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ORIGINAL ARTICLE

Overexpression of the A4-*rol*B gene from the pRiA4 of *Rhizobium rhizogenes* modulates hormones homeostasis and leads to an increase of flavonoid accumulation and drought tolerance in *Arabidopsis thaliana* transgenic plants

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Abstract

Main conclusion **Increased flavonol accumulation and enhanced drought tolerance in A4-*rol*B-overexpressing plants can be explained by the cooperative action of the SA and ROS signalling pathways.**

Abstract Clarification of function of the *A4-rol*B plast gene from pRiA4 of *Rhizobium rhizogenes* will allow a better understanding of the biological principles of the natural transformation process and its use as a tool for plant bioengineering. In the present study, we investigated whether the overexpression of A4-*rol*B gene could regulate two important processes, flavonoid biosynthesis and drought tolerance. In addition, we investigated some aspects of the possible machinery of the A4-*rol*B-induced changes in plant physiology, such as crosstalk of the major signalling systems. Based on the data obtained in this work, it can be presumed that constitutive overexpression of A4-*rol*B leads to the activation of the salicylic acid signalling system. An increase in flavonol accumulation and enhanced drought tolerance can be explained by the cooperative action of SA and ROS pathways.

Keywords Drought stress · Kaempferol · Neoplastic transformation · Quercetin · *Agrobacterium rhizogenes* · Salicylic acid

Abbreviations

DREB	Dehydration-responsive element-binding
HSF	Heat stress transcription factors
PFP	P-4-fluoro-DL-phenylalanine
PR	Pathogenesis-related
rol	*root oncogenic locus*
ROS	Reactive oxygen species
SA	Salicylic acid
TF	Transcription factor

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Introduction

The most studied inter-kingdom gene transfer is the *Agrobacterium*-mediated transformation of plants. The soil bacterium *Rhizobium rhizogenes* is a plant pathogen that colonises plant cells via the induction of specific opine-producing neoplasms called hairy roots (Lacroix and Citovsky 2019). The most studied *rol* (*root oncogenic locus*) genes (A, B, C, and D) from transferred DNA (T-DNA) of the pRiA4 are the main genetic determinants of hairy root disease and led to perturbations in plant phytohormone balance (Bulgakov 2008; Mauro and Bettini 2021). In particular, plants transformed with the A4-*rol*B gene exhibit multiple morphological abnormalities, including altered flower and leaf morphology, dwarfism and necrosis, reduced fertility, and

early ripening (Mohajjel-Shoja 2010; Kodahl et al. 2016; Otten 2018). Although several molecular mechanisms have been proposed for the action of A4-*rol*B, including an interaction with 14–3–3 proteins (Moriuchi et al. 2004), ROS metabolism perturbation (Bulgakov et al. 2012), beta-glycosidase (Estruch et al. 1991) and tyrosine phosphatase activity (Filippini et al. 1996), none of these functions of the A4-*rol*B has yet been confirmed (Schmülling et al. 1993; Nilsson and Olsson 1997; Otten 2018; Mauro and Bettini 2021).

The *A4-rol*B and *rol*C genes are part of the plast family, many of which can cause growth changes. In addition, they are known as remarkable modulators of specialised metabolism in transformed cells and plants. Heterologous expression of *rol* genes has been reported to activate the synthesis of valuable medical compounds, such as isoflavonoids, alkaloids, stilbenes, anthraquinones, and other groups of secondary metabolites (Bulgakov 2008; Mauro and Bettini 2021). Among A4-*rol* genes, A4-*rol*B is known to be the strongest effector of secondary metabolite biosynthesis (Shkryl et al. 2008). The action of A4-*rol*B is not limited to any particular biosynthetic pathway, because, in different plants, it stimulates the accumulation of a variety of compounds (Mauro and Bettini 2021). However, when expressed at high levels, A4-*rol*B can have a negative effect on cell growth and thus reduce the overall production of phytochemicals in transgenic culture (Shkryl et al. 2008; Veremeichik et al. 2019).

Overexpression of the A4-*rol*B gene in *Arabidopsis* callus culture increases the content of the main nitrogen-containing metabolites that provide protection against pathogens, indolic glucosinolates (IG), via selective activation of genes encoding MYB and bHLH transcription factors (Bulgakov et al. 2016). Interestingly, while whole *Arabidopsis* plants contain the full spectrum of indolic and aliphatic glucosinolates and flavonoids, the untransformed callus culture contained only two indolic glucosinolates (indol-3-ylmethyl and 4-methoxyindol-3-ylmethylglucosinolate), and their content was only half that of intact plants. Therefore, in this case, *Arabidopsis* callus culture can be considered a monoproducer of indolic glucosinolates (Bulgakov et al. 2016). Notably, A4-*rol*B-mediated activation of glucosinolate biosynthesis increased their content to the level of whole plants (Bulgakov et al. 2016). In *Arabidopsis*, flavonoids are presented mainly by flavonols, namely, kaempferol, quercetin, and their derivatives (Tohge et al. 2017) synthesised via the phenylpropanoid metabolic pathway. Various transcription factors (TFs) of different families regulate flavonoid biosynthesis (Petroni and Tonelli 2011). Among others, MYB11 overexpression can upregulate flavonol accumulation (Pandey et al. 2015). Blocking the flavonoid biosynthesis pathway in *Arabidopsis* callus culture is accompanied by almost total inhibition of MYB11 expression (Bulgakov et al. 2016). A4-*rol*B expressed in callus culture increased the expression of other flavonoid-associated TFs, whose expression level was equal in plants and callus. However, it could not overcome the inhibition of MYB11 expression and flavonoid biosynthesis (Bulgakov et al. 2016).

Another important issue is the effect of the A4-*rol*B gene on the regulation of the tolerance to abiotic stress treatments. Recently, we demonstrated that A4-*rol*B—expression in the transformed cell cultures of *Arabidopsis* disturbed the homeostasis of numerous proteins involved in primary metabolism, phytohormone, chaperone, and reactive oxygen species (ROS) signalling (Veremeichik et al. 2016; Bulgakov et al. 2018). There is a large amount of data indicating that A4-*rol*B can influence environmental adaptation, such as via lower accumulation of ROS (Bulgakov et al. 2012), increased peroxidase activity (Veremeichik et al. 2012; Shkryl et al. 2013), and modulation of photosynthetic activity under far-red light (Bettini et al. 2020). Interestingly, decreasing the ROS level was accompanied by the augmentation of not only the expression of ROS scavenging enzymes but also enzymes involved in ROS generation, such as respiratory burst oxidase homologs (Rbohs) and class III peroxidases (Prxs) (Shkryl et al. 2012; Veremeichik et al. 2012, 2016). This finding indicates A4-*rol*B-induced activation of the ROS signalling system with some compensatory mechanism to prevent the toxic effects of excess ROS. However, it is only possible to unambiguously assert that it improves the protection against the pathogens (Arshad et al. 2014) via ROS-dependent activation of specialised metabolite biosynthesis (Bulgakov et al. 2011; Veremeichik et al. 2016).

The phytohormone abscisic acid (ABA) plays a major role in the abiotic stress response and adaptation processes (Grant and Jones 2009), while salicylic acid (SA) is synthesised in response to the detection of biotrophic and hemibiotrophic phytopathogens at the site of infection, and then SA-dependent defence response spreads to the distal plant parts to protect undamaged tissues (Verma et al. 2016). Another important part of the action mechanisms induced by SA is flavonoid metabolism (Gondor et al. 2016). Fungal elicitors can induce an increase in SA and flavonol glycoside accumulation (Xu et al. 2009). Another plant hormone and growth regulator, auxin-class indole-3-acetic acid (IAA), acts as the main antagonist of SA (Kong et al. 2020) and ABA signalling (Liu et al. 2013).

