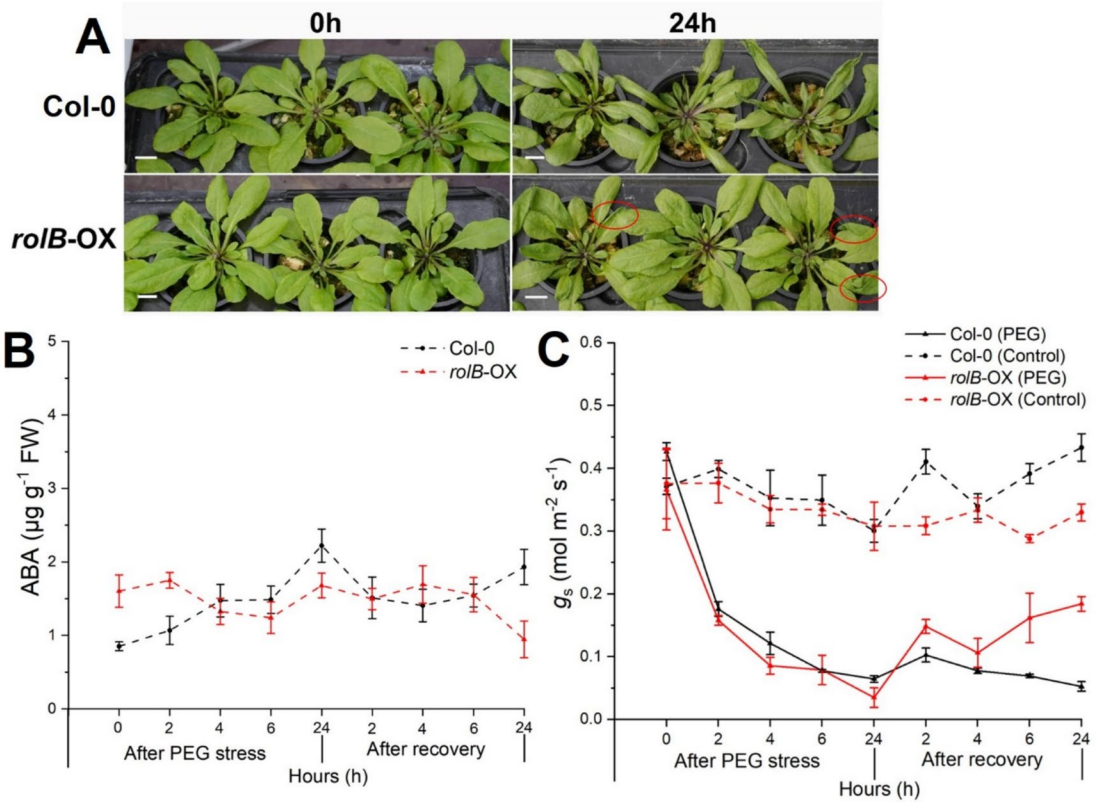

Fig. 3 Leaf phenotypes, ABA concentrations, and stomatal conductance (g_s) of Arabidopsis plants (five-week-old) during 10% polyethylene glycol (PEG) 6000 stress and recovery. (**A**) Leaf phenotypes. (**B**) Leaf ABA concentrations ($n=4$). (**C**) g_s ($n=4$). *rolB*-OX, Arabidopsis plants overexpressing A4-*rolB*. Col-0, wild-type Arabidopsis Columbia-0 ecotype. PEG indicates plants exposed to 10% PEG; Control indicates plants grow without PEG

rolB-OX dropped to 12% of its control plants at P24 and increased consistently upon recovery up to 55% of the controls at R24 (Fig. 3C).

Total antioxidant capacity of leaves under PEG treatment

The TAC in *rolB*-OX leaves was determined to investigate the effects of A4-*rolB* on TAC under PEG exposure (Fig. 4). TAC in *rolB*-OX peaked at P24, being 4.14 ± 0.58 nmol μL^{-1}. However, TAC in Col-0 did not change significantly at P24 compared to that at P0, and significantly increased thereafter, being 3.84 ± 0.59 nmol μL^{-1} at R24 (Fig. 4).