In the present study, we tried to clarify whether the A4-*rol*B gene could regulate flavonoid biosynthesis and shed light on the role of the A4-*rol*B gene in drought tolerance using the generally accepted model system, *Arabidopsis*, expressing the A4-*rol*B gene. In addition, we intended to clarify the role of A4-*rol*B in the plant immunity system as a possible mediator in the crosstalk of the major plant hormones.

Materials and methods

Plant material

A4-*rol*B-transgenic *Arabidopsis thaliana* plants of the 'Columbia' (Col0) ecotype were established by the floral dip method (Clough and Bent 1998) using the pPCV002–CaMVBT construct (Spena et al. 1987) in two independent experiments. Kanamycin selection was used to identify putative transformants of F0 plants. Seeds of antibiotic-resistant F1 lines were germinated in the presence of kanamycin. 50 well-growing kanamycin-resistant F2 plants from both transformation experiments were analyzed to determine A4-*rol*B transfer and expression. Based on both molecular analysis and A4-*rol*B-specific morphological features (dwarfism, abnormal flowering), two A4-*rol*B-transformed clones (B2 and B5) were selected from the first transformation experiments and one (B13) from the second. These three homozygous transformed clones were clonally micropropagated to obtain as much as possible seeds of homozygous transgenic F3 seeds. Kanamycin-selection assay showed 100 percent germination of F3 seeds, that pointed on the homozygous genetic status of F2 and F3 plants. Both A4-*rol*B-transgenic and wild-type (WT) plants were cultured on hormone-free Murashige and Skoog (MS) agar medium containing 1% sucrose at 24 °C with 16 h/8 h light and dark cycles for 5–10 weeks.

Experimental design

To study the effect of 4-fluoro-L-phenylalanine (PFP) as a PAL inhibitor on germination and growth of the control and A4-*rol*B-transformed plants, two series of experiments were performed. In germination assays control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13) were germinated in vitro on cultural medium supplemented with 5 mM of PFP for 7 days. To determine the effect of PFP on growth in vitro, 10-day-old seedlings of control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13) were transferred on cultural medium supplemented with 5 mM of PFP for 25 days. The photograph is representative of the experiments conducted in triplicate.

Drought tolerance of WT and A4-*rol*B-transformed *Arabidopsis* plants (F3) was studied for young and mature plants in two variants of the duration of drought. Seeds of WT and A4-*rol*B-transformed *Arabidopsis* plants (F3) were grown in soil under controlled conditions (temperature, 24/22 °C; photoperiod, 16/8 h; illumination in the day time, 3000–5000 lx; humidity, 70%) in the greenhouse for 2 and 4 weeks with regular watering every 2 days. To determine drought tolerance of young (2-week-old) and mature (4-week-old) plants watering was stopped for 20 days. Plants were photographed at 10th, 14th, and 20th days up to complete death of WT plants. Then experimental plants were rewatered and photographed again. Both experiments were repeated three times with the same results.

To study the germination of normal and F3 of the A4-*rol*B-transgenic lines (B2, B5, and B13) seeds under temperature and salinity treatments, sterile seeds were germinated in plates with agarized medium in sterile conditions. Seeds were germinated under normal cultivation conditions (24 °C), under lower (14 °C), and higher (34 °C) temperatures, as well as in the presence of 120 mM NaCl for 7 days. The experiments were conducted in triplicate.

For analysis of gene expression, we used leaf tissue of 2-, 4- and 7-week-old plants (F3 of the A4-*rol*B-transgenic lines B2, B5, and B13) growing in vitro for monitoring and excluding any possible biotic contamination. For analysis of hormones and specialized metabolites profiling 7-week-old plants (WT and F3 of the A4-*A4-rol*B-transgenic lines B2, B5, and B13) growing in vitro were used. For analysis of time of flowering, control and transgenic (F3 of the A4-*rol*B-transgenic lines B2, B5, and B13) plants were grown in soil under controlled conditions (temperature, 24/22 °C; photoperiod, 16/8 h; illumination in the day time, 3000–5000 lx; humidity, 70%) in the greenhouse for 10 weeks with regular watering every 2 days.

RNA isolation, cDNA synthesis and real-time PCR

The isolation and characterization of total DNA and total RNA as well as first-strand cDNA synthesis were carried out as described previously (Veremeichik et al. 2019). To estimate T-DNA copy number in the A4-*rol*B-transformed *Arabidopsis* plants, real-time PCR comparison of Ct values between the single-copy gene 4HPPD and the A4-*rol*B gene (primers pair according to Bulgakov et al. 2016) was conducted according to method described by Kihara et al. (2006). In this analysis plasmid DNA, carrying each gene, served as an internal control for relative quantification of target sequences. RNA samples were isolated from leaves of 4-week-old *Arabidopsis* plants. Quantitative real-time PCR (qPCR) analysis was performed using an CFX96 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as described (Veremeichik et al. 2019). Three biological replicates, resulting from different RNA extractions, were used for analysis, and three technical replicates were analysed for each biological replicate. The gene-specific primer pairs used in the qPCR are shown in Suppl. Table S1. *A. thaliana* actin (*AtAct2*) and ubiquitin (*AtUBQ10*) genes (GeneBank ac. no. NM_112764 and NM_001084884, respectively) were used as endogenous controls to normalize variance in the quality and the amount

of cDNA used in each real-time PCR experiment (Bulgakov et al. 2018). The lower expressing sample assigned the value 1 in the relative mRNA calculation using the formula $2^{-\Delta\Delta Ct}$. Data were analysed using CFX Manager Software (Version 1.5; Bio-Rad Laboratories, Inc.).

HPLC–MS analysis of *Arabidopsis* secondary metabolites

Identification and quantification of secondary metabolites in dry aerial parts of 4-week-old control and transgenic plants was performed on a 1260 Infinity analytical high-performance liquid chromatography (HPLC) instrument (Agilent Technologies, Santa Clara, CA, USA) coupled with an electrospray ionization (ESI) ion trap mass spectrometer (MS) (Bruker HCT ultra PTM Discovery System, Bruker Daltonik, Bremen, Germany) in accordance with the procedure described by us (Bulgakov et al. 2016).

HPLC–MS/MS analysis of plant hormones

Chemicals

LC–MS-grade acetonitrile and methanol were acquired from Merck (Darmstadt, Germany). MS-grade formic acid was acquired from Sigma Aldrich (Steinheim, Germany). Deionized water was obtained using a Milli-Q Simplicity water purification system (Millipore, Molsheim, France). Analytical standards (IAA, ABA, and SA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Several WT and transgenic 4-week-old *Arabidopsis* plants were frozen in liquid nitrogen and homogenized using a chilled mortar and pestle. After homogenization, the samples (200 mg) were weighed into pre-cooled vials. The resulting paste of each sample was extracted in 1 ml ice-cold 80% MeOH with 0.1% formic acid for 30 min (high speed vortex, + 4 °C), then centrifuged (20.000*g*, 15 min, 4 °C). Finally, supernatants were filtered through a syringe filter (Nylon, 0.45 µm), and used for HPLC analysis.

HPLC–MS/MS analysis and quantification of plant hormones

A Shimadzu LC-30AD HPLC system coupled with triple-quadrupole mass-spectrometer Shimadzu LCMS-8060 (Kyoto, Japan), equipped with electro-spray ionization interface was used for analysis. A Shimpack ODSII column (75 × 2 mm i.d., 2.2 µm particle size), (Shimadzu, Duisburg, Germany) was operated at 45 °C. Mobile phases were A: methanol, B: isopropanol, C: water with 2 M formic acid and 1.8 M ammonia, D: water. Eluent was pumped at constant flow rate of 0.2 ml/min with following step gradient (A%, B%, C%, D%): 0` (50, 0, 2, 48), 6` (75, 0, 2, 23), 7` (98, 0, 2, 0), 10` (98, 0, 2, 0), 10.01` (0, 0, 100, 0), 19` (0, 0, 100, 0), 19.01` (25, 0, 2, 73), 27` (stop). Injection volume was 10 µl. MS ionization parameters were: nebulizing gas flow (N_2)—2 l/min, drying gas flow (N_2)—10 l/min, heating gas (zero air) flow—10 l/min, interface, heat block and desolvation line temperatures—300, 400 and 250 °C, respectively. CID gas (argon) pressure was 270 kPa. The detection was performed by multiple reaction monitoring (MRM) in positive (indole-3-acetic acid) and negative (abscisic acid, salicylic acid) modes. External standard calibration solutions with 0.1–100 ng/ml concentration of each analysis were used to create the calibration curves. All important parameters of MRM and calibration curves necessary for HPLC–MS/MS analysis are summarized in Suppl. Table S2.

Statistical analysis

All statistical tests were performed using Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA). For comparison among multiple data, analysis of variance (ANOVA) based in the Fisher's protected least significant difference (PLSD) *post-hoc* test was employed for the inter-group comparison. Two independent categories were compared using the Student's *t* test. A difference of $P < 0.05$ was considered significant.

Results

Obtaining and characterising A4-*rol*B-overexpressing *Arabidopsis* plants

A. thaliana plants of the 'Columbia' (Col-0) ecotype were used to obtain A4-*rol*B-transgenic lines using the *Agrobacterium*-mediated floral-dip protocol (see "Materials and methods"). From the second generation F2 three A4-*rol*B-expressed clones (B2, B5, and B13) with A4-*rol*B-specific morphological features (dwarfism, abnormal flowering) were selected to obtain F3 seeds for future studies (Fig. 1a). Real-time PCR analysis showed that the haploid genomes of B2, B5, and B13 homozygous lines (F3) carried a single copy of the integrated T-DNA. The expression of the transgene was verified using qPCR analysis with gene-specific primers in F2 7-week-old and F3 2- and 7-week-old plants. We found that F2 plants lines exhibited equal and significantly higher levels of the A4-*rol*B gene expression compared to F3 plants (Fig. 1c). Among F3 plants lines, A4-*rol*B expression was 1.5–2 times lower compared to B2 and B3 lines. We have not found any difference in the A4-*rolB* expression in F3 transgenic plants

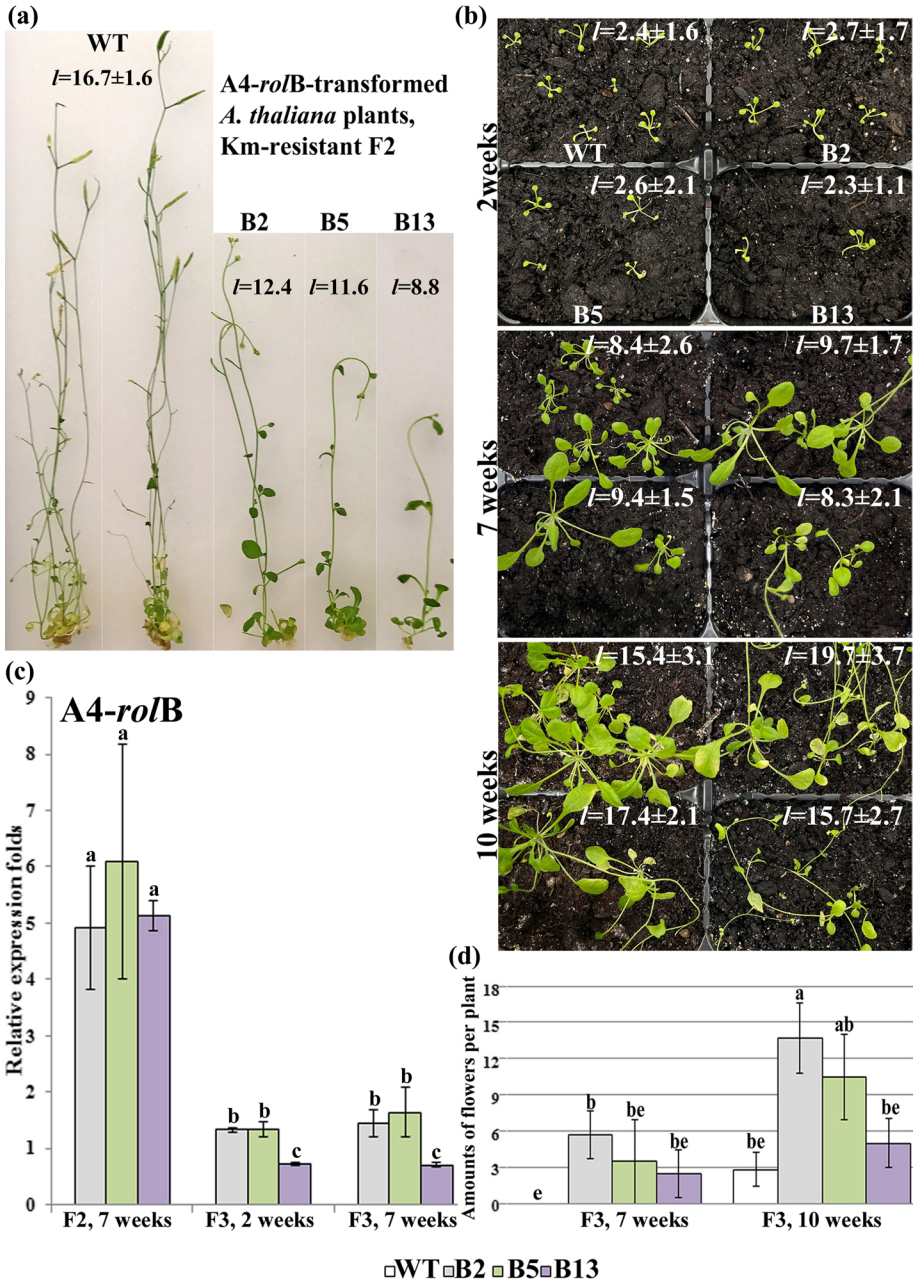


Fig. 1 **a** Morphology and length of the 7-week-old *Arabidopsis* lines growing in vitro: control (WT) and F2 of the A4-*rol*B-transgenic plants (B2, B5, and B13) *obtained* by the *floral dip*. **b** Morphology and length of control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13) during 10 weeks of cultivation in soil. **c** Relative expression of the A4-*rol*B gene in transgenic *Arabidopsis* plants was measured by real-time PCR in 7-week-old F2 plants and in F3 plants at 2 and 7 weeks. **d** Flowering time for WT and F3 A4-*rol*B expressed plants was calculated at 7 and 10 weeks in two independent experiments. The data are presented as the mean ± SE. Different letters above the bars indicate significantly different means ($P < 0.05$), Fisher's LSD

during 7-week-long growth (Fig. 1c). Notably, F3 of the B2, B5, and B13 plants showed no obvious phenotypic differences at the vegetative growth stage, while F2 plants with higher A4-*rol*B expression showed an A4-*rol*B-specific feature, such as dwarfism (Fig. 1a, b). Meanwhile, F3 A4-*rol*B-transgenic plants exhibited a faster transition into the reproductive phase and earlier flowering compared to wild-type (WT) plants. The statistical significance of earlier flowering was shown for B2 and B5 plant lines compared to WT plants in two independent experiments (Fig. 1d).