Expression patterns of *PIPs* under PEG treatment

Accumulated evidence indicates that *PIPs* have high impacts on plant hydraulic conductivity [18, 64]. In this study, expression profiles of *PIPs* were analyzed in leaf and root tissues of *rolB*-OX before and after PEG stress, to investigate the potential effect of A4-*rolB* on transcript levels of *PIPs*. In the leaves, the expression levels of *PIPs* (except for *AtPIP1,3* and *AtPIP2;5*) were lower (by 0.4–0.6 folds) in *rolB*-OX compared to Col-0 before PEG exposure (at P0). After PEG treatment, the expression levels of *PIPs* were generally up-regulated in *rolB*-OX, with the exception of *AtPIP1;2* and *AtPIP1;3.* However, in Col-0 plants, only four *PIPs* (*AtPIP1;1*, *AtPIP2;1*, *AtPIP2;2* and *AtPIP2;5*) showed a significant upregulation in expression levels after PEG stress (Fig. 5).

In the roots, before PEG treatment (i.e., at P0), the expressions of *AtPIP1;2*, *AtPIP1;3*, and *AtPIP2;2* were significantly lower by 0.5–0.6 folds in *rolB*-OX than in Col-0. After PEG stress, the transcription levels of *PIPs* (except for *AtPIP1;3* and *AtPIP2;2*) were upregulated in the roots of both genotypes, although the upregulation in *AtPIP2;5* and *AtPIP2;7* was not significant in Col-0. Nevertheless, the observed upregulation became irregular with 24-h stress recovery (Fig. 6). Notably, the expression of *AtPIP2;5* and *AtPIP2;7* was dramatically increased by 24 h of PEG stress in roots of *rolB*-OX, being 5.7- and 3.3-fold, respectively, of those in Col-0 plants (Fig. 6G, H).

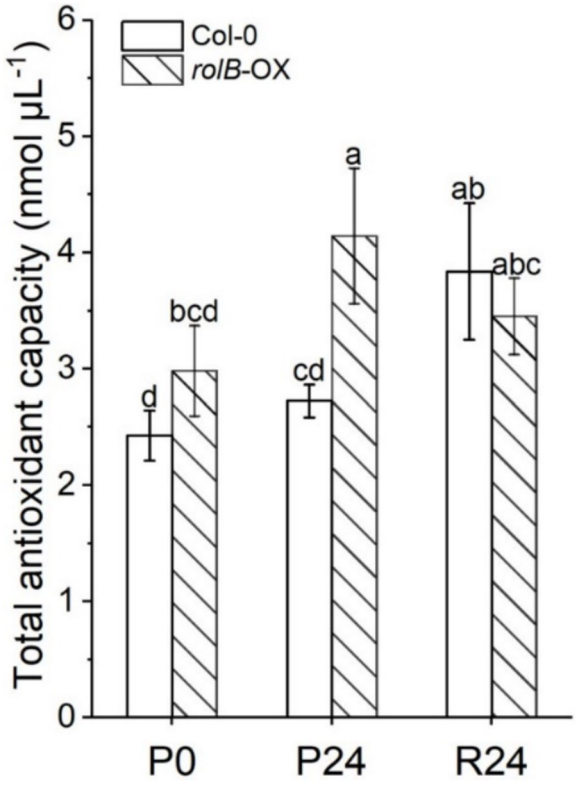

Fig. 4 Total antioxidant capacity of Arabidopsis plants (five-week-old) under 10% polyethylene glycol (PEG) 6000 stress and recovery. *rolB*-OX, Arabidopsis plants overexpressing A4-*rolB*. Col-0, wild-type Arabidopsis Columbia-0 ecotype. P0, before PEG treatment; P24, upon 24 h of PEG stress; R24, after 24 h of stress recovery. Values are mean ± SD ($n = 4$), different letters on the top of each column indicate significance among genotypes and treatments by Duncan test at $p \leq 0.05$ level

Discussion

A4-*rolB* induces dwarfing phenotypes and weakened root growth in Arabidopsis

Arabidopsis overexpressing A4-*rolB* exhibited a pronounced dwarfing phenotype compared with control plants (Col-0) (Fig. 1), which confirmed the result from Kodahl et al. [30], and the observation on other *rolB* overexpressing plant species such as *Kalanchoë blossfeldiana* [20]. In addition, weak root growth was noticed in *rolB*-OX, as exemplified by its significantly lower total root length (59% of Col-0) and smaller root surface area (63% of Col-0) (Fig. 2). The weak root growth in *rolB*-OX plants could, at least in part, be attributed to *rolB*-induced leaf necrosis, which was reported by Kodahl et al. [30]. This is due to the fact that root growth is typically dependent on the functioning of source leaves. However, this observation contradicts the results in *rolB*-overexpressing apple, where *rolB* triggered enhanced root growth, including increased root number and fresh root weight [45, 66]. Hence, it suggests that the effect of *rolB* gene on root phenotype could be species-specific.