A4-*rol*B perturbs salicylic acid and ROS signalling pathways

To address how A4-*rol*B modulates the hormonal balance, we focused on the signalling pathways of ABA, IAA, and SA. First, we tested if A4-*rol*B could modify the contents of ABA and IAA phytohormones but did not observe any significant differences between the control and A4-*rol*B-transformed lines (Fig. 2). In contrast, the level of free SA was significantly increased by 1.7- and 1.4-fold in the B2 and B5 lines, respectively, while staying unchanged in the B13 line.

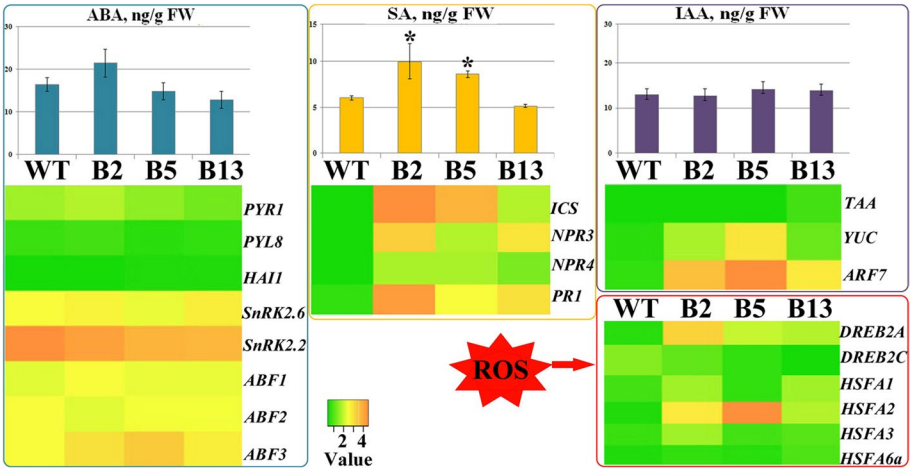


Fig. 2 Plant hormones profiling and expression of marker genes in control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13). The abscisic acid (ABA), salicylic acid (SA), and indole-3-acetic acid (IAA) contents were measured using HPLC in two independent experiments with three replicates. The data are presented as the mean ± SE. mRNA levels of ABA-dependent and ROS dependent, excluding *HSFA6a*, stress-related genes, and genes involved in SA and IAA signalling pathways measured by real-time PCR in three independent biological replicates. qPCR of each biological replicate was performed in three technical replicates. Data are presented as heatmaps computed based on qPCR data as $2^{-\Delta\Delta Ct}$. Different colours indicate significantly different means ($P < 0.05$), Fisher's LSD. Stars above the bars indicate significantly different means compared to the control WT plants, Student's *t* test ($P < 0.05$)

Next, we examined the expression of genes encoding proteins involved in the metabolism of the aforementioned phytohormones. The Knotted ABA signalling system in a simple scheme consists of negative regulators, PYLs (protein serine/threonine phosphatase 2Cs, PP2Cs), positively regulators, SnRK2s (SNF1-related protein kinase 2), and transcription factors, including ABRE (ABA-responsive element [AREB] binding protein) binding factors (ABFs) (Grant and Jones 2009). No significant differences between WT and A4-*rol*B plants were observed for genes involved in ABA signalling. Presumably, A4-*rol*B can positively influence the expression of the main transcription factors regulated by ROS, such as dehydration-responsive element-binding (DREBs) and heat stress transcription factors (HSFs), which play an important role in the ABA-independent response to different abiotic stressors (Guo et al. 2016; Agarwal et al. 2017; Katano et al. 2018). In particular, *DREB2A* was upregulated 1.9–2.9-fold and the level of expression of *HSF2A* was activated by 2.1–4.2-fold in all A4-*rol*B-transgenic lines (Fig. 2).

Isochorismate synthase (ICS; EC 5.4.99.6) synthesises SA in response to biotrophic and hemibiotrophic infections. NON-EXPRESSOR OF PR GENEs (NPRs) are among the key regulatory elements in SA-induced systemic acquired resistance that provide the induction of pathogenesis-related (*PR*) genes, which increase resistance to a wide range of pathogens (Verma et al. 2016). Significant activation was observed for genes involved in SA signalling in all transgenic plants. The highest rate of activation was observed for *ICS* and *PR1* transcripts, in which the level of expression was increased by 2.3–4.5-fold and by 2.2–3.4-fold, respectively. A moderate increase in transcript abundance was detected for *NPR3* (from 2.1- to 3.3-fold) and *NPR4* genes (from 1.8- to 2.1-fold) in all lines (Fig. 2). In addition, we have analysed the impact of A4-*rol*B on the expression of biosynthesis genes of IAA, *TAA1* (L-tryptophan aminotransferase), and *YUC* (monooxygenase-like proteins), and auxin response factor *ARF7* (Sharma et al. 2015). *YUC* and *ARF7* genes mediating IAA biosynthesis and signalling, showed slight and moderate increases in all transgenic lines from 1.4- to 2.4-fold and from 2.2- to 3.2-fold, respectively.

These results indicate that A4-*rol*B-mediated perturbation in the SA signalling pathway, as well as upregulation of ROS-dependent dehydration- and heat stress-inducible genes, may contribute to drought tolerance in transgenic *Arabidopsis* plants. It is known that SA is produced in plants cell by two independent ways: from phenylalanine and from chorismic acid via action of PAL and ICS enzymes (Verma et al. 2016). To determine whether PAL-dependent SA biosynthesis could be the main target for A4-*rol*B, or whether this activation could be a secondary A4-*A4-rolB* effect, we inhibited growth of plants with p-fluoro-DL-phenylalanine (PFP). Germination (Fig. 3a) and growth (Fig. 3b) assays in the presence of 5 mM PFP showed 70% inhibition of germination and full inhibition of growth for WT plants. Germination of the F3 A4-*rol*B-expressing plants B2 and B5 was not inhibited, while germination of the low-expressed B13 line was slightly inhibited (Fig. 3a). To test long growth of plants in the presence 4-fluoro-L-phenylalanine, WT and F3 A4-*rol*B-transgenic seeds were germinated and grown on the MS/2 medium for 10 days. Then, 10-day-old WT

and A4-*rol*B-expressing seedlings were transferred on the MS/2 medium supplemented with 5 mM of 4-fluoro-L-phenylalanine for 25-day-long growth. Long growth of WT plants in the presence of PFP showed strong growth inhibition, whereas growth of the A4-*rol*B-expressing plants was reduced, but viable (Fig. 3b).

Expression pattern of *ICS1* and *PAL1* at the earlier (2 weeks) and later (7 weeks) growth stages showed low and equal expression level in 2-week-old WT and A4-*rol*B-expressing plants and strong (more than 10 times for *ICS1* and 20 times for *PAL1*) activation of expression in the 7-week-old plants; moreover, we showed that the expression of these genes was more than doubled in the A4-*rol*B-transformed plants at this stage (Fig. 3c). In the B13 line expression of *ICS1* and *PAL1* was slightly but statically significant lower than in the B2 and the B5 lines. The absence of the differences in gene expression of the main SA and flavonoids producers ICS1 and PAL1, at the earlier stage of growth in the WT and A4-*rol*B-transformed plants, indicated a possibility that these effects are rather secondary. Notably, in addition, the probability of such an assumption is also highlighted by the same expression of the transgene at earlier and later periods (Fig. 1c).

Flavonoid biosynthesis in A4-*rolB*-transgenic plants

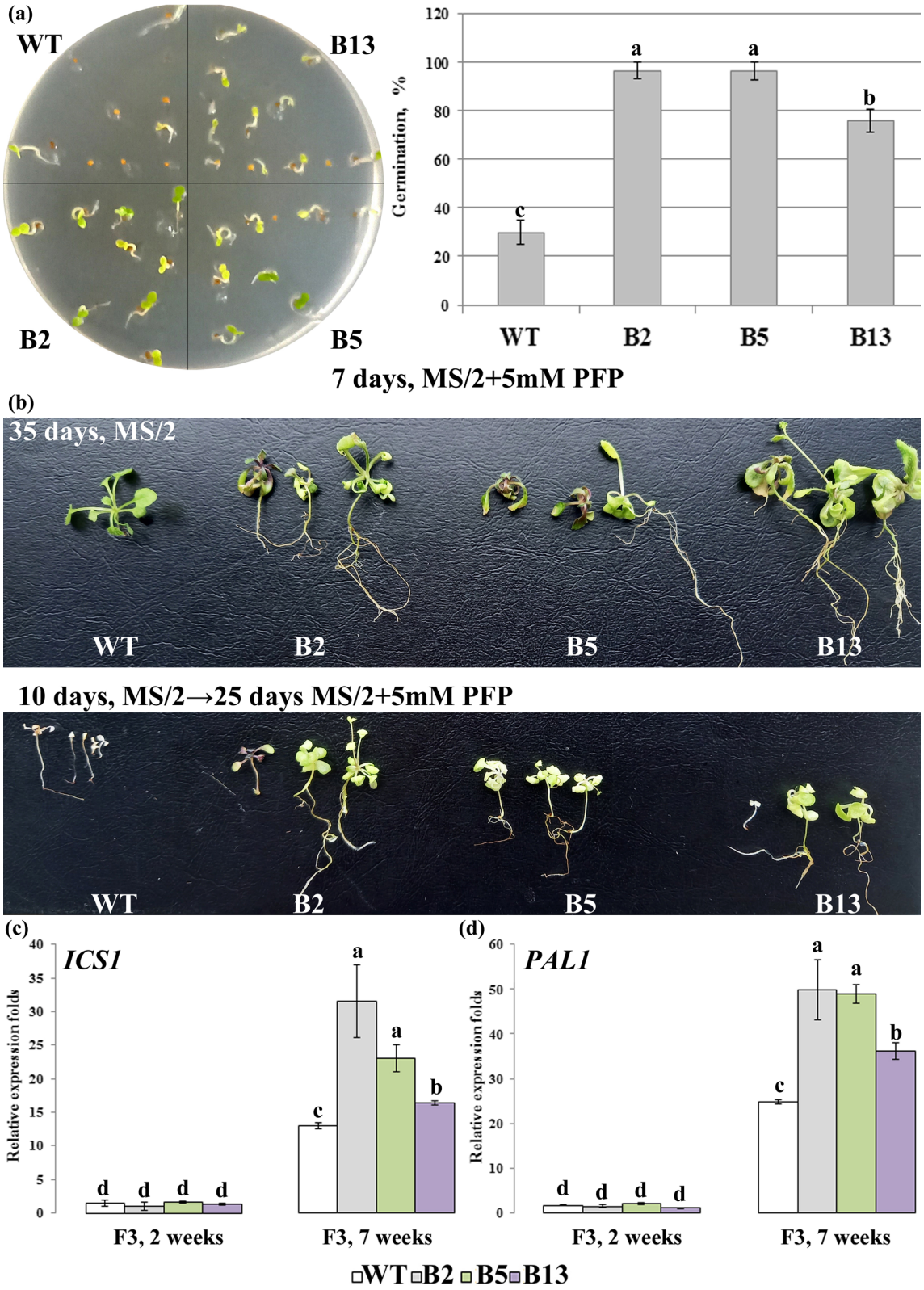
To study the influence of A4-*rol*B expression on the accumulation of specialised compounds in transgenic *Arabidopsis* plants, we performed HPLC–MS analysis of aerial parts of 4-week-old WT, B2, B5, and B13 lines. The content of glucosinolates (GSLs) was similar in all studied plant lines (Suppl. Table 3), with a prevalence of aliphatic GSLs over indolic GSLs. At the same time, HPLC analysis revealed that the A4-*rol*B gene provoked remarkable alteration in the accumulation of flavonoid compounds (Fig. 4). Kaempferol, quercetin, and isorhamnetin derivatives represented major secondary metabolites in all studied plants. Kaempferol glycosides accumulated at relatively high levels in *Arabidopsis* and were highly regulated by A4-*rol*B (Fig. 4a). In particular, kaempferol hexose dideoxyhexose-2 levels were 7.1–10.4-times higher than those in the control line. A moderate level of activation was observed for kaempferol hexose deoxyhexose with a 3.9–4.5-fold increase and kaempferol dideoxyhexose with a 3.2–4.7-fold upregulation. Despite these changes, all transgenic lines demonstrated the same ratio of kaempferol glycosides as WT plants.

Our data show that A4-*rol*B overexpression provoked a substantial increase in the levels of two quercetin derivatives in transgenic plants (Fig. 4b). The highest level of activation was observed for quercetin hexose deoxyhexose-3, which increased by 9.5–11.5-fold in A4-*rol*B-expressing lines (Fig. 4b). The same level of activation of isorhamnetin (3-methylquercetin) hexose deoxyhexose biosynthesis was noted for all A4-*rol*B-transgenic lines (Fig. 4b). Two lines, B2 and B13, accumulated statistically significant higher amounts of quercetin hexose dideoxyhexose-1, while no difference in the accumulation of this metabolite was observed for the B5 line compared to both transgenic and WT plants. Interestingly, A4-*rol*B-induced perturbations led to changes in quercetin glycoside ratios in the studied *Arabidopsis* lines. For example, quercetin deoxyhexose was a predominant compound in WT plants, representing 80% of the total quercetin glycoside content and its content was not changed in transgenic plants compared to WT. In A4-*rol*B-expressing plants quercetin hexose deoxyhexose-3 dominated 57–67% of the total quercetin glycosides. On average, A4-*rol*B overexpression in *Arabidopsis* plants led to a statistically significant increase in the content of kaempferol derivatives by 4.3-fold and quercetin derivatives by 3.3-fold compared to the control WT plants.