Enhanced ABA concentration and total antioxidant capacity, and improved stomatal recovery in *rolB*-OX

The *rolB* gene has been widely reported to enhance stress resistance in plants or plant cells, e.g., drought and oxidative stress [7, 59]. This is supported by recent research, which elucidates the interaction between *rolB* and TOPLESS repressor proteins, thereby inducing immune reprogramming [22]. Nevertheless, how *rolB* alters plant physiology to affect stress resistance in plants is still poorly understood. To reveal *rolB*'s role in osmotic stress, we investigated the phenotypes and physiological responses of *rolB*-OX under polyethylene glycol (PEG) treatment. As PEG treatment processed, ABA concentrations generally increased in Col-0 line along with decreased stomatal conductance (g_s) (Fig. 3B, C and Table S2), which is in line with the common observation that ABA enhancement induces stomatal closure [5, 6]. Interestingly, *rolB*-OX had dramatically higher ABA levels (1.93 times that of Col-0) before onset of the PEG stress (at P0), coinciding with its significantly lower g_s (only 80% of Col-0) (Fig. S1 and Table S2). This suggests a promoting effect of A4-*rolB* on ABA levels, and thus inhibiting g_s under well-watered conditions. Additionally, *rolB*-OX maintained relatively stable ABA levels compared to Col-0 during PEG treatment, although its g_s was sharply decreased by osmotic stress (Fig. 3B, C and Fig. S1). This result indicates that A4-*rolB*-mediated modulation in ABA concentrations is not the only factor regulating g_s. Therefore, it is proposed that A4-*rolB* may confer other mechanisms such as plant hydraulic regulation under stress, since hydraulic signalling is, in addition to ABA, another key factor regulating stomatal behavior [13, 56]. Based on the above, A4-*rolB* gene affected stomatal regulation in Arabidopsis by mediating an increase in ABA levels.

ABA induces antioxidant defense or oxidative damage by regulating ROS levels, thereby mediating plant stress responses such as drought [32, 65]. After PEG treatment, although ABA levels did not significantly increase in *rolB*-OX at P24 compared to that at P0 (Fig. S1A), *rolB*-OX exhibited less leaf wilting symptoms compared to Col-0 (Fig. 3A), implying its better maintenance of hydraulic integrity/leaf turgor than the Col-0. This could be likely attributed to a higher antioxidant defense triggered by A4-*rolB* gene, as exemplified by the significantly higher total antioxidant capacity (TAC) in *rolB*-OX than the Col-0 under PEG stress (at P24) (Fig. 4). This corroborates early findings documenting that *rolB* possesses potent antioxidant capabilities in plants and plant tissues [7, 38]. Given the dual effects of ABA in inducing