Next, we examined the transcriptional levels of key enzymes participating in the regulation of flavonoid biosynthesis using qPCR analysis. Essential A- and B-rings of the flavonoid skeleton (i.e., C6–C3–C6) of naringenin chalcone are produced by the enzyme chalcone synthase (CHS, CHS, EC 2.3.1.74) from one p-coumaroyl-CoA and three malonyl-CoA molecules. Hydroxylation of naringenin and synthesis of dihydrokaempferol, a precursor of kaempferol and quercetin, is catalysed by flavanone 3b-hydroxylase (F3H, EC 1.14.11.9) (Nabavi et al., 2020). The transcript abundance of chalcone synthase (*CHS*), flavonone-3-hydroxylase (*F3H*), and flavonol-specific transcription factor MYB11 (*MYB11*) was considerably increased in the A4-*rol*B-expressing plants (Fig. 4c). These genes showed 3.1–3.7-fold up-regulation for *CHS* and 2.0–2.9-fold up-regulation for *F3H* in all transgenic lines, while *MYB11* expression was activated only 1.7–2.4-fold. Taken together, the results demonstrate that A4-*rol*B-induced accumulation of flavonoid compounds correlated with the activation of biosynthetic and regulatory genes controlling the biosynthesis of flavonoids.

A4-*rol*B confers drought tolerance in transgenic *Arabidopsis* plants

The overaccumulation of flavonoids leads to an increase in drought tolerance of plants (Nakabayashi et al. 2014). Drought tolerance of WT and A4-*rol*B-transformed *Arabidopsis* plants (F3) was studied for young and mature plants in two variants of the duration of drought (see "Materials and methods"). To determine drought tolerance of young 2-week-old plants, watering was stopped for 20 days (Fig. 5a). For mature 4-week-old plants watering was stopped for 14 days (Fig. 5b). Experimental plants were photographed at 10th, 14th, and 20th days up to complete death of WT plants (Fig. 5). At the end of the period of water deficit, all plants displayed symptoms of dehydration,



including the wilting of leaves and stems. Afterwards, plants were rewatered, and recovery was checked on the next day. Surprisingly, the control plants were severely damaged and failed to revive, whereas the A4-*rol*B-expressing plants demonstrated improved drought tolerance and regained growth. In both experiments, the drought tolerance of the B13 plants

◂**Fig. 3** **a** In vitro germination assay in the presence of 5 mM of 4-fluoro-L-phenylalanine of control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13. **b** In vitro growth of 10-day-old seedlings transplanted on the MS/2 medium supplemented with 5 mM of 4-fluoro-L-phenylalanine for 25 days (lower part) and 35 days on the control MS/2 medium (upper part). The photograph is representative of the experiments conducted in triplicate. **c** Relative expression of the *ICS1* and *PAL1* genes in *Arabidopsis* plants was measured by real-time PCR in WT and A4-*rol*B-transgenic F3 plants at 2- and 7-week points. The data are calculated as $2^{-\Delta\Delta Ct}$ and presented as the mean ± SE. Different letters above the bars indicate significantly different means ($P < 0.05$), Fisher's LSD

was lower compared to that of the two other lines (Fig. 5a, b).

To assess whether A4-*rol*B plays a functional role in plant responses to other abiotic stressors, we examined seed germination of WT, B2, B5, and B13 lines under abiotic stress conditions. Control and A4-*rol*B-expressing seeds were plated and germinated under normal (24 °C), lower (14 °C), and higher (34 °C) temperatures, as well as in the presence of 120 mM NaCl for 7 days (Suppl. Fig. S1). Both the control and transgenic lines showed significant growth inhibition under cold treatment, while heat stress completely abolished their germination capacity. Similarly, salt stress resulted in growth inhibition in all studied lines in a similar manner.

Discussion

The most studied example of inter-kingdom gene transfer is the *Agrobacterium*-mediated transformation of plants, which is used as a main approach in both fundamental and practical areas of plant bioengineering (Guo et al. 2018). The A4-*rol*B plast gene is one of the main genetic determinants of the *R. rhizogenes*-mediated HGT. Better understanding of functional peculiarities of A4-*rol*B both with respect to the primary action and to various modifications, such as growth changes and modifications in metabolism, can lead to useful biotechnological applications of the A4-*rol*B gene and clarify how this is of benefit to the bacterium. One of the most applicable and studied effects of the A4-*rol*B gene from the pRiA4 plasmid is the activation of specialised metabolite biosynthesis in transgenic plants and cell cultures (Bulgakov 2008). In different cases where RolB was used, the biosynthesis of different metabolite groups, such as terpenes, N/S-containing compounds, and phenolic compounds (Fang et al. 2016), was activated (Mauro and Bettini 2021). In the present study, for the first time, we described A4-*rol*B-induced activation of flavonol biosynthesis in *Arabidopsis* plants. On average, A4-*rol*B overexpression in *Arabidopsis* plants led to an increase in the content of kaempferol derivatives by 4.3-fold and quercetin derivatives by 3.3-fold compared to the control WT plants. Flavonoid-related TF expression in A4-*rol*B-transformed *Arabidopsis* cells in our previous (Bulgakov et al. 2016) and present studies have indicated the important role of MYB11 in A4-*rol*B-mediated regulation of flavonol biosynthesis. Finding effective approaches to activate flavonoid biosynthesis is relevant in the field of plant biotechnology due to such beneficial properties of these compounds as anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties (Nijveldt et al. 2001; Tohge et al. 2017).

In the present study we have shown that expression of the A4-*rol*B was twofold decreased in the F3 homozygous plants compared to F2 plants (Fig. 1). This reduction in transgene expression was accompanied by a loss of the A4-*rol*B-specific morphological trait, such as dwarfism, which are consistent with other published data and could be explained by epigenetic silencing (Dehio and Schell 1994; Kodahl et al. 2016). The overaccumulation of flavonoids can stay in physiologically comfortable limits and promote protection to biotic and abiotic stress due to their antioxidative properties as ROS scavengers (Nakabayashi et al. 2014; Treml and Šmejkal 2016; Singh et al. 2021; Wang et al. 2020). Our data show an increased accumulation of flavonoids accompanied by an increase in the resistance of A4-*rol*B transformed *Arabidopsis* plants to drought (Fig. 4). The high level of drought tolerance of A4-*rol*B-expressing *Arabidopsis* plants allowed them to remain alive after almost 2 weeks without water, while WT plants died. To clarify the physiological properties of the A4-*rol*B-expressing plants we studied the A4-*rol*B-dependent effects on hormone homeostasis. Schmülling et al. (1993) determined phytohormone levels in A4-*rol*B-transgenic tobacco and showed only minor variations in IAA, ABA, and gibberellic acid content. The A4-*rol*B-dependent behaviour of transgenic tobacco protoplasts is not a consequence of modifying the intracellular auxin concentration but likely results from changes in the auxin perception pathway (Delbarre et al. 1994). In addition to these data, there are no recent investigations concerning phytohormone levels in A4-*rol*B-transformed plants (Mauro and Bettini 2021). In the present study, we evaluated endogenous hormone levels in A4-*rol*B-expressing *Arabidopsis* plants using HPLC–MS/MS analysis of ABA, IAA, and SA. Among these, only the SA level was significantly increased in A4-*rol*B-transformed *Arabidopsis* plants in a dose-dependent manner (Fig. 2). We did not find any difference in the content of ABA and IAA. However, the expression of the major auxin responsive factor, ARF7, was increased in A4-*rol*B plants, while the expression of genes encoding enzymes of IAA biosynthesis did not change significantly. This finding confirms the presumption of Carmi et al. (2003) that A4-*rol*B can activate the IAA-dependent process through the IAA-independent pathway (Carmi et al. 2003). One possible explanation is the existence of a safety mechanism of the whole plant against A4-*rol*B-induced

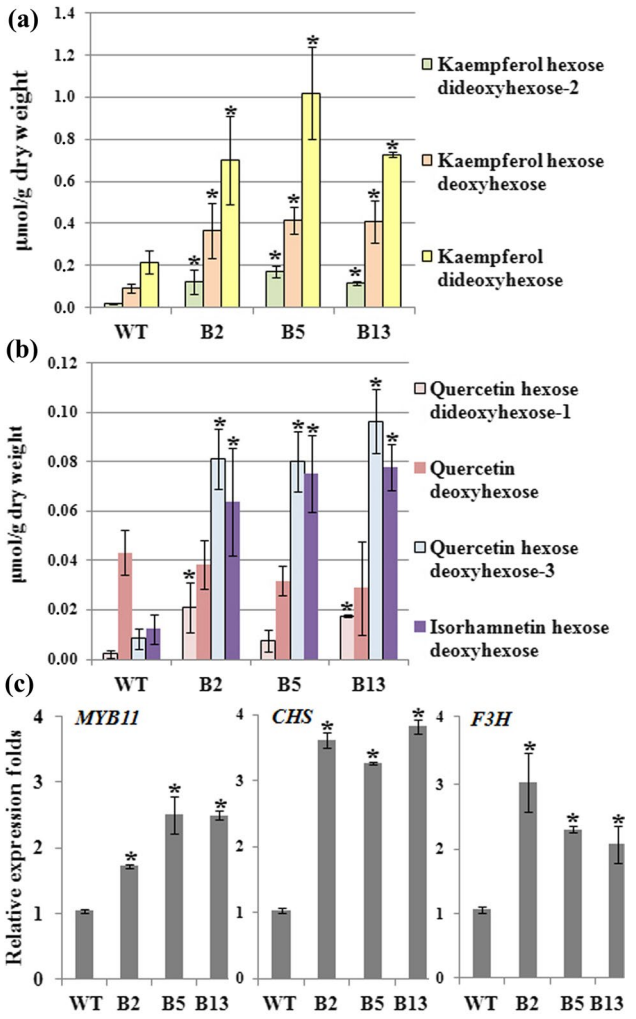


Fig. 4 Accumulation of flavonoids and transcriptional levels of key enzymes participating in their regulation in control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13). Contents of kaempferol (**a**), quercetin (**b**), and their derivatives were measured using HPLC. mRNA levels (**c**) of chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), and flavonol-specific transcription factor *MYB11* measured by real-time PCR in three independent biological replicates and qPCR of each biological replicate was performed in three technical replicates. The data are presented as the mean ± SE, based on qPCR data calculated as $2^{-\Delta\Delta Ct}$. Stars above the bars indicate significantly different means ($P < 0.05$) compared to control WT plants, Student's *t*-test

activation of the SA-signalling pathway to prevent growth inhibition (Kong et al. 2020). In other words, it needs to be clarified in future whether *A4-rolB* can directly or indirectly modulate IAA signaling. Or are the observed changes in IAA transduction an antagonistic response of the host plant to excessive activity of the SA signaling pathway?

The steady-state level of SA was significantly increased in *Arabidopsis* plants with high levels of A4-*rol*B expression. Moreover, qPCR analysis of the expression of the gene encoding the key enzyme of SA biosynthesis, *ICS*, showed a high level of positive correlation between transgene expression and SA content (Figs. 1, 2). A4-*rol*B expression in the B13 line was almost twofold less than that in the B2 and B5 lines; *ICS* expression was 2.5-fold higher than that of the WT, while in the B2 and B5, the *ICS* expression level was five- and fourfold higher than that in the WT, respectively. SA content in the B13 line was equal to that of WT plants. This observation indicated that A4-*rol*B expression led to activation of the SA biosynthesis in a dose-dependent manner. In addition to SA biosynthesis, our data reveal the activation of gene expression in the major markers of the SA-signalling pathway, such as NPRs and PR1, in A4-*rol*B transformed plants. Overall, it can be supposed that A4-*rol*B overexpression led to an increase in SA biosynthesis and consequently to the activation of the SA-dependent signalling pathway, which upregulated flavonol biosynthesis as a defence against pathogens. These data agree with the observation that A4-*rol*B increased resistance to pathogenic fungi in transformed tomato plants (Arshad et al. 2014). However, our experiment with PAL inhibitor PFP and analysis of the expression pattern of *ICS1* and *PAL1* as the main SA and flavonoids producers, indicate the possibility that these effects are rather secondary. Assumed (Fig. 6), A4-*rol*B primarily activates another system, perhaps it can be involved in growth regulation or in a calcium signaling system (Shkryl et al. 2020), which in turn induces ROS (Veremeichik et al. 2016).

In the present study, we did not observe any changes in ABA biosynthesis, reception, and signalling, phosphatases, kinases, or transcription factors. However, not only ABA signalling can regulate plant responses to abiotic stress treatment. ROS signalling plays an important role in this process (Mittler 2017). ROS metabolism changes in A4-*rol*B-expressing plant cells were described previously (Bulgakov et al. 2012, 2018; Veremeichik et al. 2012, 2016; Shkryl et al. 2013). Previously, we described that A4-*rol*B-dependent activation of NADPH-oxidase expression in A4-*rol*B-expressing plant cells (Veremeichik et al. 2016), accompanied by activation of ROS scavenger enzymes, resulted in balanced intracellular ROS levels under control conditions and oxidative stress treatment, such as paraquat, menadion, and high light (Bulgakov et al. 2012), and reduced apoptosis-like symptoms in transformed plant cells (Gorpenchenko et al. 2012). ROS are supposed to be a modulator of DREB group 2 and some HSFs (Ohama et al. 2017). *Arabidopsis* plants that expressed the A4-*rol*B gene showed increased expression of the *DREB2A* gene, which regulates drought and heat tolerance but not cold tolerance (Sakuma et al. 2006). In contrast, there were no changes (Fig. 2) in the expression levels of *DREB2C* and *DREB2C*-regulated *HSFA3* (Chen et al. 2010). Among *HSFs*, only the expression of the downstream master regulator *HSFA2* was significantly increased in A4-*rol*B-expressing plants. HSFA2 was