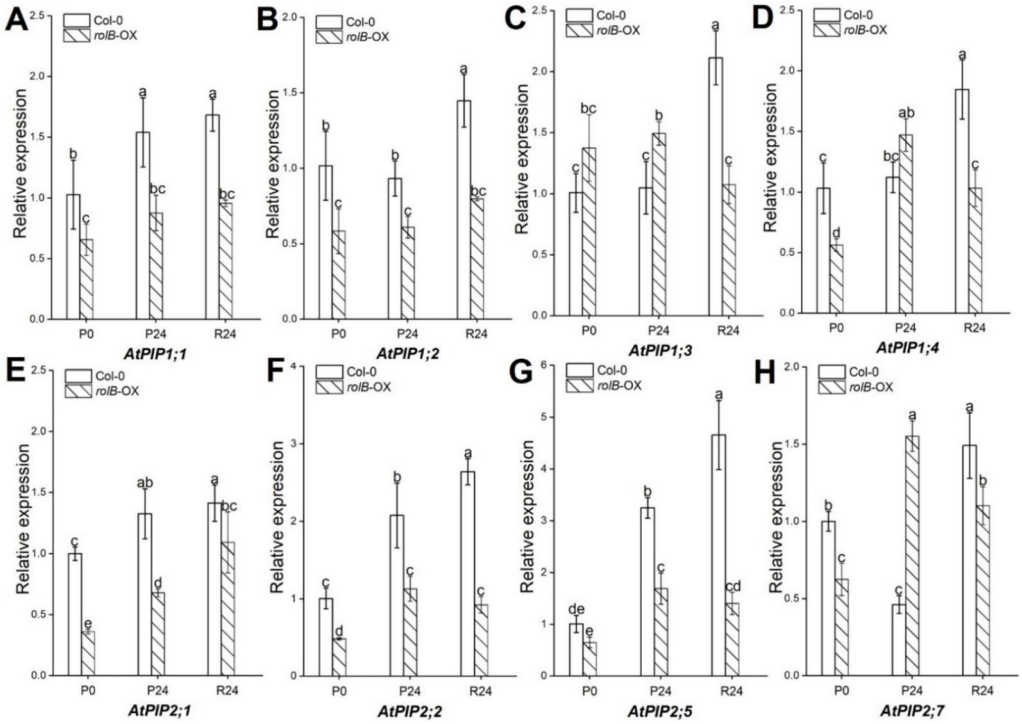

Fig. 5 Expression patterns of genes encoding plasma membrane intrinsic proteins (*PIPs*) in leaves of Arabidopsis plants (five-week-old) under 10% polyethylene glycol (PEG) 6000 stress and recovery. *rolB*-OX, Arabidopsis plants overexpressing A4-*rolB*. Col-0, wild-type Arabidopsis Columbia-0 ecotype. P0, before PEG treatment; P24, upon 24 h of PEG stress; R24, after 24 h of stress recovery. *At* indicates *Arabidopsis thaliana*. *PIP1;1*, plasma membrane intrinsic protein 1;1. *PIP1;2*, plasma membrane intrinsic protein 1;2. *PIP1;3*, plasma membrane intrinsic protein 1;3. *PIP1;4*, plasma membrane intrinsic protein 1;4. *PIP2;1*, plasma membrane intrinsic protein 2;1. *PIP2;2*, plasma membrane intrinsic protein 2;2. *PIP2;5*, plasma membrane intrinsic protein 2;5. *PIP2;7*, plasma membrane intrinsic protein 2;7. Values are mean ± SD ($n = 4$), different letters on the top of each column indicate significance among genotypes and treatments by Duncan test at $p \leq 0.05$ level

antioxidant defense and oxidative damage in stressed plants [14, 32, 44], the induced antioxidant capacity in *rolB*-OX after PEG stress could be related to its moderate ABA levels (only 68% of Col-0 at P24) (Fig. S1A), which was adequate to stimulate antioxidant defenses while remaining insufficient to induce oxidative damage. However, this was not the case in Col-0, where excessive ABA accumulation (1.52 times of *rolB*-OX) may have triggered severe oxidative damage [3, 63], resulting in significantly lower TAC in Col-0 compared to *rolB*-OX. Before onset of PEG treatment, an appropriate ABA concentration had been achieved in *rolB*-OX, being the same level as that at P24 (Fig. S1), to activate plant antioxidant defense. This was supported by the increased TAC in *rolB*-OX at P0 compared to Col-0 (although not significant) (Fig. 4). Consequently, this activated antioxidant defense before stress may have facilitated PEG stress resilience in *rolB*-OX as described above, ultimately leading to its higher recovered g_s after stress recovery (55% recovery of its control at R24) (Fig. 3C and Fig. S1B). Based on the above, we propose that A4-*rolB*-mediated increase in ABA concentrations could be linked to the enhanced osmotic stress resistance in *rolB*-OX, probably through modulating plant antioxidant capability.

In addition, given the high impact of *PIPs* expression on plant hydraulic conductivity [13, 33, 64], the better maintenance of hydraulic integrity in *rolB*-OX leaves under PEG stress may be related to the markedly upregulated expression of *PIP2,5* and *PIP2;7* (3.3–5.7-fold of Col-0) in root tissues (Fig. 6G, H). This is due to the higher expression of *PIPs* in roots, which implies enhanced root hydraulic conductivity [18], thereby improving water transport from roots to leaves under stress conditions. However, this needs to be elucidated through further research.

Conclusion

In this study, A4-*rolB* induced a pronounced dwarfing phenotype and weakened root growth as shown by the decreased root length and root surface area in *rolB*-OX compared to Col-0. In addition, A4-*rolB* overexpression induced a better plant hydraulic integrity, as exemplified by less severe leaf wilting, and resulted in an increase in ABA levels and TAC as well as a better recovery of g_s in Arabidopsis after PEG stress.

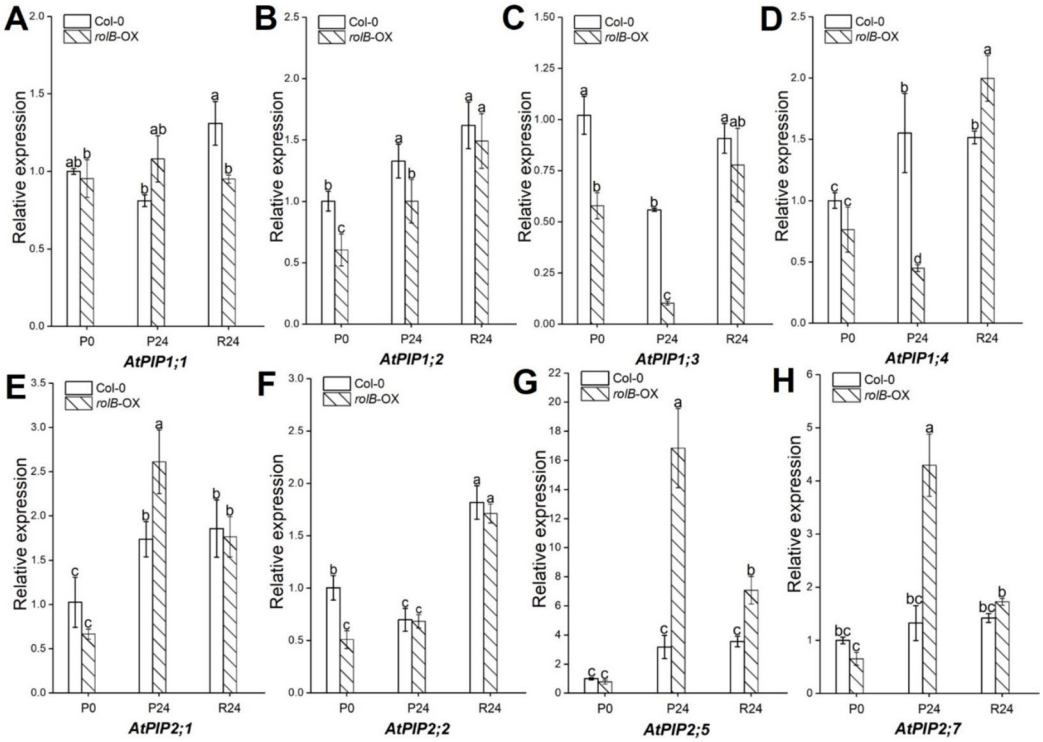

Fig. 6 Expression patterns of genes encoding plasma membrane intrinsic proteins (*PIPs*) in roots of Arabidopsis plants (five-week-old) under 10% polyethylene glycol (PEG) 6000 stress and recovery. *rolB*-OX, Arabidopsis plants overexpressing A4-*rolB*. Col-0, wild-type Arabidopsis Columbia-0 ecotype. P0, before PEG treatment; P24, upon 24 h of PEG stress; R24, after 24 h of stress recovery. *At* indicates *Arabidopsis thaliana*. *PIP1;1*, plasma membrane intrinsic protein 1;1. *PIP1;2*, plasma membrane intrinsic protein 1;2. *PIP1;3*, plasma membrane intrinsic protein 1;3. *PIP1;4*, plasma membrane intrinsic protein 1;4. *PIP2;1*, plasma membrane intrinsic protein 2;1. *PIP2;2*, plasma membrane intrinsic protein 2;2. *PIP2;5*, plasma membrane intrinsic protein 2;5. *PIP2;7*, plasma membrane intrinsic protein 2;7. Values are mean ± SD ($n = 4$), different letters on the top of each column indicate significance among genotypes and treatments by Duncan test at $p \leq 0.05$ level

It can be concluded that A4-*rolB* promotes osmotic stress resistance in Arabidopsis by mediating enhancement in leaf ABA concentrations and TAC and affecting stomatal regulation. Moreover, the association between ABA and TAC and its effect on the osmotic stress resistance of *rolB*-OX was thoroughly discussed in this study. These findings reveal novel physiological effects of *rolB* on plant stress resistance and lay the foundation for further elucidating the role of *rolB* in plant responses to abiotic stress.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06675-8.

Below is the link to the electronic supplementary material.Supplementary Material 1: Figure S1. Leaf ABA concentrations and stomatal conductance (g_s) of Arabidopsis plants (five-week-old) under 10% PEG (6000) stress and recovery.

Supplementary Material 2: Table S1. Primer sequences for qRT-PCR analysis.

Supplementary Material 3: Table S2. Average values of g_s and ABA of Arabidopsis plants before and after 10% PEG (6000) treatment.

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Authors' contributions
XC, HL, and FL conceptualized the research. XC, HL, and FL planned the experiments and contributed to the interpretation of the results. XC performed the experimental work and data processing, and BF assisted with experiment execution and data analysis. XC wrote the original manuscript and organized the figures and tables. HL, FL, and BF provided critical feedback to the original manuscript editing. All authors contributed to the article and approved the submitted version.

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Data availability
All data supporting the findings of this study are available within the paper and within its supplementary data published online.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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