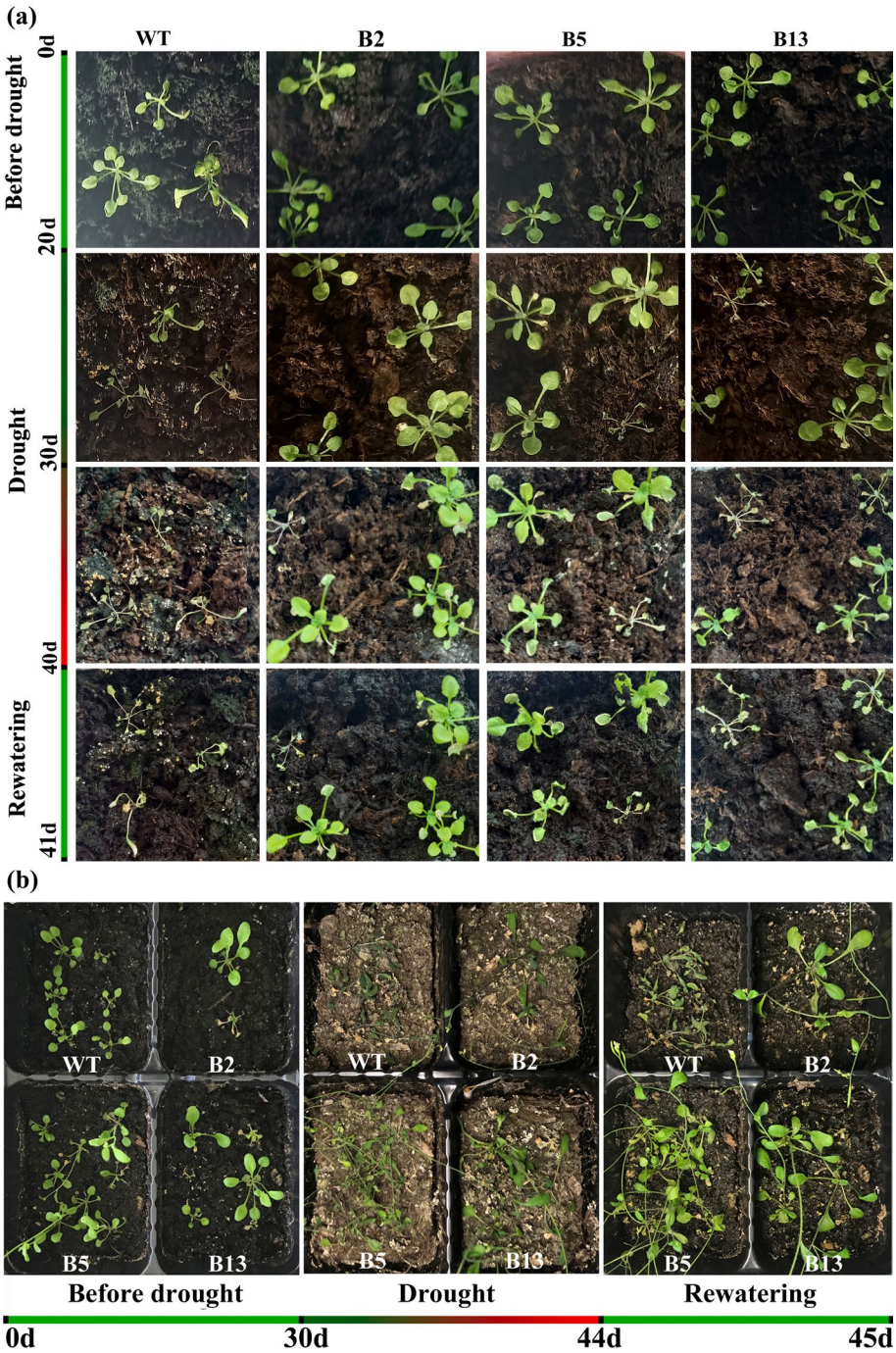


Fig. 5 Drought stress tolerance tests of control (WT) and F3 of the A4-*rol*B-transgenic *Arabidopsis* lines (B2, B5, and B13). 20-day-old (**a**) and 30-day-old (**b**) plants before and after 40- and 14-day-long drought stress, respectively, and recovered after rewatering. The photograph is representative of the experiments conducted in triplicate

described as a connecting link between SA and ROS signalling via the ethylene signalling pathway (Katano et al. 2018) and as a key regulator in response to several types of environmental stress (Nishizawa et al. 2006), including anoxia (Banti et al. 2010) through reprogramming of the transcriptome via *HSFA2*-dependent enhancement of gene expression encoding ROS scavenger enzymes (Shekhawat et al. 2021). *HSFA2* expression is directly regulated by HSFA1s, and H_2O_2 is an alternative way of regulating HSFA2 target gene expression, as well as *HSFA2* transcripts (Nishizawa et al. 2006). Moreover, HSFA2 is closely connected with the heat shock protein HSP70/chaperone network (Jacob et al. 2017). Previously, it was shown that A4-*rol*B can induce the expression of chloroplast HSP70s (Bulgakov et al. 2018), which, together with HSFs, play an important role in plant defence (Ogawa et al. 2007; Dickinson et al. 2018).

The expression of flavonoid-related TF and biosynthesis genes is regulated through stress-induced HSF action (Wang et al. 2020). Considering A4-*rol*B-induced steady-state activation of ROS-signalling (Bulgakov et al. 2012,

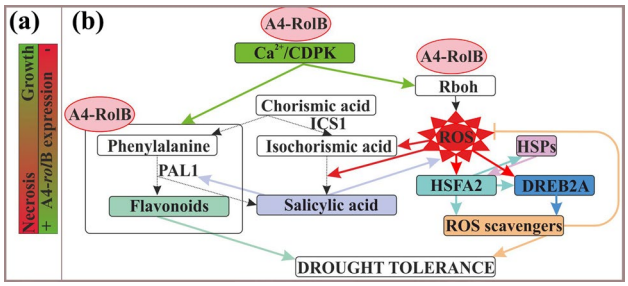


Fig. 6 Simplified model suggesting the possible location of A4-RolB action in the plant cell. The present model is based on a present and previous studies of the effect of the A4-*rol*B expression on the plants morphology and physiology. **a** Schematic representation of correlation of the A4-*rol*B expression level with growth and necrosis. **b** Network of Ca^{2+}/CaM/Calmodulin/CDPK, SA and ROS signaling systems and their contribution and role in the drought tolerance. A4-RolB in the red oval designates locations of the described molecular effects of the A4-*rol*B expression. A full description of the depicted processes with references are given in the Discussion section

2018; Veremeichik et al. 2012, 2016; Shkryl et al. 2013), it can be presumed that A4-*rol*B overexpression should lead to increased resistance to other abiotic stress treatments. However, we did not find any differences in the resistance of WT and A4-*rol*B-transgenic seeds in the simple germination test to other abiotic stressors, such as high and low temperatures and high salinity. Previously, we showed that the effect of individual *A4-rolB* overexpression on salt tolerance in *R. cordifolia* calli was significantly lower than the effect of individual *rol*C genes or combined expression of *rol*ABC and pRiA4 WTs (Shkryl et al. 2008; Bulgakov et al. 2010). Furthermore, there is limited data on abiotic stress tolerance in *A4-rolB*-expressing plants. Constitutive expression of the *A4-rolB* gene in whole tomato plants reduced fruit yield (Arshad et al. 2014), while fruit-specific expression enhanced it and could prevent yield reduction under abnormal temperatures (Shabtai et al. 2007). However, based on these data, it is difficult to reveal the role of A4-*rol*B as a modulator of abiotic stress tolerance in plants. In summary, we suggest that there is a need for additional and more detailed investigation of thermotolerance of A4-*rol*B-expressing plants, as long or short treatment and heat shock pretreatment.

Based on the data obtained in this and our previous work, it can be presumed that constitutive overexpression of A4-*rol*B under the strong 35S promoter led to the activation of SA and ROS signalling systems (Fig. 6). This conclusion is consistent with previously obtained data on A4-*rol*B-dependent activation of both ROS generation (Veremeichik et al. 2016; Wang et al. 2014) and ROS detoxification (Bulgakov et al. 2012), which together result in damage-avoiding ROS homeostasis (Bulgakov et al. 2012). An increase in flavonol accumulation and enhanced drought tolerance can be explained by the cooperative action of these two pathways. The significant role of the A4-*rol*B gene in trending areas of research, such as plant drought tolerance, may draw attention and launch a new wave of detailed research on A4-*rol*B-induced effects and their underlying molecular mechanisms.

Author contribution statement GNV, TVR, VPB, YNS Conceptualization, Datacuration, Project administration, Supervision, Validation, Visualization,Writing—original draft. VPB, YNS Resources, Funding acquisition.TVR, VPG, PVV Investigation, Methodology, Formal Analysis. SAS,EVB, YAK, AAK, DVB Investigation.

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Data availability The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All the authors whose names appeared on the submission approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that the questions related to the accuracy of integrity of any part of the work were appropriately investigated and resolved.

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